PROGRESS IN MEDICINAL CHEMISTRY 20

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Preface

It gives us pleasure to present six reviews in the present volume. Chapter 1 covers the problem of the chemotherapy of leprosy and stresses the slow progress that has been made in recent years, chiefly because of the failure to obtain suitable models for testing new agents. After dapsone, clofazimine and the rifamycins looked promising but resistance still remains an obstacle.

Anthraquinones are widely distributed in nature and have been extensively used in the dyestuff industry. Recently, certain simple synthetic hydroxylated anthraquinones have been claimed to possess low but reproducible anti-tumour properties and these are described in Chapter 2.

Existing strategies for the elimination of viruses from vegetatively propagated stock are evaluated in Chapter 3. The impetus for improvement of genetic engineering techniques and protoplast fusion will, in the long term, result in advances in the production of resistant varieties.

Since the introduction into clinical practice in 1960 of chlordiazepoxide (Librium), the benzodiazepines have become the most frequently prescribed of all psychotropic drugs. Chapter 4 concentrates on structure-activity relationships among the 1,4 and 1,5 compounds which lack cyclic structures on two of their faces.

Many data have accumulated over the past two decades concerning the behaviour of chelating agents in biological systems and greater emphasis has to be placed now on the comparative biochemistry, distribution and effects on cells of these agents. This topic is reviewed in Chapter 5 and illustrates the unparalleled therapeutic potential of these compounds.

A common structural feature of the early histamine H_2-receptor antagonists was the imidazole ring. Chapter 6 considers the possibility that the basic heterocyclic ring may not be essential for this blocking activity by discussing the properties of ranitidine, a furan compound.

Authors of these reviews have to devote much time and effort to their preparation and we are indebted to them for their patience and consideration. We are also grateful to the owners of copyright material we have included. Finally, as always, we acknowledge with thanks the full co-operation of the staff of our publishers.

January 1983

G.P. Ellis
G.B. West

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Progress in Medicinal Chemistry – Vol. 20, edited by G.P. Ellis and G.B. West

1 The Chemotherapy of Leprosy

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INTRODUCTION

This review presents a critical assessment of the chemotherapy of leprosy referring to both ancient and more recent drugs. In the light of the growing understanding of *Mycobacterium leprae* and the disease of leprosy, suggestions are made about possible ways new drugs might be developed. However, the chemotherapy of leprosy cannot sensibly be discussed without first presenting a brief summary of the major features of the disease.

HISTORY AND EPIDEMIOLOGY

Leprosy is commonly regarded as one of the oldest diseases known to mankind [1, 2]. It is a disease of skin and peripheral nerves caused by *Mycobacterium leprae* which presents in different ways. Many early descriptions of leprosy, as, for example, the Old Testament writing circa 1300 B.C., probably included specific leprosy together with many other skin diseases. The disease was widely disseminated and accurately described in India from around 600 B.C. and in Japan circa 400 B.C. The earliest indisputable skeletal evidence comes from a Coptic mummy dated 500 B.C. Leprosy was widespread in Europe, reaching its peak in the 13th and 14th centuries, although the real extent of the disease may have been exaggerated by incomplete and faulty diagnosis [2]. Today leprosy is largely confined to a broad equatorial band covering Africa,

India, South East Asia and South America. In the main, the countries are those of the developing world, although endemic foci exist in parts of continental U.S.A. (Texas, Louisiana). Global estimates of the disease range from 10 to 20 million, but the incidence varies considerably from 0.1 to 20–50 per thousand of the population (*Figure 1.1*). Approximately 30—50% of all the people with leprosy are thought to be in the Indian sub-continent. It is estimated that only 20% of all leprosy sufferers are receiving any form of treatment [3]. The epidemiology of leprosy has been the subject of a recent international conference [3a].

It was confidently predicted in the 1950's [2] that leprosy would be totally eradicated within 20 years. Today there is a widespread impression amongst many people, including scientists concerned with drug use and development, that leprosy is a disease of the past which has largely been controlled by modern drugs. The facts are distinctly otherwise.

The treatment of leprosy sufferers has long been the object of certain missionary, charitable and voluntary societies. The Leprosy Mission (formerly the Mission to Lepers) was founded in 1874. In 1931 The International Leprosy Association was formed, bringing together many voluntary and private organi-

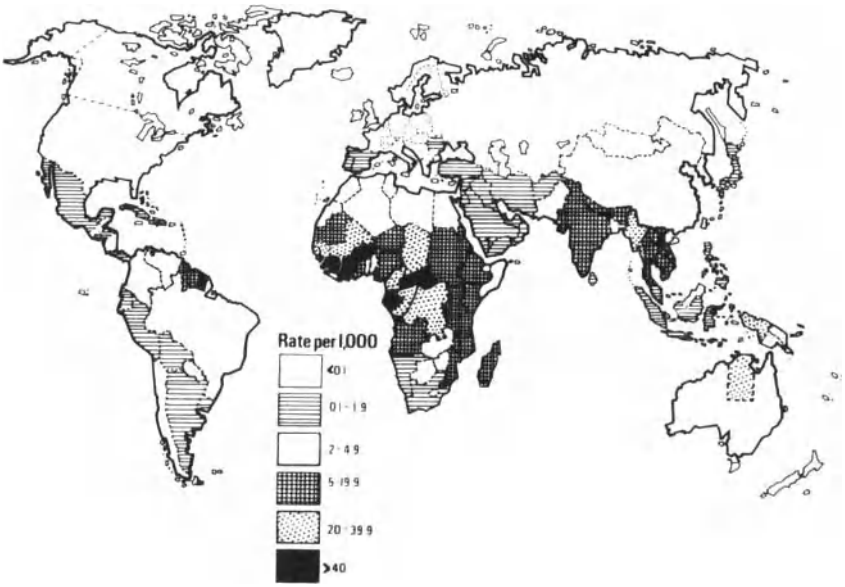


Figure 1.1. World distribution of leprosy [2] (W.H.O. data).

sations concerned with the treatment and rehabilitation of leprosy sufferers. The All India Leprosy Association was founded in 1929. In 1974 the World Health Organization together with the United Nations Development Programme and the World Bank began to establish the Special Programme for Research and Training in Tropical Diseases (SPRTTD). The six target diseases of SPRTTD are malaria, schistosomiasis, filariasis, trypanosomiasis, leishmaniasis and leprosy. The programme is being developed under the direction of Scientific Working Groups (SWGs). There are two SWGs concerned with leprosy, IMMLEP (Immunology of Leprosy), initiated in 1974, and THELEP (Chemotherapy of Leprosy), initiated in 1976. The immunology of leprosy and the search for a vaccine are largely outside the scope of this review but are the object of intensive study and debate [4]. The early results of the field trials of the vaccine are very encouraging [4a, 4b]. A useful review of the immunological aspects of leprosy has recently appeared [4c, 4d].

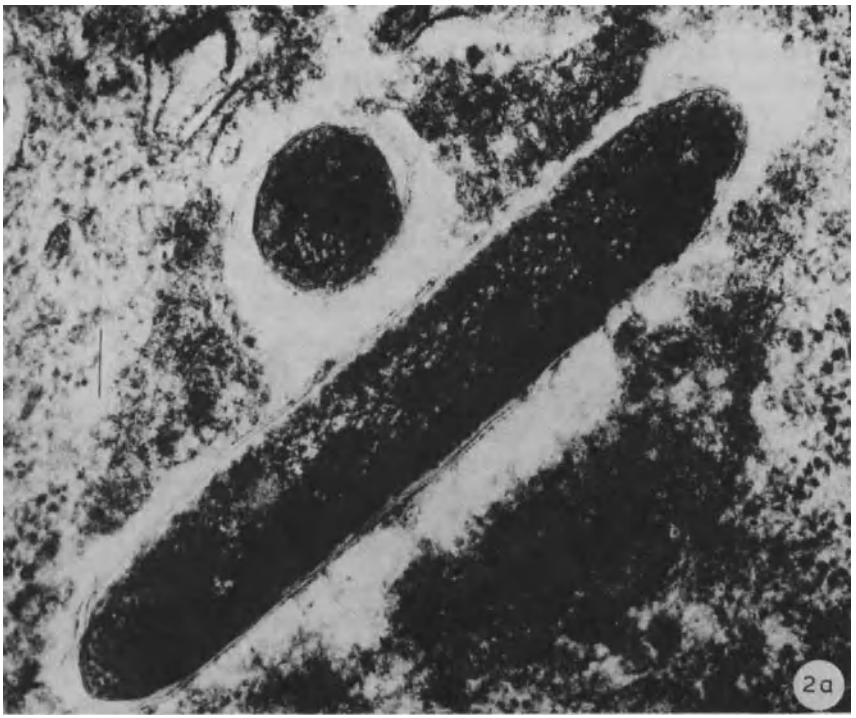
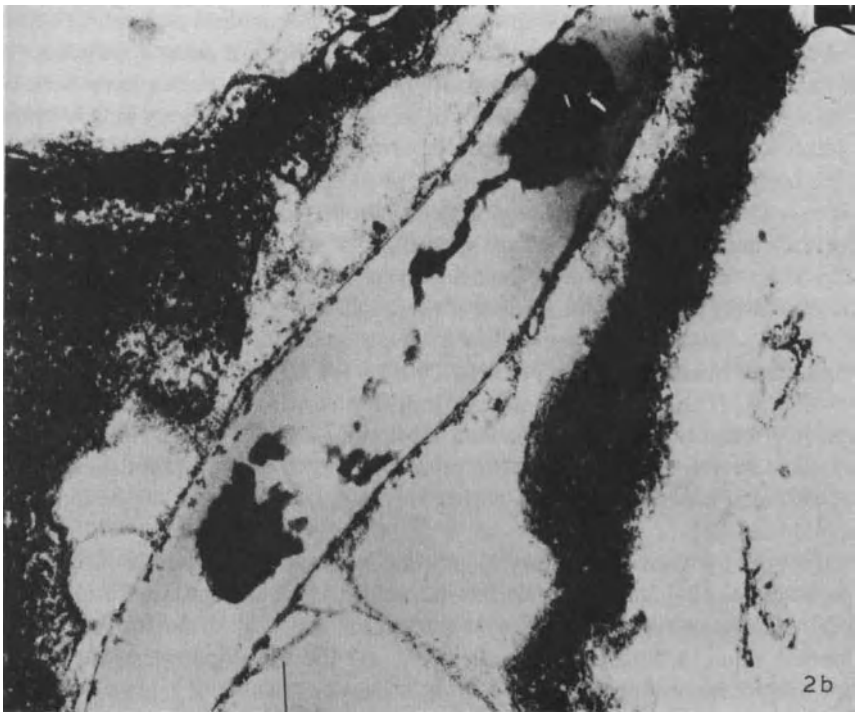


Figure 1.2 a. Electron micrograph of viable solid-staining M. leprae *bacillus, longitudinal and cross sections. b. Electron micrograph of* M. leprae *bacillus from patient undergoing dapsone therapy; note loss of cell contents. Both photographs by courtesy of Leprosy Mission.*

THE ORGANISM

Mycobacterium leprae is an obligate intracellular parasite with high infectivity and low pathogenicity and a predilection for skin, mucous membranes and peripheral nerves [5]. It was first identified in the skin nodules from leprosy patients by the Norwegian physician and scientist Gerhard Henrik Armour Hansen in 1873. Leprosy is therefore sometimes referred to as Hansen's disease or Hanseniasis. As a consequence of his observations, Hansen suggested, in 1879, that micro-organisms might be the cause of chronic infectious disease in man [6]. It is a curious twist of history that *M. leprae*, which is generally recognised as the first bacterium to be identified in man and which also provides the foundation of the germ theory of disease, should be such an unusual organism.

Today it remains one of the least understood pathogenic bacteria, although



the closely related and intensively studied *M. tuberculosis* affords a useful but partial model.

The major features of *M. leprae* may be summarized as follows:

1. It is a large (6–8 × 0.5 μm), acid-fast (Ziehl-Neelsen method) bacillus (*Figure 1.2*) which occurs as solid or fragmented rods often in large clumps (globi) (*Figure 1.3*, p. 25).

2. The organism shows different degrees of solid staining (*Figures 1.2, 1.3*). Solid-stained bacilli are regarded as viable. In 1962 the morphological index (MI) was introduced [7]. This expresses the viability of an isolate or inoculum as the average percentage of morphologically normal solid-staining or deeply staining bacilli. The MI is widely used in classifying the type of leprosy, evaluating the efficacy of drug therapy and the viability of inoculi used in mouse footpad tests (see below). Some reservations have been expressed as to whether all non-solid staining bacteria are dead or simply in a modified quiescent state [8, 9], and the index must be evaluated carefully [10, 10a].

3. The natural host, and until recently the only unequivocal source of *M. leprae*, is man [11, 11a]. Claims to identify a similar natural infection in chimpanzees [12, 13] and armadillos [14, 15] and other species have been or are being investigated [11a]. Inoculi of *M. leprae* will grow very slowly in the mouse footpad, whilst the nine-banded armadillo, an animal with a low body core temperature, has recently been found to develop a disseminated form of leprosy [16, 17] when inoculated with *M. leprae* from leprosy patients. Thus, for the first time, relatively large quantities of the bacilli are now available. This is an important advance, particularly in the development of a vaccine and in facilitating fundamental studies of the bacillus.

4. *M. leprae* is extremely slow growing, even for a mycobacterium; the replication rate is of the order of 10–20 days [4b, 18].

5. (a) The organism cannot be cultured *in vitro*. No claim to have successfully cultured *M. leprae* has been substantiated [9, 19–19b]. This is a major obstacle to a detailed study of the organism and to the rational development of chemotherapeutic agents. A variety of novel growth media are being investigated [20–23].

(b) The organism can survive *in vitro* for about 9–14 days in simple synthetic media [24] and for about the same time when incorporated into human macrophages suspended in a simple medium [25, 26]. This has useful but limited value in biochemical studies [27] and the development of rapid tests for drug-resistant organisms [28].

(c) Recent studies indicate that the organism can survive outside the human body for 14–28 days depending on the temperature and relative humidity of the environment. In moist presterilized soil at room temperature, *M.*

leprae is claimed to remain viable for up to 45 days [29]. This obviously has great significance when considering how the disease is transmitted.

6. The biochemistry and structure of *M. leprae* are under active investigation.

(a) The general structure of the cell wall is thought to be similar to that of other mycobacteria [30, 31] (*Figure 1.4*) and provides a target for selective drug design. Major components include a repeating arabinogalactan polymer bearing mycolic acid residues, (*Figures 1.5, 1.6*) a peptidoglycan polymer of *N*-acetylglucosamine and muramic acid cross-linked by peptide bridges made up of various amino acids including α,ε-*meso*-diaminopimelic acid (DAP), a non-mammalian substance [32] (*Figure 1.7*). The outermost surface of the cell wall involves peptidoglycolipids (mycosides) (*Figure 1.8*), peptidolipids and glycolipids which form rope-like structures.

In agreement with this general pattern, the mycolic acids from *M. leprae* have been found to belong to the group associated with other mycobacteria, (*Figure 1.6*), rather than with the smaller molecules obtained from corynebacteria and nocardia [30, 31a]. They account for a large proportion of the covalently bound lipids of the cell wall [31a]. Mycolic acids have been found in leprous tissue [33]. The antigenic properties of mycobacteria are associated with the mycosides. Although *M. leprae* is described as only weakly antigenic [34], a specific antigen from the bacillus has recently been identified as a mycoside probably of the C group [35, 35a] (*Figure 1.8*).

The divergences from the general pattern are associated with the rather unstable acid fastness of *M. leprae* which is readily extracted with pyridine [18, 36]. This property may reflect the large amount, about 25%, of extractable non-covalently bound lipid in the cell wall [31a]. Electron microscope studies suggest that the cell wall of *M. leprae* may be smoother than those of many other mycobacteria and have fewer strap-like appendages [37]. The most unusual feature of the cell wall is the very high glycine, as opposed to L-alanine, content. It has been suggested that glycine replaces L-alanine in the peptidoglycan structure (*Figure 1.7*). This amino acid exchange is usually associated with a decrease in cell wall stability [31a].

(b) The respiratory chain in *M. leprae* has been shown to be unexceptional in that both succinate and NADH serve as substrates which are oxidized via various cytochromes ($a + a_3$, b, c and o) which use oxygen as the terminal electron acceptor [38, 39].

(c) Recently workers in one laboratory have claimed to have identified a unique diphenoloxidase enzyme system in *M. leprae* [40]. The existence of such an enzyme has continued to be a matter of controversy [41, 41a]. However, such a putative enzyme system, which utilizes L-dihydroxyphenylalanine

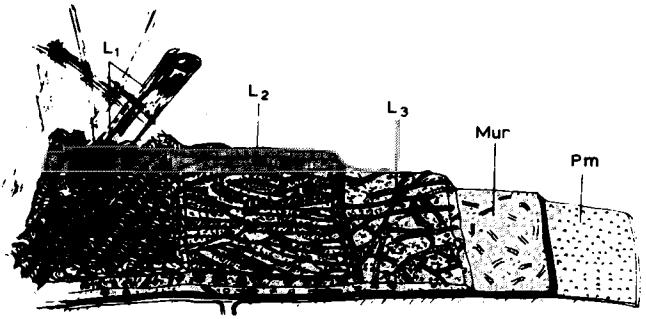


Figure 1.4. The mycobacterial cell wall generalized from electron micrographic data. Pm, plasma membrane; Mur (murein or peptidoglycan). L_3–L_1, fibrous rope-like layers of increasing complexity composed of glycolipids and peptidoglycolipids including the mycolic acid moieties. The strap-like appendages on L_1 may be less evident in M. leprae. *Adapted from Ref. 30 with permission.*

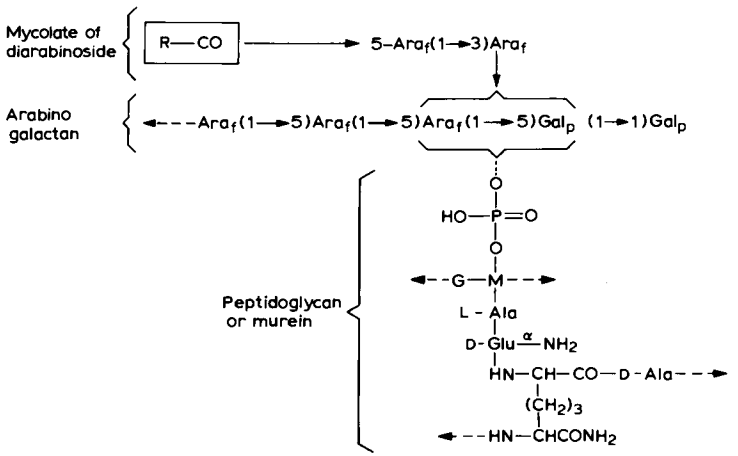


Figure 1.5. The arabinogalactan polymer of the mycobacterial cell wall attached to both the outer glycolipid moiety and the inner peptidoglycan residues, from Ref. 3 with permission.

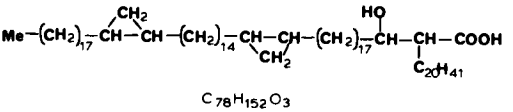


Figure 1.6. A mycolic acid recently identified in M. leprae *[30, 31].*

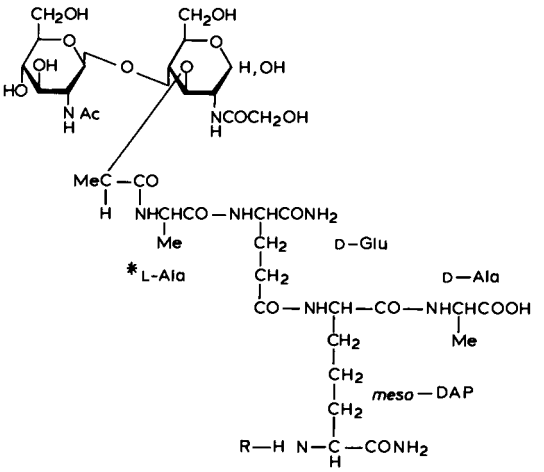


Figure 1.7. Mycobacterial peptidoglycan consisting of N-*acetylglucosamine, muramic acid,* L-*alanine,* D-*glutamic acid,* meso-*diaminopimelic acid and* D-*alanine, R = another unit beginning with an interpeptide bridge between the* meso-*DAP shown and a second* meso-*DAP linked in turn to* D-*Glu-*L-*Ala, etc. [30, 31]** M. leprae *is exceptional amongst mycobacteria in that glycine replaces* L-*Ala in the peptide chain.*

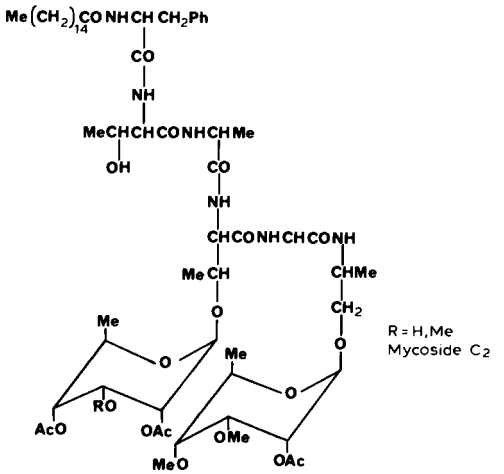


Figure 1.8. Mycoside C_2 *from* M. avis *illustrating the peptidoglycolipid nature of these compounds which are important as surface antigens in mycobacteria, from Ref. 30 with permission.*

(L-DOPA) as substrate, provides a new and accessible target for specific and selective drug design. The implications for the design of new drugs are considered later in this review.

(d) Radiolabelled thymidine as well as L-DOPA have been shown to be incorporated into *M. leprae* [24–28a].

(e) Recently, γ-glutamyl transferase activity has been found in *M. leprae* [41b], which has also been reported to have a superoxide dismutase but not catalase enzyme system [41c].

7. The response of *M. leprae* to drug therapy is unusual. The organism is uniquely sensitive to 4,4′-diaminophenylsulphone (dapsone). It is also sensitive to rifampicin and clofazimine, both of which are active against *M. tuberculosis*. In addition to the development of drug resistance, which is considered later in this article, there is the phenomenon of microbial persistence, which occurs with all the present drugs even after prolonged therapy, for example, after 12 years of dapsone therapy or 5 years of rifampicin therapy. These persistent organisms are usually found in macrophages but do occur in other tissues. They survive chemotherapy with combinations of two or three drugs, for example, clofazimine + dapsone + thiambutosine. After passage through the mouse, they have been found to be fully sensitive to dapsone [42–44]. Persistent organisms may resemble bacterial spores in their low metabolic activity [42]. The problem of persistent organisms in leprosy has recently been reviewed and presents a formidable challenge to chemotherapy in the future [44a].

THE DISEASE

Leprosy is essentially a chronic disease and a great 'mimicker' of many other diseases. Principally it affects the skin, mucous membrane and peripheral nerves, but the eyes, bone, muscle and endocrine, reticuloendothelial and haematopoietic systems may also be involved [45, 45a]. The primary direct effects of leprosy can lead to extensive and very damaging secondary effects (*Figure 1.9*) [46].

Leprosy is now thought to be transmitted principally via nasal discharges (cf., tuberculosis), although the role of various insects in facilitating dispersion may be important [47]. Evidence is beginning to accumulate that the nasal mucosa is the primary site of attack by *M. leprae* [48].

The classification of leprosy has always been difficult, especially in the very early stages. Indeterminate leprosy frequently presents as small hypopig-

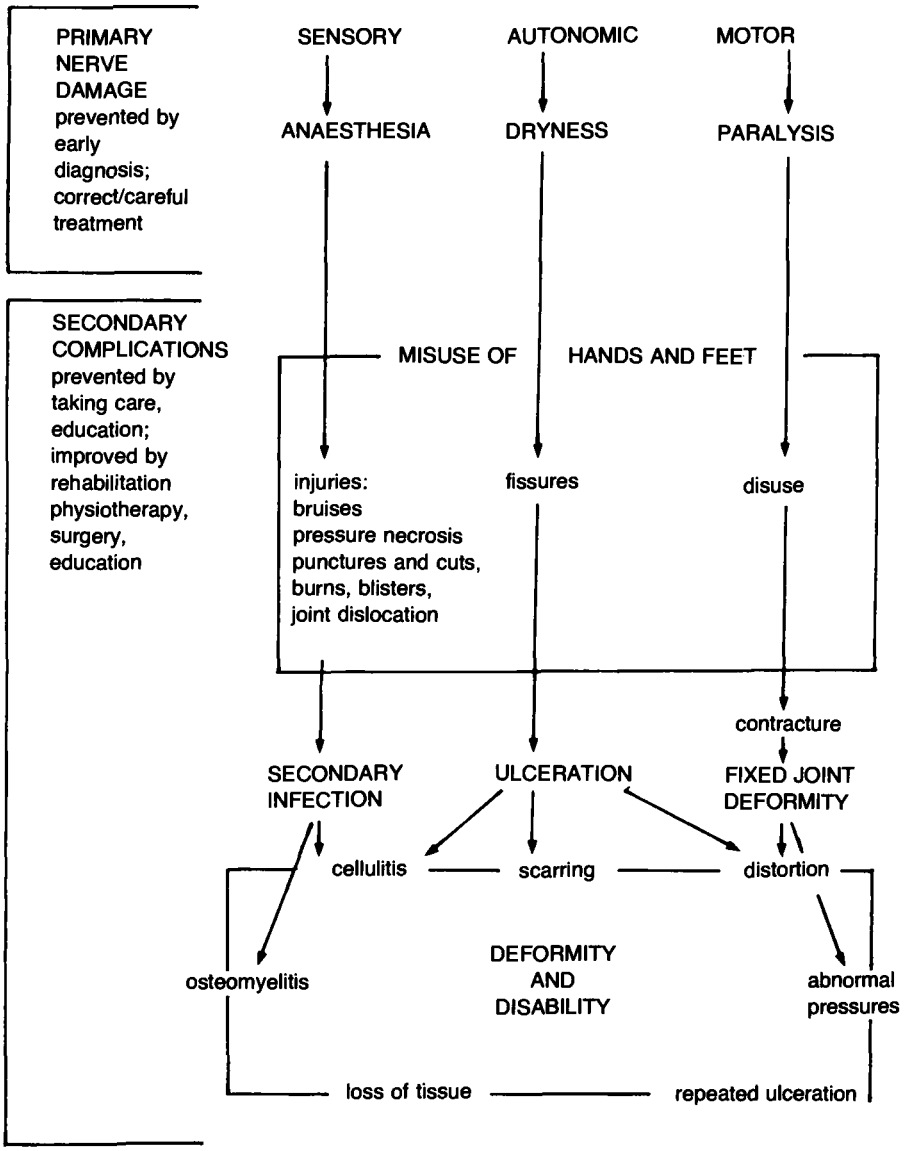


Figure 1.9. The pathogenesis of leprosy following nerve damage.

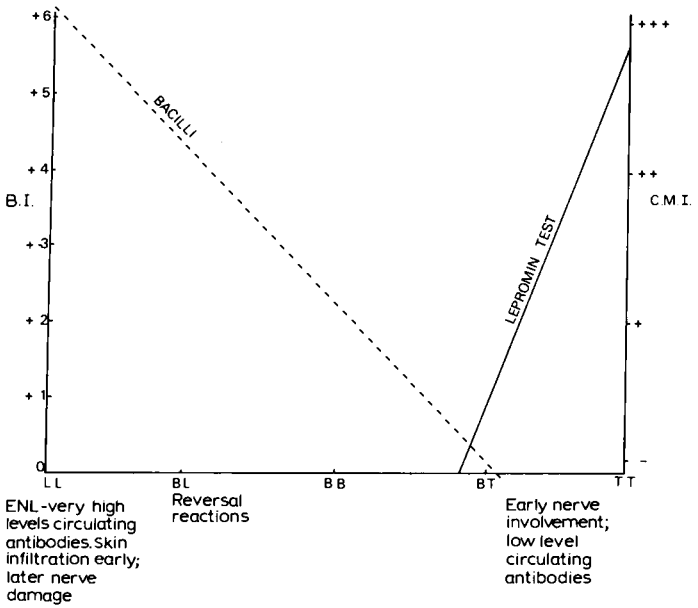


Figure 1.11. A summary of the major immunological and bacteriological features of leprosy.

mented lesions (*Figure 1.10*, p. 25) which may be self-healing or develop into a more recognizable form of the disease. Established leprosy is now classified on a five-point scale extending from the polar lepromatous leprosy (LL), through various intermediate or borderline (dimorphous) states designated as borderline lepromatous (BL), borderline borderline (BB), borderline tuberculoid (BT), to polar tuberculoid leprosy (TT) [49, 50]. Subsequently, the classification of LL has been sub-divided into polar (LLp), a stable immunological condition, and sub-polar (LLs), an unstable immunological state [4, 50] (*Figure 1.11*). A similar bipolar spectrum for tuberculosis has recently been reported [50a]. Each classification is associated with various clinical, bacteriological and immunological features. Two bacteriological measurements are usually made. These are, first, the bacterial index (BI), which is a measure of the number of bacilli present in various sites in the patient [51, 52]. A logarithmic scale varying from 0 to 6+ is generally used. In lepromatous leprosy, as many as 10^9 bacilli/g of tissue may be found. The second is the morphological index (MI) described earlier. This assesses the proportion of viable bacilli which are present in a particular sample [7–10]. The major immunological assessments involve estimating the level of cell-mediated immun-

ity (CMI) by the lepromin test. The results can be expressed qualitatively using a scale ranging from +++ to − [53, 54]. *M. leprae* appears to be a powerful immunogen [54a], although it is only weakly antigenic [34]. The level of circulating antibodies is also a useful immunological parameter, being highest in lepromatous leprosy (*Figure 1.11*). Fluorescent antibody tests on leprosy patients and members of their family and community have recently been performed. These show that a large number of people, up to 50% of contacts with no clinical signs of leprosy, do have circulating antibodies to *M. leprae* [4, 4b], i.e., there is a high level of natural immunity. This evidence agrees with the description of *M. leprae* as an organism of high infectivity but low pathogenicity [5]. Some illustrative clinical examples of the different types of leprosy are shown in *Figures 1.10, 1.12–1.18* (colour plates). These illustrations are by no means exhaustive and comprehensive texts [1, 2, 18] should be consulted for further details.

The lepromatous leprosy patient is clearly of great concern, both in terms of effective chemotherapy (reduction of the bacterial load) and as a major source of infection within the community. The proportion of lepromatous to tuberculoid patients varies considerably, from 10 to 70%, in different parts of the world [55, 56] and also within the same region [57]. Generally, more men than women are affected by leprosy [3a] and the disease is associated with extensive hormonal disturbance linked to impotence and gynaecomastia [57a–57c].

Other major immunological features of leprosy, with consequences for drug therapy, are the reactional states which are usually acute and may be severe or relatively mild. The two major acute reactions are reversal reactions (type I reactions) and erythema nodosum leprosum (ENL, type II reactions) [5, 58]. ENL is associated with lepromatous leprosy. In LL there are large numbers of circulating antibodies present together with massive amounts of antigenic bacilliary material. The precipitation of the immune complex and possibly other factors [4b, 4c] is thought to initiate ENL which presents as a fever accompanied by numerous painful erythematous nodules and may also involve other organs, particularly the kidney, testes [59], lymph nodes and joints (arthritis). Immune complex glomerular nephritis is recognized as a major life-threatening aspect of leprosy which is associated with the antibody response of the patient but not necessarily with ENL [60].

Reversal reactions are commonly associated with an upgrading of the immunological state of the patient from a defective to an efficient CMI. Unfortunately, this change may be associated with extensive inflammation, ulceration and nerve damage [5]. Reversal reactions associated with a downgrading of the patient's immunological response have also been described

[58], but other workers have not substantiated this claim [4b]. The immunopathology of nerve damage in leprosy has been described [60a]. Both type I and type II reactions may occur spontaneously or during drug therapy. Any association with drug therapy provides a powerful disincentive for patient compliance both in presenting for treatment and in self-medication.

Stoner [61] has recently presented an interesting hypothesis in which he examines the immunology of leprosy and the disease classification (*Figure 1.19*). He suggests that the immunological response of the host may not be due to any major deficiency in the host defence mechanisms, e.g., genetic factors, or lack of transfer factor [4, 4b], but rather to various escape mechanisms of *M. leprae*. The organism penetrates non-defensive cells, particularly Schwann cells, and also smooth muscle fibres and endothelial cells which cannot be activated by an immunological mechanism to destroy the invading parasite. *M. leprae* may also block the phagosome-lysosome fusion necessary for its destruction in cells normally capable of destroying bacteria, e.g., macrophages. Above all, *M. leprae* may serve as a powerful immunosuppressant causing the generation of suppressor cells in the host and thereby subverting and largely reversing the normal immune response [62]. Stoner's ideas are consistent with the conflicting and tentative claims that vaccination with *M. leprae* or BCG [4b, 63, 64, 64a] and drug therapy [65] may upgrade

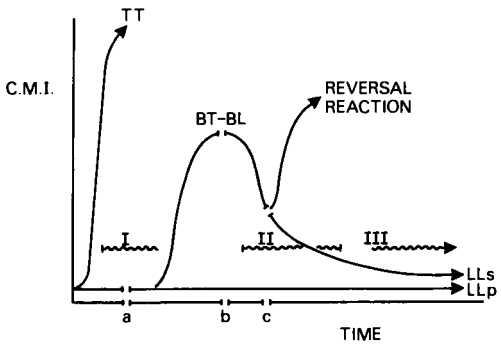


Figure 1.19. Phases of immunosuppression associated with the immunological spectrum of leprosy. In TT there is no suppression of an efficient and rapid CMI response. Borderline patients (BT-BL) after an initial immunosuppressant phase I (lasting 2–5 years) develop an effective CMI response which may be upgraded by an ensuing reversal reaction (frequently found 6–18 months after commencement of therapy) or in a second phase, II, of immunosuppression lead to a deficient CMI which remains ineffective during a further phase of immunosuppression, III. The patient is now lepromatous (LL_s). Some lepromatous patients do not progress through a borderline condition but are thought to remain totally and irreversibly suppressed from the first (LL_p); adapted from Ref. 61 with permission.

the immunological response of leprosy patients, since both may effectively reduce the number of *M. leprae*, but by different mechanisms, and thereby the associated immunosuppression. Stoner's proposals have recently been criticized [61a, b]. However, there is clear evidence that the generally increased immunological instability (immunosuppression?) associated with pregnancy, particularly the third trimester, is a cause of relapse in leprosy patients and may lead to active leprosy in women with no previous clinical history [61c, d].

DRUG-TESTING SYSTEMS

The peculiar properties of both *M. leprae* and its infection in man mean that the usual methods of evaluating antimicrobial agents cannot be used. There are three clearly accepted ways of evaluating current and potential antileprotic drugs.

TESTS INVOLVING OTHER MYCOBACTERIA

Most frequently *M. tuberculosis* is used as an *in vitro* screening system [42, 56] which can be followed by *in vivo* evaluation of any active compounds against the infection in mice. The *in vitro* tests are both rapid and cheap. Many drugs active against *M. tuberculosis* in this system are also active against *M. leprae*. However, dapsone shows relatively weak activity in this test and clofazimine, whilst active *in vitro*, is less active *in vivo*. In contrast, many proven antitubercular drugs show little or no activity against *M. leprae* in mouse footpad or in man [66] – see later sections of this review.

Other cultivable mycobacteria have been proposed as alternative organisms. *M. marinum* was selected from a range of mycobacteria as a suitable model organism. The response of the infection in rats to current antileprotic drugs indicated some similarities with leprosy chemotherapy, but indicated that dapsone alone was not a very effective drug [67]. Various strains of *M. scrofulaceum* have been proposed as an *in vitro* model, but again dapsone has shown only low activity [68]; other mycobacteria, *M. lufu*, *M. smegmatis* ATTC 607, *M. intracellulare*, are mentioned in succeeding sections of this review.

M. lepraemurium (rat leprosy) has been widely used in comparative immunological and bacteriological studies [52, 69, 70]. A great variety of organisms have been examined as possible sources of antigenic material and for use in vaccine preparations [71, 72].

THE MOUSE FOOT PAD

This model was introduced in 1960 [73, 74] and is now established as the definitive method for evaluating antileprotic drugs. It is also widely used in bacteriological studies, including drug resistance, and in immunological investigations [75]. The World Health Organisation is currently seeking to standardize foot-pad procedures world-wide [76]. *M. leprae* is not a natural pathogen in mice, but inoculations of the bacillus (10^3–10^4 organisms) into the foot pad show, after a long lag period, a normal logarithmic growth pattern before reaching a plateau [77]. The process is, however, very slow, taking 12–15 months. The time of the experiment can be reduced to 6–9 months if irradiated thymectomised or nude (athymic) mice are used [78–81]. These, however, show a more disseminated form of the disease. In normal mice the organisms remain located in the foot pad and at the end of the experiments the animals are killed and the foot pads harvested and examined for the relative numbers of both total (BI) and viable (MI) organisms. The distinction between bactericidal and bacteriostatic drug action can be made using the foot-pad technique [82] by observing the delay in multiplication of *M. leprae* when the drug is administered in the log phase of growth: the kinetic method [82, 83]. Antileprotic drugs are usually administered in the diet of laboratory animals and their antibacterial activity is most frequently expressed as that percentage, w/w of the compound in the feed, required to inhibit growth; for example, for dapsone, it is 0.0001%. In spite of its proven value and extensive development, the mouse foot pad remains a slow and expensive method of evaluating new drugs and is used only when more rapid, if less precise, *in vitro* methods indicate promising activity against *M. leprae*. The foot-pad test has been criticized on the grounds that it gives positive results with compounds which prove to have little or no activity against *M. leprae* in man [68].

The armadillo is too difficult to breed [84] and too expensive to maintain for use in routine drug testing. A variety of other mammals are being investigated for this purpose, including the Korean chipmunk and the common hedgehog [9, 84, 85].

IN VITRO TEST USING *M.LEPRAE*

The incorporation of radiolabelled L-DOPA and/or thymidine into *M. leprae* sustained in synthetic media [24–28a, 86] provides a rapid direct method for testing current and potential antileprotic drugs. Although the methods are at present lacking in precision and sensitivity, they clearly identify very active compounds, but less active compounds might be missed.

CHEMOTHERAPY

A number of short reviews covering various aspects of the chemotherapy of leprosy have appeared regularly over the last decade [3, 42, 87–92]. The treatment of leprosy is described in detail in several comprehensive textbooks [1, 2, 93, 94], and brief accounts of drug therapy are given in two recent major textbooks of pharmacology and therapeutics [95a, 96]. All these publications agree in identifying the major, front-line, drugs as dapsone, clofazimine and rifampicin. A variety of second line drugs are recognized as useful therapeutic agents, including thiambutosine, prothionamide, ethionamide and thiacetazone. The question arises, "If this armamentarium is available, why is the WHO so concerned about the present and future chemotherapy of leprosy?".

The concern of the WHO arises from three observations:

(i) *M. leprae* is known to persist in patients both during and after intensive and extensive chemotherapy with any of the known antileprotic drugs, alone or in combination [42–44].

(ii) Dapsone monotherapy has been the mainstay of the global treatment of leprosy patients, and now dapsone resistance, first suspected in the 1950's and confirmed in 1964 [97, 98], has become widespread geographically. The degree of resistance varies in different areas between 10 and 30% of all leprosy patients being treated [99–103]. The development of resistance to dapsone provides a classical study. Undoubtedly, resistance has been a consequence of prolonged treatment with a single drug over a long period with irregular and low dosage. Patient non-compliance has certainly been an important factor [104, 105] in the story.

(iii) Primary dapsone resistance, i.e., leprosy caused by dapsone-resistant organisms, has been identified in a number of studies [102, 103, 106, 107]. A recent study in Ethiopia found that sixteen out of thirty-one (55%) new, previously untreated, cases of leprosy were due to resistant organisms [102]. The THELEP panel concludes, "These facts clearly indicate that unless there is substantial improvement in leprosy chemotherapy within the next 5 to 10 years, then the possibility of controlling leprosy by chemotherapy may be seriously jeopardized".

The objectives adopted by THELEP are summarized as:

(a) finding better ways of using existing drugs;
(b) promoting the development of new drugs;
(c) assessing the needs for improved chemotherapeutic methods.

With these objectives in mind, the major antileprotic drugs will first be considered, then second-line and other drugs, followed by an examination of the possibilities for the development of new drugs [108].

FIRST-LINE DRUGS

THE SULPHONES

M. leprae is uniquely sensitive to 4,4′-diaminodiphenyl sulphone (DDS, dapsone; 1, *Table 1.1*). In mice, levels in feed of 0.0003% [109] and 0.00001% [87] are bacteriostatic and at a level of 0.0001% the delay in multiplication of the organism is found to be 91 days using the kinetic method devised by Shepard [82, 109]. The MIC in mouse plasma is less than 5 ng/ml, indicating that *M. leprae* is over 100-times more sensitive to dapsone than is any other mycobacterium so far examined. A similar efficacy is found in man, where 1 mg/day has been found to be clinically effective [110]. Depot therapy with 4,4′-diacetylaminodiphenyl sulphone (DADDS; 3, *Table 1.1*) as an intramuscular injection of 225 mg, given every 11 weeks, releases 2.4 mg/day of dapsone [87].

Dapsone was first synthesized in 1908 and was investigated in the late 1930's as part of the explosive developments in chemotherapy following the identification of the antimicrobial action of prontosil. It was shown to be active in experimental streptococcal and tuberculosis infections, but was considered too toxic for human medicine. Following the recognition of its activity against rat leprosy [111, 112], dapsone was evaluated in small-scale clinical trials.

Initially, the doses of dapsone employed proved to be too toxic, but later studies with the *N*-substituted derivatives (13, 14) [113], and the realization that these compounds were metabolized to dapsone, established dapsone as the most important drug in the treatment of leprosy [113]. It was cheap and could be made available in large quantities. From the late 1940's, dapsone has been used on a world-wide scale as almost the only effective medicine in treating leprosy. This continuous monotherapy has no doubt been a major factor in the emergence of dapsone-resistant organisms [94, 97–99]. Dapsone does not have any bactericidal activity against the ubiquitous persister organisms which are a major target for new drug development. Diaryl sulphones can be synthesized by a variety of well-established routes which are illustrated for dapsone in *Figure 1.20*. Nearly all the compounds described in *Tables 1.1–1.4* were prepared by these routes [114–121]; derivatization such as acylation and azomethine formation was by standard routes. Significant amounts of inactive by-products have been identified in dapsone complying with pharmacopeial monographs [119, 122].

Structure-activity relationships

All changes, so far made, in the structure of dapsone have resulted in inactive or marginally active compounds. The analogues (2–4, 11–14, *Table 1.1*) function as prodrugs which are metabolized to dapsone [87, 111, 123–128]. The

Table 1.1. 4,4′-DIAMINO SULPHONES WITH MONOSUBSTITUTION ON THE AMINO GROUP(S)

4-R^1NH-$C_6H_4SO_2C_6H_4$-4′-NHR^2

No.	R^1	R^2	*Activity*[p]	*Reference*
1[a]	H	H	A	
2[b]	Ac	H	AMD	87,109,120–122,130
3[c]	Ac	Ac	AMD	87,120–123, 126, 146
4[d]	HCO	HCO	AMD	87, 126
5[e]	$HOOC(CH_2)_2CO$	H	A?	111
6	$R^1 = R^2$ = hydnocarpoyl		AO/I	111
7	$R^1 = R^2$ = dihydrohydnocarpoyl		A?	111
8	$H(CH_2)_n$ $n = 1–6$	H	AO/A/I	87, 109, 130
9[f]	R^3OOCCH_2	H		
	R^3 = H, Na, Me	H	AMD?/I	111, 130
10	$HO(CH_2)_n$ $n = 2, 3$	H	AO/A	111, 130
11[g]	NaO_2SCH_2	CH_2SO_2Na	AMD	3, 147, 148
12[h]	NaO_3SCHR^4 R^4 = H, Me, Ph	CHR^4SO_3Na	AMD	111, 125, 127, 128, 149, 150, 153, 154
13[i]	NaO_3SCH | $HOCH_2(CHOH)_4$	$CHSO_3Na$ | $(CHOH)_4CH_2OH$		3, 111, 124, 125, 127, 128, 147, 149–153
14[j]	NaO_3SCH | $NaO_3SCHPhCH_2$	$CHSO_3Na$ | $CH_2CHPhSO_3Na$	AMD	3, 111, 125, 127 128, 147, 150
15	H_2N	NH_2	I	111, 155
16[k]	HO	H, CHO, Ac	AO/I	130, 131, 156–159
17[l]	HO	OH	I	
18a	[m]	[m]	see text	139–141, 144, 145, 161
18b[n]	[o]	[o]	A	139–141, 144, 145, 161

[a]Dapsone, DDS; [b]MADDS; [c]DADDS; [d]DFS; [e]Succisulphone; [f]Sulphone Cilag; [g]Sulphoxane sodium; [h]Diasone, R^4 = H; [i]Glucosulphone sodium; [j]Solapsone; [k]DDS-NOH; [l]DHDS; [m]2,4-Dioxo-1,2,3,4-tetrahydropyrimidine-5-sulphonyl; [n]Diuciphon; [o]2,4-Dioxo-6-methyl-1,2,3,4-tetrahydropyrimidine-5-sulphonyl. [p]A = active; AMD = active after metabolism to DDS; AO = active against other micro-organisms; I = inactive.

hydnocarpoyl derivative (6) incorporating two active compounds has not been widely used [111], but hydrolysis *in vivo* may be very slow [111, 129]. In rabbits, glucosulphone sodium (13) and some related methanesulphonic acid derivatives are reported not to be metabolized after intravenous (i.v.) injection [128]. The 4,4′-amino groups, or groups metabolized to them, must be retained (but see discussion on (18), *Table 1.1*, below). The replacement of one or both amino groups has been studied (*Tables 1.1* and *1.2*), but all the compounds investigated were found to be inactive against *M. leprae* [109]. Compounds (16) and (25) were active against a laboratory mycobacterium [130] and (16) has been claimed as the active metabolite of dapsone [131]. The metabolism of (23) to, and the contamination of (25) with, dapsone [109] accounts for their reported activity [87]. Substitution in the aryl rings at positions other than the 4,4′-sites gives inactive compounds (*Table 1.3*) except for promacetin (32) [3, 111, 132]. This compound has not found wide acceptance [111] and has not been rigorously investigated. If its activity can be clearly demonstrated, then it provides the opportunity of using the 2-position as an area of bulk tolerance

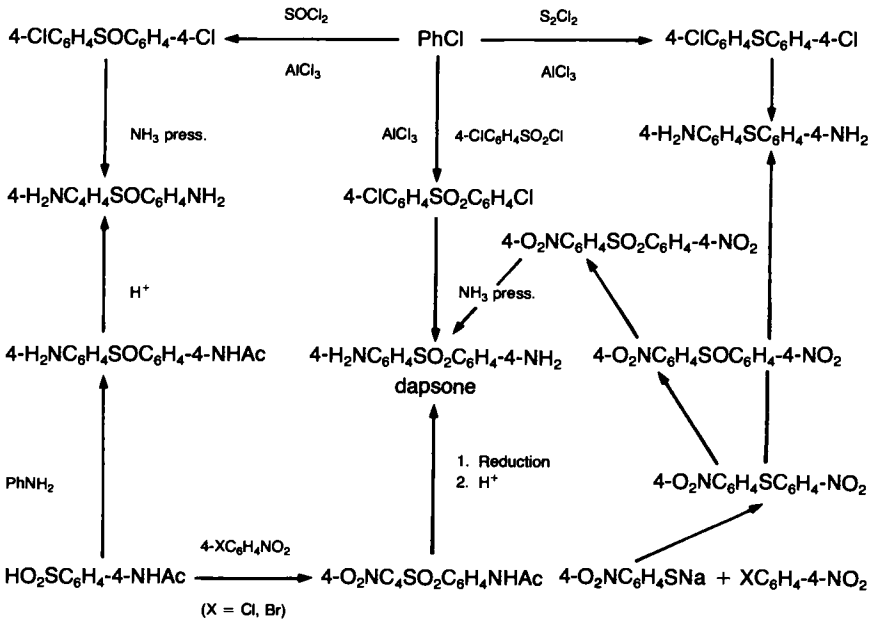


Figure 1.20. A summary of the synthetic routes to dapsone and its analogues (see also Tables 1.1–1.4*) [114–121].*

Table 1.2. 4,4′-DISUBSTITUTED SULPHONES

$4\text{-}R^1\text{-}C_6H_4SO_2C_6H_4\text{-}4'\text{-}4'\text{-}R^2$

No.	R^1	R^2	*Activity*[a]	*Reference*
19	AcNH	$N{=}CHR^3$ (a) R^3 = Ph; (b) R^3 = 4-$AcNHC_6H_4$; (c) R^3 = 2-OH-3,5-Cl_2-C_6H_2	AP/AMD	90, 135, 137, 138, 162
20	H_2N	$NH(CH_2)_nH$ n = 2–5	I	130
21	AcNOAc	AcNOAc	A?	160
22	R^4NH R^4 = H, HCO, Ac	N=O	AMD/I	87, 109, 122, 130, 159
23	R^4NH R^4 = H, HCO, Ac	NO_2	AMD/I	87, 109, 122, 130, 159
24	H_2N	H	I	87, 109, 119, 122, 130
25	H_2N	OH	AO/A?	109, 122, 130
26	H_2N	OMe	I	87, 109, 130
27	H_2N	F, Cl or Br	I	87, 109, 119, 122, 130
28	H	H	I	109
29	$H_2NCSNHN{=}CH$	$CH{=}NNHCSNH_2$	NT	163
30	HO	OH	A/I	109, 130, 164
31	Cl	Cl	NT	114

[a]See footnote *Table 1.1*, AP = active against plasmodia; NT = not tested.

Table 1.3. DIPHENYL SULPHONES WITH SUBSTITUENTS ADDITIONAL OR ALTERNATIVE TO THOSE AT THE 4,4′-POSITIONS

No.	*Substituents in ring A*	*Substituents in ring B*	*Activity*[a]	*Reference*
32	$2\text{-}SO_2NNa\text{-}4\text{-}NH_2$ (Ac)	$4'\text{-}NH_2$	A	2, 111, 132, 150
33	$2\text{-OH-}4\text{-}NH_2$	$2'\text{-OH-}4'\text{-}NH_2$	AO/I	109, 117, 134, 165
34	$2\text{-OEt-}4\text{-}NH_2$	$2'\text{-OEt-}4'\text{-}NH_2$	I	117, 165
35	3-Me-4-NHAc	4′-NHAc	AP	135
36	$2\text{-}NH_2$	$4'\text{-}NH_2$	I/AO	87, 109, 119, 122, 130
37	$3\text{-}NH_2$	$4'\text{-}NH_2$	A/I	87, 109, 122, 130
38	$3\text{-}NH_2$	$3'\text{-}NH_2$	I	87, 109, 130

[a]See footnotes *Tables 1.1* and *1.2*.

Table 1.4. COMPOUNDS DERIVED BY THE REPLACEMENT OF THE SULPHONE GROUP IN DAPSONE

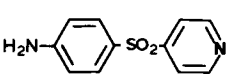
4-R^1NH-C_6H_4-X-C_6H_4-4′-NHR^2

No.	R^1	*X*	R^2	*Activity*[a]	*Reference*
42	H	SO	H	AMD	87, 109, 118, 166
43	R^3CO R^3 = H, Me	SO	COR^3	AO/I	118, 122
44	H	S	H	AMD/I	87, 109, 122, 155, 167
45	H	CH_2	H		
46	H	CO	H	I	87, 109
47	H	NH	H	I	87, 109, 166
48	H	O	H	I	87, 109
49	H	Se	H	I	168
50	H	–	H	I	168
51	H	SO_2-SO_2	H	I	109
52	H	S-S	H	I	109

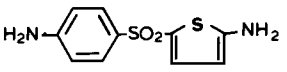
[a]See footnote *Table 1.1.*

[133] which could be used in the development of new compounds. The bis-sulphone (33) was found to be twice as active as dapsone against *M. tuberculosis* [117] and another laboratory bacillus [134], but it is inactive against *M. leprae* [109]. Compound (35) is active against plasmodia species only [135], whilst (38) has been found to be sufficiently contaminated with dapsone [109] to explain its reported activity [87].

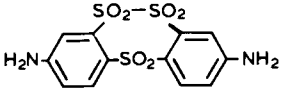
Replacement of the sulphone group (*Table 1.4*), except by sulphoxide [136, 109] or sulphide [109] which are probably metabolized to dapsone, gave inactive compounds. The polysulphone (39) was inactive.



(40)



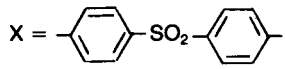
(41)



(39)

Those sulphones (40, 41) in which one 4-aminophenyl ring has been replaced by a heterocyclic ring are inactive, although earlier, compound (41) had been used in the treatment of leprosy [87, 109, 111]. *Table 1.5* lists a series of repository sulphones which are equipotent with DADDS against *M. leprae* and *P. berghei* [90, 137, 138].

Table 1.5. REPOSITORY SULPHONES ACTIVE AGAINST *M. LEPRAE* AND *PLASMODIA spp.* [169]



No.	
53	$AcNHXN{=}CHC_6H_4\text{-}4\text{-}CH{=}NXNHAc$
54	$(H_2NXNHCH{=}CHCH{=}N\text{-}4\text{-}C_6H_4)_2SO_2$
55	$(CH{=}NXN{=}CH\text{-}4\text{-}C_6H_4)_n$
56	$(NHXN{=}CHCH{=}CH)_n\text{-}n$HCl

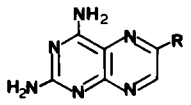
A recent development in this field is the announcement by Russian workers of diuciphon (18a) [139–141]. This compound is claimed to be some 4-times less toxic than dapsone and to be effective in all forms of leprosy, including dapsone-resistant cases. This latter claim is somewhat surprising and, if it is confirmed, suggests that the uracil moiety as well as the dapsone structural unit is responsible for the action of this drug. Certainly, metabolic transformation to dapsone is unlikely, as is *N*-hydroxylation of the exocyclic nitrogen to give an active metabolite [142]. The uracil moiety could serve as an inhibitor of *de novo* uracil synthesis. 6-Methanesulphonyluracil blocks formation of orotic acid ribose monophosphate [143]. Another possible target enzyme is thymidylate synthetase. Compounds reported as salts of dapsone and 6-methyluracil [144, 145] have also been claimed to be active antileprotic drugs, but more careful comparative studies need to be done to substantiate these claims.

Mode of action

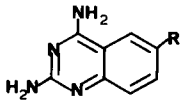
It is generally recognized that dapsone acts primarily as an antimetabolite of *p*-aminobenzoic acid (pAB) and blocks the synthesis of dihydrofolic acids in much the same way as do sulphonamide drugs [3, 109, 111, 130, 170–173]. This is supported by the finding of cross-resistance between dapsone and the long-acting sulphonamides [170–172] and by the partial reversal of the action of dapsone by pAB and its glutamyl derivative, pABG [170, 172].

However, there are interesting differences. In the first place, resistance to dapsone can be overcome by an increase in the dose much smaller than that required for sulphonamides to overcome sulphonamide resistance. The folate synthetase enzyme, therefore, appears to differentiate dapsone from sul-

phonamides [109, 170]. Secondly, dihydrofolate reductase (DHFR) inhibitors are generally inactive against *M. leprae* and are not synergistic with either dapsone [87, 174, 175] or sulphonamides [170, 174]. The diaminopteridines (57) have been found to bind strongly to mycobacterial DHFR, and one compound (57, R = Me_2CHCH_2) is being tested in the mouse foot pad. These compounds, however, have only a weak antimicrobial action, which may be due to poor penetration through the mycobacterial cell wall [176].



(57) R=alkyl, aralkyl



(58) R= a, 3,4-dichlorobenzylamino
b, 2-naphthylsulphonyl
c, 3,4-dichlorophenylacetamido

2,4-Diaminoquinazolines (58) are powerful inhibitors of microbial DHFR's [90] and although (58a) was found to be inactive in earlier studies [170, 174], other derivatives, especially (58b, c) were subsequently claimed to be active [173, 177, 178]. However, synergism with dapsone could not be clearly demonstrated [173, 178] and, surprisingly, low doses of these compounds were claimed to be more effective than higher doses [178]. Generally, inhibitors of DHFRs are presently regarded as less promising antileprotic drug candidates [179]. In contrast, dapsone serves as a classical pAB antimetabolite against plasmodia species and is synergistic with pyrimethamine [90, 146]. It seems, therefore, that the folate synthetase-dihydrofolate reductase pathway in *M. leprae* is unusual in its binding characteristics and/or its accessibility to the drug [109].

4-Aminophenyl-4′-hydroxyaminophenyl sulphone (DDS-NOH, 16), a metabolite of dapsone, shows some antimycobacterial activity [130] and has recently been found to be a potent generator of superoxide and hydrogen peroxide, which are bactericidal to *S. aureus*, and also to improve the metabolic (glucose oxidation) and bactericidal (phagocytosis) activities of polymorphonucleocytes (PMNs) obtained from patients with chronic granulomatous disease [131]. However, dapsone and other sulphones may owe their antimalarial activity, against the intraerythrocytic parasite, to an inhibition of glucose uptake and/or adenosine transport through the red blood cell membrane [180], an effect not antagonised by pAB. The significance of these observations in the treatment of leprosy has yet to be determined, although a glucose oxidase-peroxidase enzyme 'cocktail' has been shown to kill *M. leprae in vitro* [180a]. Dapsone has also been shown to have significant anti-inflam-

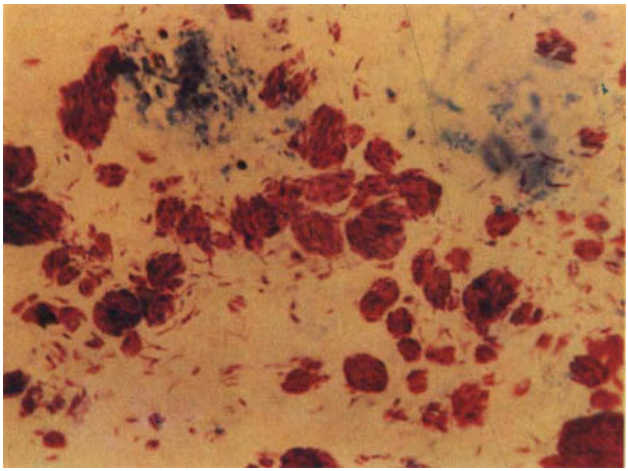


Figure 1.3. Nose blow from lepromatous leprosy patient showing characteristic globi (clumps) of bacilli and some separate organisms Ziehl-Neelsen stain [2].

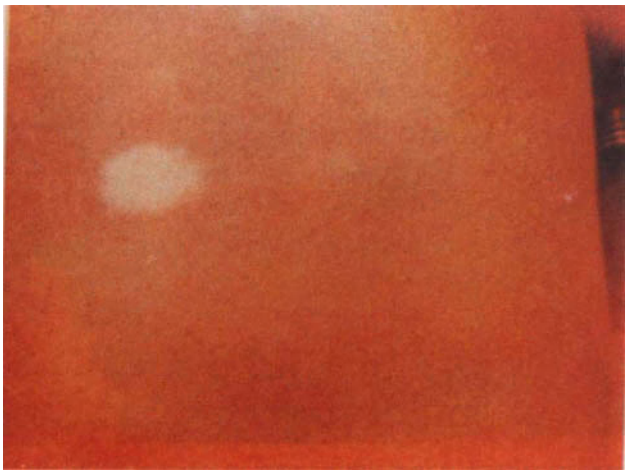


Figure 1.10. Indeterminate leprosy lesion [2].

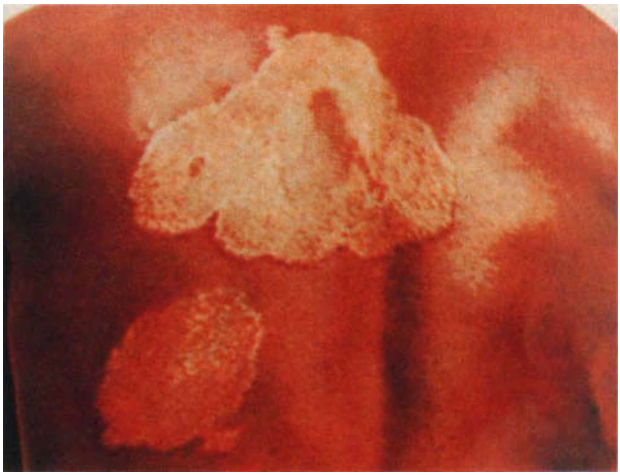


Figure 1.12. Characteristic borderline tuberculoid leprosy lesions with well-defined raised edges and developing central 'immune area' [2].

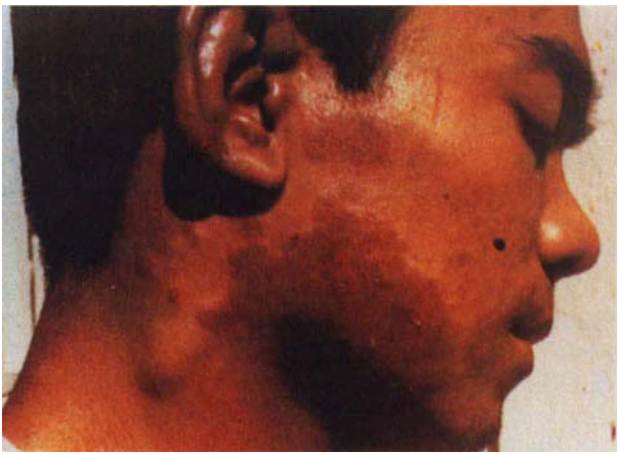


Figure 1.13. Borderline leprosy lesions of ear and cheek with massive enlargement of the great auricular nerve [2].

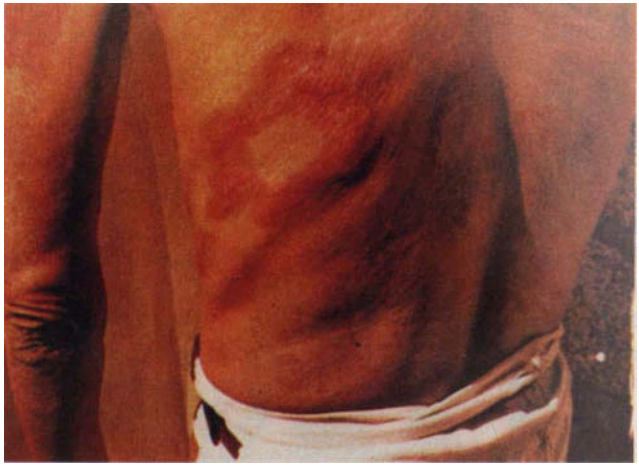
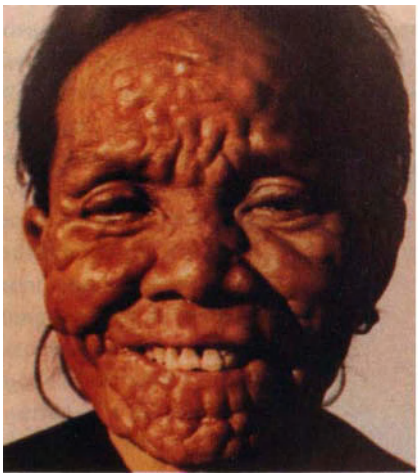


Figure 1.14. Borderline leprosy lesion with central 'immune area' [2].



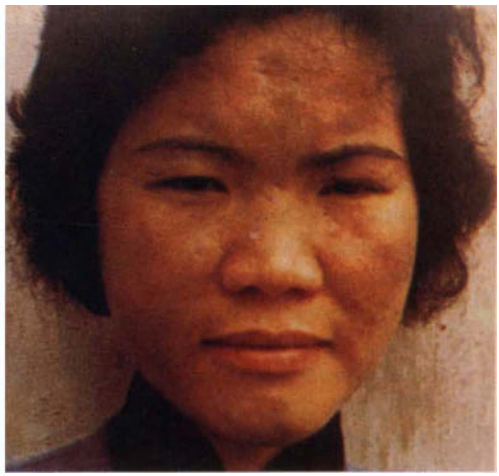


Figure 1.15. (Left) Diffuse lepromatous leprosy with leonine face and extensive nodulation [2].

Figure 1.16. (Right) Diffuse lepromatous leprosy, highly bacilliferous but no nodulation [2].

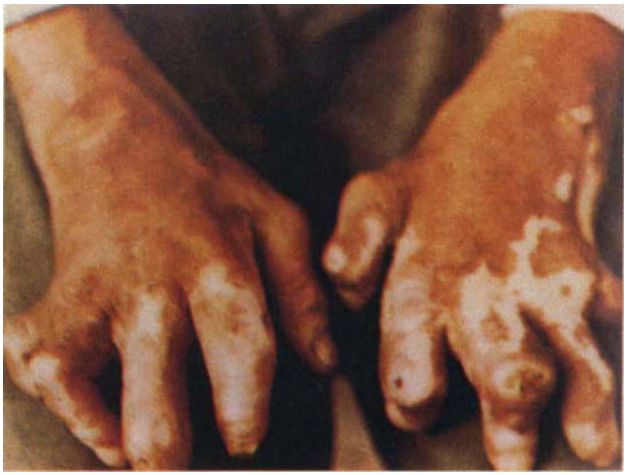


Figure 1.17. Claw hands with contractures and scarring resulting from nerve damage and subsequent scarring [2].

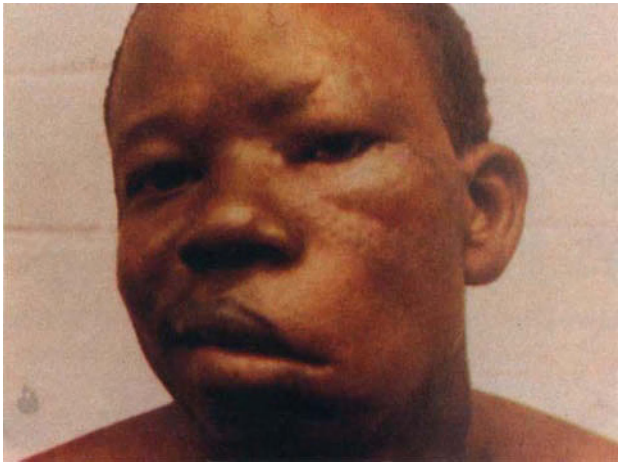


Figure 1.18. Left facial paralysis associated with an extensive tuberculoid lesion and subsequent scarring [2].

matory properties [172, 181, 182] and is being investigated in a variety of disease states involving autoimmune reactions and immune complex formation [172]. Dapsone accumulates in lysosomes and appears to exert a specific membrane-stabilizing effect, thereby inhibiting the release of lysosomal enzymes. This effect is offered as a possible explanation of its action in these diseases [172]. The significance of these findings to the mode of action of dapsone in leprosy requires further investigation. In contrast, dapsone has recently been reported to have immunostimulating properties (increased neutrophil motility) both *in vitro* and *in vivo* [183]. This effect occurs only at high dose levels (100 mg) and is similar to that of levamisole. This immunostimulatory effect may be involved in the pathogenesis of ENL, but the high dose level makes it unlikely [183]. Circulating antibodies to dapsone have recently been detected in sera from some leprosy patients, but these are not thought to be important in ENL [184].

Overall, the action of dapsone may be two-fold, a direct bactericidal action coupled with some modulation(s) of the host-response system. Further work is required to evaluate the significance of these different effects.

The suggestion that dapsone exerts some direct physicochemical effect on membrane function by a nonspecific mechanism reflecting its lipophilicity can be criticized [170]. Nonspecific drug action is associated with relatively high concentrations of a drug [170, 185]. In contrast, the very low concentrations at which dapsone exerts its effects are characteristic of an interaction with a specific target system. The toxic effects of dapsone, which usually arise at high dosage, greater than 10 mg per day, include lysis of erythrocyte membranes, particularly in patients deficient in glucose-6-phosphate dehydrogenase (G-6PD), and may reflect a nonspecific action. However, dapsone is not highly lipophilic, log *P* circa 1.3 (ethylene dichloride) [170], 1.7 (ethyl acetate) [159] and 0.9 (chloroform) [159], and would not be expected to accumulate excessively in membranes. Indeed, the log *P* values are only slightly higher than those of established antitubercular drugs [186], and suggest rapid distribution throughout the body tissues, in agreement with the theoretical, 20-compartment, model examined by Hansch. In this model, transport into the twentieth compartment is most rapid as log *P* approaches zero [187]. Recently, an extensive quantitative structure-activity relationship (QSAR) study of sulphones has been reported [188]. In this study, the best equation obtained for a series of 4-aminophenyl-4′-substituted phenyl sulphones (cf. *Table 1.2*, (19), $R^1 = NH_2$, R^2 = various substituents) against *M. smegmatis* 607 *in vitro* was the following:

$$\log \frac{1}{\text{MIC}} = -1.22R - 0.21f - 2.21 \qquad \begin{matrix} n & r \\ 16 & 0.86 \end{matrix} \qquad (1)$$

MIC being the minimal inhibitory concentration, R, the Swain and Lupton resonance parameter, and f, the hydrophobic fragmental constant associated with each substituent. The equation indicates that antimycobacterial activity is increased by electron-releasing substituents (negative R term) which are also hydrophilic (negative f term). The size of the coefficients of R and f shows that biological activity is very sensitive to changes in R (large coefficient) but less sensitive to changes in f (small coefficient). Strongly electron-releasing substituents would increase the charge density on the sulphone oxygen atoms through conjugation, a factor recognized qualitatively by earlier investigators. The very high activity of dapsone is consistent with the analysis in Equation (1) which indicates that dapsone has the optimal or near-optimal structure for this class of compounds. The equation was used to predict the activity of the 2-hydroxyethylamino compound (24, but $R^2 = NHCH_2CH_2OH$). However, Equation (1) would also suggest that 4-aminophenyl-2-(5-aminothienyl) sulphone (41) might be an active compound. The reported inactivity of this compound may therefore be worth further investigation.

An elegant study of the action of sulphonamides and dapsone in both intact and cell-free systems of *E. coli* has been reported [188a]. These studies show that both dapsone and sulphonamides act as specific antagonists of *p*-aminobenzoic acid [188b, c]. Their activity is due to very strong specific binding to dihydropteroic acid (DHPA) synthetase, which is the key step in their mode of action. 'False' folate analogues of both dapsone and the sulphonamides were formed in cell-free systems, but their formation is not rate-determining. In *M. kansaii*, a similar type of activity has been demonstrated. Furthermore, with *E. coli* a pronounced synergism occurred with dapsone or sulphonamides and a dihydrofolate reductase inhibitor. This study is being extended to *M. lufu* and *M. leprae*. The results are awaited with interest.

Absorption, distribution and metabolism

Dapsone is almost totally absorbed following oral dosage [91, 146]. It is a very weak base, pK_a circa 1.0 [170], and is exclusively present as the neutral molecule, except in the stomach. A study with ^{14}C-labelled dapsone has shown that 90% of the dose is excreted in the urine and 10% in the faeces [159]. The drug is well distributed throughout the body tissues and blood [87, 91, 146, 172] and accumulates in leprous tissue [189, 189a]. It is about 73% bound to blood proteins at a single site [146]. Dapsone is excreted in the biliary circula-

tion. The important pharmacokinetic parameters, which are best described by an open two-compartment model [128, 146], are summarized in *Table 1.6*. The disposition of the drug is altered by pyrimethamine, which displaces dapsone from its plasma protein binding site, thereby increasing the volume of distribution and decreasing the maximum plasma levels [146]. An important therapeutic indicator is the ratio of peak serum levels to the minimal inhibitory concen-

Table 1.6. PHARMACOKINETIC PARAMETERS OF DAPSONE [146]

Parameters	*Value*
$t_{1/2}$ (h) (half-life)	27.5 ± 0.33
Cl (ml h^{-1} kg^{-1})	38.4 ± 10.9
V (kg^{-1})	1.53 ± 0.52
C_{max} (μg ml^{-})	1.875 ± 0.188

Table 1.7. MINIMAL INHIBITORY CONCENTRATION (MIC) AGAINST *M. LEPRAE*, PEAK SERUM CONCENTRATIONS, DURATION OF COVERAGE AND BACTERICIDAL ACTIVITIES OF CURRENT ANTILEPROSY DRUGS [109]

Drug	*MIC (μg/ml)*	*Dosage (mg)*	*Ratio peak serum MIC*[a]	*Duration for which serum concns. exceed MIC (days)*[b]	*Bactericidal activity*[c]
Rifampicin	0.3	600	30	1	+++
Dapsone	0.003	100	500	10	+
Acedapsone	0.003[d]	225	15	200	–
Ethionamide	0.05	500	60	1	++
Prothionamide	0.05	500	60	1	++
Thiacetazone	0.2	150	8	2	–
Sulphamethoxypyridazine[e]	30	1000	3	3	–
Thiambutosine	0.5	1500	1	<1	–

[a] Ratio of peak serum concentration in man after a single dose to MIC determined in the mouse.

[b] Serum concentrations in man after a single dose.

[c] Purely bacteriostatic; +, ++, +++, relative degrees of bactericidal activity.

[d] Acedapsone is inactive against *M. leprae* but is converted to dapsone – the figures for MIC and peak serum concentration refer to the value for dapsone.

[e] Cross-resistant with dapsone.

trations (MIC) of a drug; for dapsone, this is 500. This figure demonstrates the outstanding efficacy of dapsone compared with other antileprotic drugs. A comparison of various therapeutic data, for the most widely used antileprotic drugs, is given in *Table 1.7* [190].

The metabolism of dapsone has been studied by several groups, but many metabolites have yet to be unequivocally identified. About 15% of the total dose is excreted in urine [191] and only a small amount of *N*-acetylation occurs. Dapsone is polymorphically acetylated in man, but this does not modify the pharmacokinetic parameters [146]; monoacetyldapsone (MADDS, 2; *Table 1.1*) is a repository form of dapsone. The major metabolite is *N*-hydroxydapsone (16, *Table 1.1*) which is probably excreted as both the *N*- and *O*-glucuronides together with other conjugates [120, 159, 192]. A recent and extensive study has shown that up to 33% of the total dose may be excreted as *N*-hydroxydapsone or one of its derivatives. A summary of the various metabolic transformations is given in *Figure 1.21*. In the rat, dapsone inhibits drug metabolism affecting both cytochrome *P*-448 and *P*-450 enzymes [192a].

Side-effects

Numerous toxicological side-effects have been described, including gastrointestinal irritation, haemolysis [192b], especially in G-6PD-deficient patients, which is occassionally severe, leading to anaemia, hepatitis, dermatitis, nausea and vomiting, reversible peripheral neuropathy, optic atrophy [192c], rashes,

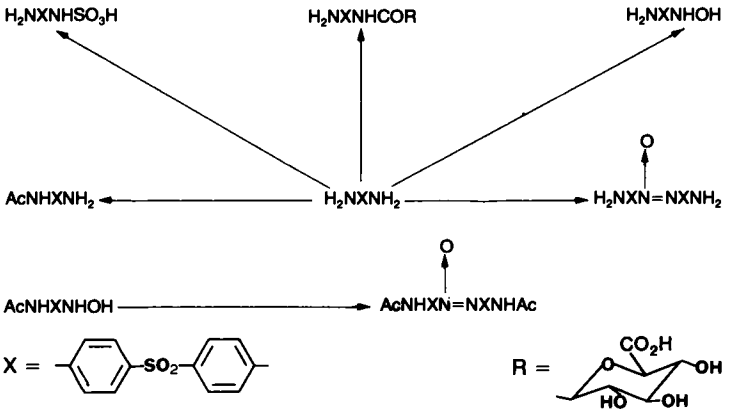


Figure 1.21. A summary of the major metabolic routes for dapsone [191, 192].

drug fever and, debatably, even psychosis [94, 96, 192d]. The involvement of dapsone in ENL and reversal reactions is contentious, but these now appear to be a consequence of the disease process rather than a direct effect of therapy. However, drug therapy may accelerate these reactional states [61, 193]. A major study has been made of the methaemoglobinaemia associated with dapsone treatment [157, 158]. A mechanism involving *N*-hydroxydapsone has been proposed (*Figure 1.22*), and a proposal that it may be carcinogenic [158] has been investigated. High doses of dapsone over the whole lifetime of an animal are reported to be strongly [194] or weakly [195] carcinogenic in rats, but dapsone and metabolites (1–3, 16, 17, *Table 1.1*) did not give positive Ames test, although both the sulphoxide (42) and the sulphide (44) in activated systems were mutagenic [196]. Dapsone was found not to modify the activity of other chemical carcinogens [195] and an epidemiological study has shown that leprosy patients exhibit a lower incidence of cancer than does the control population [196]. Despite this extensive catalogue of dangerous side-effects, only three deaths have been reported over 17 years' monitoring in the United Kingdom [196a].

SULPHONAMIDES

Early workers found that sulphonamides had little or no activity against leprosy [111, 136]. Among a large number of compounds prepared at this time were

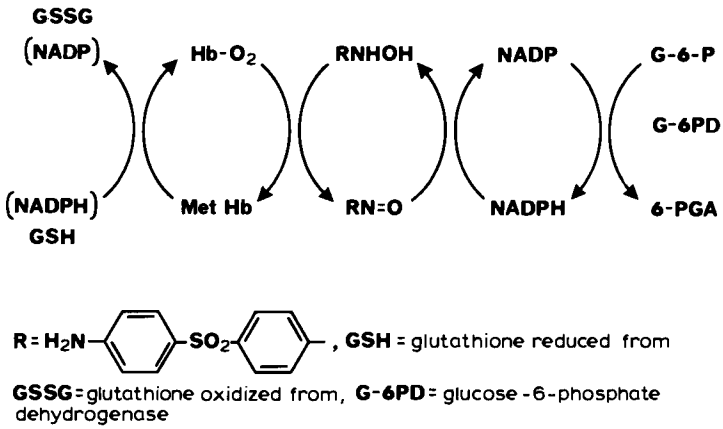


Figure 1.22. A scheme illustrating the way in which methaemoglobin formation is coupled with the oxidation of N*-hydroxydapsone (adapted from Refs. 157, 158).*

the N^4- and N^1-chaulmoogroyl and the N^1-hydnocarpyl derivatives [197]. These compounds were inactive for reasons which are now obvious. The introduction of long-acting sulphonamides, which bind strongly to plasma proteins, led to the identification of a number of these compounds as potential antileprotic drugs (*Table 1.8*). However, even the best compounds are about 10,000-times less active than dapsone [91] (*Tables 1.7* and *1.8*). Furthermore, the cost and toxicity of these compounds is much greater than dapsone and they evoke cross-resistance to dapsone. Their use in therapy can no longer be justified [89]. The recent QSAR study [188a] mentioned above clearly shows that MIC values for a number of new sulphanilamides correlate significantly with pK_a values, Equation (2):

$$\log \mathrm{MIC}_{o,m,p} = 0.67pK_a - 0.24D - 4.74 \qquad n = 18 \quad r = 0.95 \qquad (2)$$

Table 1.8. SULPHONAMIDES WITH ANTILEPROTIC ACTIVITY

$4\text{-}N^4H_2C_6H_4SO_2N^1HR$

Activity is expressed: (a) as % w/w in mouse chow; (b) as MIC μg/ml in mouse footpad.

R	*Activity*		*References*
	a	*b*	
6-Methoxy-3-pyrazinyl (sulphamethoxypyrazine) its N^4-acetyl derivative	0.01	30	87, 136, 170, 179, 198
2,6-Dimethoxy-4-pyrimidyl (sulphadimethoxine)	0.01	20	91, 109, 111, 136, 170, 199
5-Methoxy-2-pyrimidyl (sulphamethoxydiazine)			111, 136
5,6-Dimethoxy-4-pyrimidyl (sulphadoxine)	0.01–0.04	35	87, 91, 109, 170, 200–202
1-Phenyl-5-pyrazolyl (sulphaphenazole)			111, 136
4,5-Dimethyl-2-isoxazolyl (sulphamoxole)	0.1–1.0		174

$MIC_{o,m,p}$ = minimal inhibitory concentration, mol l^{-1}, for a range of sulphanilamides derived from *o*-, *m*-, and *p*-substituted anilines; D = a dummy parameter having values of 1 for *o*-substituted and zero for *m*- and *p*-substituted derivatives.

A similar equation was obtained in cell-free systems for the inhibition of DHPA synthesis, Equation (3):

$$\log i_{50,o,m,p} = 0.46pK_a - 0.33D - 2.47 \qquad \begin{matrix} n & r \\ 18 & 0.97 \end{matrix} \qquad (3)$$

$i_{50,o,m,p}$ = concentration, mol l^{-1}, causing a 50% inhibition of DHPA synthesis. These equations indicate that these sulphonamides exert their antibacterial effect by inhibiting folate synthesis. Furthermore, they demonstrate that biological activity increases with increasing acidity of the sulphanilamide. Some compounds which were very strongly ionized, to over 90%, were much more active in cell-free systems, which suggests that transfer into the bacterial cell may be a limiting factor with some of these compounds. The results from the projected studies with mycobacterial systems are awaited with interest. Sulphonamides may yet prove to be useful therapeutic agents in leprosy.

IMINOPHENAZINES

These compounds are derivatives of 2-anilino-5-phenyl-3,5-dihydro-3-iminophenazines (*Figure 1.23* and *Table 1.9*). They are frequently described as riminophenazines or rimino compounds (rimino being a contraction of R-imino [3, 203]). The most important compound in the series is clofazimine, B663 (66i, *Table 1.9*) which is widely used in the treatment of dapsone-resistant leprosy. The story of the development of these compounds, which is a fascinating combination of science and serendipity, has been well told by the late V.C. Barry [203], who began his comprehensive studies in 1948. The interesting synthetic routes to iminophenazines are outlined in *Figure 1.23*. The oxidative coupling of 2-aminodiphenylamines (59) gives substituted phenazines (60, 61). The ratio of these two isomeric compounds varies with the nature of the oxidizing agent; with ferric chloride, (60) predominates, whereas with *p*-benzoquinone the yield of (61) is greatly increased [203]. When the reaction is carried out in the presence of a carbonyl compound, with benzoquinone as the oxidizing agent, imidazolophenazines (62) are formed. These undergo catalytic reduction and subsequent re-oxidation to give substituents on the nitrogen atoms at positions 2 and 3 (63). A second route involving the coupling of 2-aminodiphenylamines (59) with substituted quinoneimines (64) makes avail-

able somewhat differently substituted iminophenazines (65) (see also *Table 1.9*). These difficult and expensive syntheses, which gave only moderate yields, were a major obstacle to the development of the iminophenazines as, first, antitubercular, and subsequently, antileprotic compounds.

The first compounds (66a, b) were very active *in vitro* but showed reduced activity *in vivo* and were found to be highly toxic. The introduction of chlorine atoms into the molecule greatly increased the *in vivo* activity, especially when linked with the introduction of lipophilic alkyl substituents on the imino nitrogen atom; compare (66c, d) with (66i–k). Clofazimine (66i) was selected as the best compound for further evaluation and its activity against *M. lepraemurium* [204] led in 1962 to its introduction for the treatment of leprosy [136, 205].

Subsequently, clofazimine has been found to be 10–100-times more active against *M. leprae* (MIC 0.1–1.0 mg/kg) than against *M. tuberculosis* [206]. In mice, the drug has a pronounced bacteriostatic action [206–208] at concentrations of 0.0001–0.001% in mouse chow [206, 207, 209, 210]. Clofazimine is now established, with rifampicin, as a front-line drug in the treatment of dapsone-resistant leprosy. A second important property of clofazimine, rapidly recognized by leprologists, is its pronounced anti-inflammatory action, which is valuable in controlling ENL whilst continuing effective chemotherapy [210]. Although it was recommended for the treatment of reversal reactions [210],

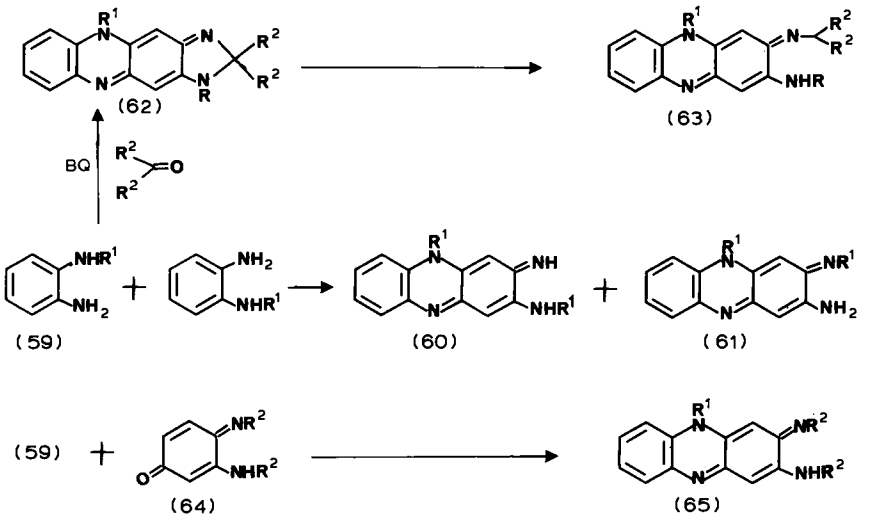
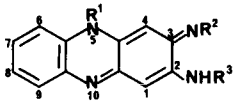


Figure 1.23. Synthetic routes to substituted iminophenazines. BQ = p-*benzoquinone.*

Table 1.9. SOME ANTITUBERCULAR AND ANTILEPROTIC IMINOPHENAZINES [203]



No.	*(code)*	R^1	R^2	R^3	*Activity comments*
66a	(B283)	Ph	H	Ph	High *in vitro* activity with moderate *in vivo* activity: toxic
66b	(B595)	Ph	Ph	H	
66c	(B628)	4-ClPh	H	4-ClPh	Very high in vivo activity
66d	(B629)	4-ClPh	4-ClPh	H	High *in vivo* activity
66e	(B670)	Ph	$CHMe_2$	Ph	low[a]
66f	(B775)	Ph	CH_2CHMe_2	Ph	moderate[a]
66g	(B669)	Ph	C_6H_{11}	Ph	
66h	(B720)	Ph	$(CH_2)_2NEt_2$	Ph	low[a]
66i	(B663)	4-ClPh	$CHMe_2$	4-ClPh	very high[a]
66j	(B673)	4-ClPh	C_6H_{11}	4-ClPh	very high[a]
66k	(B749)	4-ClPh	$(CH_2)_2NEt_2$	4-ClPh	moderate[a]
66l	(B710)	Ph	Ph	Ph	low[a]
66m	(B1912)	Ph	C_6H_{11}	Ph	very high[a] 8-chloro substituent

[a] *In vivo* activity.

recent studies show that it is of doubtful value [211] or possibly exacerbates the condition [212]. The conclusions of a 10-year study (1965–75) of clofazimine in the treatment of leprosy show that doses ranging from 100 mg twice a week to 100 mg four times a day are acceptable. The higher doses are used to control reactional states. No resistant organisms have emerged and clofazimine alone is claimed to be as effective as dapsone-plus-clofazimine therapy [210, 213]. However, persister organisms remain uneffected, even after 10 years therapy [44–46, 214].

Structure-activity relationships

Three main areas of molecular modification have been explored [203]. In the first, the structure of clofazimine (66i) was varied by introducing additional chlorine atoms into the molecules at positions 1, 6, 7 and 8. The result was loss in activity, except for the 8-chloro derivative, which was equipotent with

clofazimine. The second series was based on the triaryl analogue (66l). A variety of derivatives with a chloro- or methoxy substituent in various positions were examined, but showed, at best, only modest activity. Among these compounds, two of the most active, (66, 7-OMe, R^1 = 4-$MeOC_6H_4$, R^2 = R^3 = Ph, and 7-OMe, R^1 = Ph, R^2 = R^3 = 4-$MeOC_6H_4$) did not contain any chlorine atoms. The third molecular modification involved variations in R^2 coupled with changes in R^1 and R^3 and the introduction of various substituents at positions 6 and 8. Generally, R^2 had to be alkyl or, better, cycloalkyl and R^1, R^3 aryl or substituted aryl for optimum activity, compare (66g and i) and (66e and i). When hydrophilic salt-forming groups were introduced at R^2 (66h, k), activity was much reduced. However, when an 8-chloro substituent (66m) was introduced into the moderately active (66g), a dramatic increase in potency occurred. This latter compound, also known as B1912, has been widely investigated and found to be equipotent with clofazimine in mice [207]. Interestingly, its tissue levels are lower and serum levels higher than those of clofazimine, despite its estimated greater lipophilicity (see below for further comments). Four further analogues (66f, g, h, k) have recently been examined [215]; three of them (66f, g, k) had one-tenth the activity of clofazimine, whilst the other (66h) was about 100-times less potent. Rather surprisingly, the more hydrophilic compounds (66h, k), which would be expected to be excreted more rapidly, had much the longest half-lives, these being similar to that of clofazimine.

Mode of action

The earliest studies [216] showed that the drug is strongly adsorbed by micro-organisms, but how much of the drug penetrates the bacterial cell is not clear. Under anaerobic conditions, clofazimine is decolourized by micro-organisms, but the colour is rapidly restored in the presence of oxygen. This reversible redox system (*Figure 1.24*) involving the *p*-quinonoid system has a redox potential of approximately 0.18 V and is capable of diverting up to 20% of respiratory hydrogen. This effect alone is not enough to account for its powerful bacteriostatic properties. However, hydrogen peroxide formed during the oxidative regeneration of (66i) would cause severe damage if the reaction occurred, to any marked extent, within the cell (*Figure 1.24*). In agreement with these proposals, catalase-deficient *M. tuberculosis* species and catalase-containing organisms in the presence of azide (a catalase inhibitor) were found to be more susceptible to clofazimine. Furthermore, in semi-solid media, inhibition of growth of *M. tuberculosis* was more effective at the surface (high oxygen tension) than at some distance below the surface (lower oxygen tension) [216].

More recently [217–220], extensive studies with clofazimine (66i) and (66m) have shown that both compounds interact strongly and selectively with deoxyribonucleic acids from various sources (mycobacterial, other bacteria, yeast, human, synthetic poly(G)). The reaction is specific for guanine in single-stranded DNA and guanine-cytosine (G-C) pairs in double-stranded DNA. Binding is thought to involve the 2-amino group of guanine [217], presumably through hydrogen bonding to the cation of clofazimine (pK_a 8.35) which would be expected to be the predominant form (90%) at pH 7.4, and is associated with a red shift of the 495 nm band of the compound [218].

Intercalation is probably not involved, since clofazimine, unlike intercalating drugs such as hycanthone, does not displace methyl green from DNA-dye complexes [218]. Furthermore, models show that the 4-chlorophenyl ring at position 5 is not co-planar with the phenazine ring and would thereby prevent intercalation [217]. In double-stranded DNA, binding to guanine results in the disruption of DNA template function [217, 221], causing inhibition of RNA formation and subsequent inhibition of protein synthesis. In yeasts, there is cross-resistance with chlorimipramine [221], a known inhibitor of mitochondrial function. DNA polymerase repair and excision enzymes in *E. coli* are not affected [217]. Binding of clofazimine, as measured by the extent of the red shift, increases as the proportion of G-C in the DNA increases. It is possible that the higher G-C content of mycobacterial DNA provides a basis for the selective action of the drug against *M. leprae*. On the other hand,

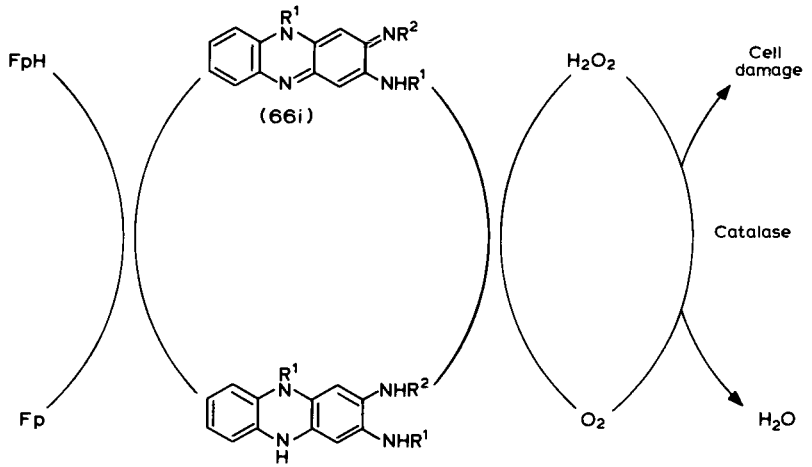


Figure 1.24. The generation of hydrogen peroxide by the oxidation of clofazimine.

selectivity may be due to specific carrier molecules on the macrophages which first bind the drug before both drug and carrier molecule undergo phagocytosis, followed by phagolysosomal digestion which results in the intracellular liberation of clofazimine in the cell containing the leprosy bacillus, i.e., clofazimine is a lysosomotrophic chemotherapeutic agent [218, 222].

Although clofazimine is not mutagenic in the Ames test, it does show antimitotic effects in cell cultures of dividing skin fibroblasts, but it seems unlikely that this is related to its antileprotic activity [218].

On the other hand, (66m) binds to DNA 4–6-times more strongly than does clofazimine and it is a powerful metaphase inhibitor in fibroblast cultures [219]. It also crosses the placental barrier and shows extensive nucleotoxic effects in pregnant mice [220]. Although other workers could not obtain similar results [223], an explanation of these discrepancies has been offered [224] and it seems prudent to undertake any further development of (66a) with extensive preclinical studies [224].

Absorption, distribution, metabolism

Clofazimine is an extraordinary chemotherapeutic compound. It is probably the most lipophilic drug administered orally to man, with an estimated log P of 7.45. However, the reported pK_a of 8.35 indicates that it is 90% ionized under physiological conditions. Other workers [221] suggest it is less strongly basic. The earliest studies in mice revealed a very variable therapeutic effect which was associated with irregular absorption from the gut. A micronized form of the drug gave more regular and complete absorption [203]. The dose in man varies with the aims of therapy and professional judgment, but a common dosage regiment would vary from 100 mg (chemotherapy) to 400 mg (chemotherapy and control of ENL) daily of a microcrystalline suspension of the drug in an oil-wax base presented as a capsule [210]. Even with the control of formulation, the amount of drug absorbed and other pharmacokinetic parameters remain very variable [206, 210, 213, 225] depending on the individual patient (or volunteer) variations, the dosages, and the extent of therapy. A two-compartment model has been proposed [213] to accommodate these observed variations; with short-term, low-dosage regimens $t_{1/2}$ is 7 days but with long-term, high-dosage regimens $t_{1/2}$ is 70 days [206, 210, 213, 225]. Undoubtedly, a large proportion of the dose is recovered in the faeces, figures varying from 4% to 74% depending on the particular investigation [206], but in patients, up to 33% [210], 4%–33% [206] or 50% [225] is recovered. No drug is detected in the urine in the first 24 h and then only low levels equivalent to not more than 0.1% of the dose are detectable over 24 h [206, 225]. Plasma

levels also remain low 0.2–0.5 μg/ml [206, 210, 225], but increase with the dose and at 300 mg/day are of the order of 1.0–3.0 μg/ml [206, 210, 225]. All these observations indicate a substantial accumulation of the drug by patients. It has been estimated that a patient receiving 100 mg/day on continuous therapy will accumulate up to 10 g of clofazimine [206]. The drug initially remains concentrated in the intestinal mucosa but slowly distributes itself throughout the body and accumulates at various sites, particularly the skin, gastrointestinal tract, spleen, liver, reticuloendothelial cells, kidney and pancreas [210, 213]. The drug occurs as crystalline desposits in various cells, particularly macrophages [203] during long-term therapy and causes increased pigmentation of the skin and the cornea [210, 213, 226]. Clofazimine has been detected in lymph nodes almost 4 years after the drug has been withdrawn [227], and has recently been identified as an intraneural deposit [227a]. The drug does not penetrate the blood-brain barrier but crosses the placenta, causing pigmentation of the foetus. It is reported not to be teratogenic [210].

All the above studies have been concerned with measurements involving the unchanged drug. Recently, three major pigmented urinary metabolites have been identified, but details of their structure are not yet available. They are not mutagenic [228].

Side-effects

The useful anti-inflammatory effects of clofazimine already referred to are described in a recent study as immuno-inhibitory and may be the basis of a possibly beneficial pharmacological interaction with the immunostimulant dapsone in the treatment of ENL [228a].

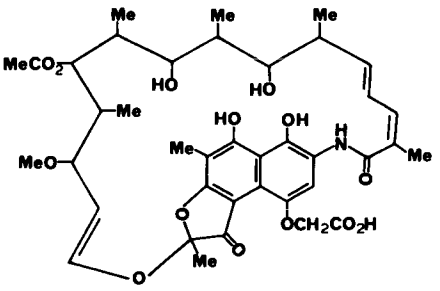
The unusual properties of clofazimine still make the monitoring of side-effects a matter of concern [229]. Undoubtedly, the major side-effect is 'clofazimine small bowel', which occurs in a high proportion (30–48%) of patients on long-term therapy receiving more than 100 mg/day of the drug [213, 230]. The condition appears to be associated with the deposition of the drug in the intestinal mucosa, particularly the ileum. X-ray studies [213, 230, 231] show oedema and effacement of the mucosa. Reduction of the dose leads to a return to normal small bowel structure. Other gastrointestinal disturbances (diarrhoea) and abdominal pain are also reported and have given rise to unnecessary surgical intervention [232]. One unusual case of eosinophilic enteritis has also been reported [233]. Another report associates the severe abdominal pains and subsequent extensive tissue damage as the possible cause of death in some patients [234], but other workers report no fatalities with the drug [230]. Another important side-effect is increased skin pigmentation [213,

230, 234], which may involve increased melanin formation as well as drug deposition [213]. This effect, whilst mainly cosmetic in the early stages, may lead to ichthyosis with extensive scaling of the skin (snake or fish skin appearances) which requires topical treatment [213, 234]. Phototoxicity has also been observed with the drug. The cornea is a common site of drug deposition and corneal xerosis also occurs. A recent study indicates that eye damage does not occur due to drug deposition [226]. The importance of other reported side-effects, such as increased fasting blood-sugar levels [213], has yet to be established. An incidental and surprising observation is that isoniazid reduces the severity of clofazimine side-effects [234], but this has not been further investigated.

Despite the many possible undesirable side-effects of clofazimine, a recent 12-year (1966–78) review of 31 cases expresses the opinion that no other drug currently available for the treatment of leprosy could have achieved such good results [234a].

RIFAMYCINS

These are natural and semi-synthetic compounds, belonging to the ansamycin group of antibiotics which possess an aromatic ring system spanned by an aliphatic chain. The final steps in the total synthesis of rifamycin have been reported [235]. Their chemical, biological and clinical properties have been the subject of a number of recent reviews [3, 235–239]. The natural compounds are obtained as metabolites from the fermentation broths of *Nocardia mediterranea*, a soil organism originally classified as *Streptomyces mediterranei*. In the presence of sodium barbitone, rifamycin B (67) is produced almost exclusively and can be isolated as a pure crystalline compound [3, 237] from which a large variety of semi-synthetic derivatives may be prepared [3, 237–239]. The more



(67)

important synthetic routes are outlined in *Figure 1.25*. Many rifamycins have a potent bactericidal action against mycobacteria, many Gram-positive and some Gram-negative bacteria. At higher doses, some also show antiviral and antineoplastic activity [236–238]. From the hundreds of semi-synthetic derivatives that have been investigated, rifampicin (68) has emerged as a major chemotherapeutic agent, particularly for the treatment of tuberculosis and leprosy. It has a powerful bactericidal action coupled with good pharmacokinetic properties [3, 96, 236a, 239]. Rifamycin SV (69, R=H) was first used to treat leprosy [240], but its unpredictable absorption from the gut, necessitating parenteral administration, coupled with its somewhat lower *in vivo* activity, made it an unsatisfactory compound for wide-scale use [136]. Shortly afterwards, rifampicin was first used to treat leprosy [241]. The results of a carefully controlled 9-year (1968–77) study of the use of rifampicin in the treatment of lepromatous leprosy [242] show that it has a unique, rapid bactericidal action which causes a dramatic fall in the MI (and thereby infectivity) within 14 days and is accompanied by marked clinical improvement. There was no concomitant increase in the clearance of dead bacilli, and ENL was somewhat more common than usual (but probably not significantly so) in the patients in the study. Persister organisms, at first thought to be reduced to undetectable

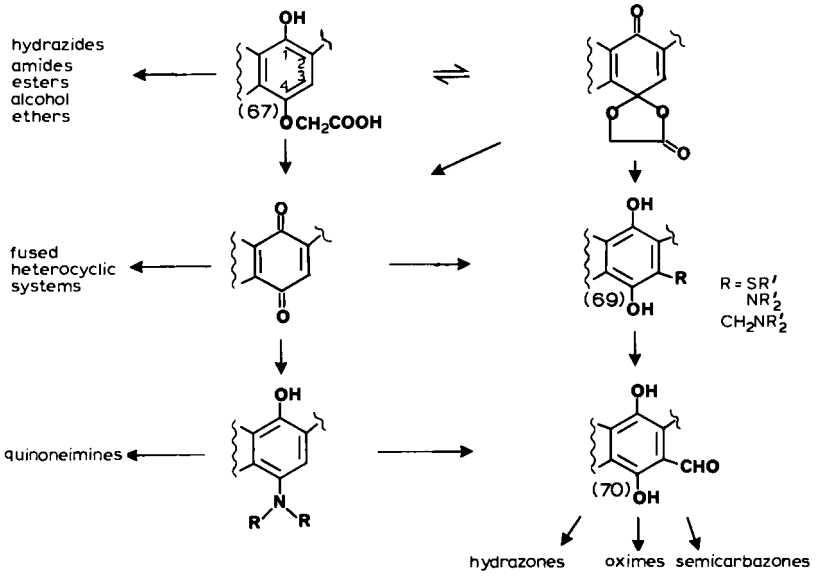


Figure 1.25. The major synthetic routes to rifamycin derivatives.

levels, were found to be present even after 5 years of continuous therapy. Although resistant strains of *M. leprae* did not emerge during this study, previous workers had expressed concern about the development of resistance. In 1976 resistant organisms had been isolated and positively identified in the mouse foot pad [243]. The resistance is of the single-step type [238, 243] and has occurred as little as 3 years after the commencement of monotherapy [98, 214]; the multiple-step resistance associated with dapsone therapy [243] appears to develop more slowly [244]. In combination with other antileprotic drugs [241], rifampicin may be given continuously or, in larger doses, intermittently [245, 246]. An initial report on the use of rifampicin in paucibacillary leprosy is less encouraging [247].

Structure-activity studies

The hundreds of rifamycins that have been synthesized and examined for a variety of biological actions cannot be fully summarized here; an extensive review has appeared [237]. The minimal requirements for activity are as follows: the presence of two polar groups (either hydroxyl and hydroxyl, or carbonyl and hydroxyl) at the 1 and 8 positions of the naphthalene ring; two free hydroxyl groups at positions 21 and 23 in the ansa chain together with a conformation of this chain which holds these four sites in a particular relationship [3, 237]. The postulated binding of rifamycins to DNA-dependent RNA polymerase involves extensive hydrogen bonding at these four sites and is consistent with an area of bulk tolerance [133] at the 3,4-positions, which are the major areas for the introduction of substituents into the rifamycin molecule. The introduction of a free carboxyl group into the rifamycin molecule gives compounds which are inactive because they cannot penetrate the bacterial cell membrane although they can bind with the isolated polymerase enzyme [3, 237]. The major variations in the semi-synthetic rifamycins are summarized in *Figure 1.26*. Many compounds were found to retain activity against mycobacteria, but not infrequently activity *in vitro* was much greater than *in vivo*. Irregular absorption following oral dosage in mice was not uncommon. No simple relationship between structural changes and antimycobacterial activity was apparent [237, 238]. Some long-acting rifamycin derivatives of undisclosed structure are being investigated and are among the more promising potential new drugs [214, 248].

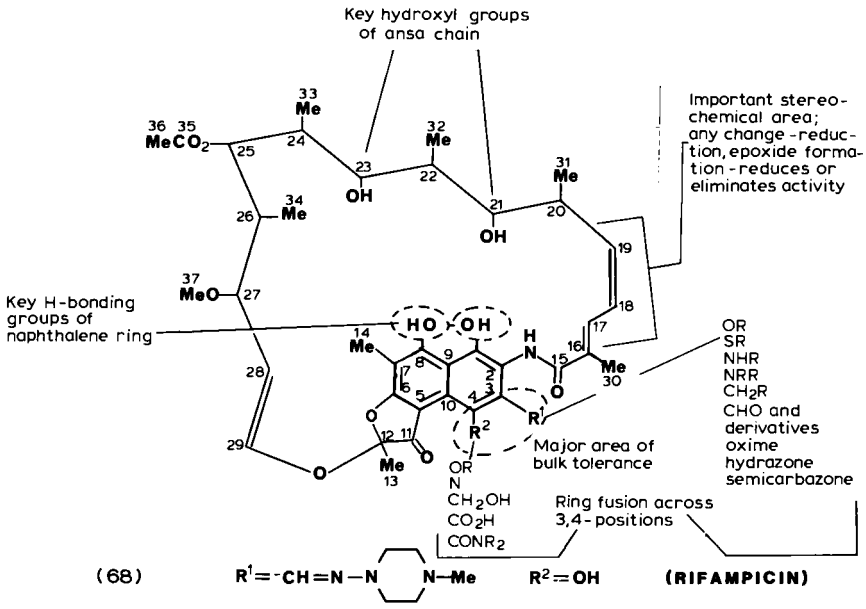


Figure 1.26. Summary of the major structure-activity relationships in rifamycins.

Mode of action

It is generally agreed that the bactericidal effects of rifampicin (and other rifamycins) are a result of specific, selective and strong but reversible binding to DNA-dependent RNA polymerase. This enzyme blockade rapidly inhibits binding to DNA and subsequent initiation of the messenger RNA chain, causing a pronounced inhibition of protein synthesis [3, 96, 236, 238]. The action on the bacterial enzymes is highly selective and occurs *in vitro* at concentrations of 0.01 μg/ml; the mammalian enzyme is unaffected at concentrations 10,000-times greater. The specific site of binding has been shown to be the β-subunit of the polymerase enzyme of *E. coli* and is greatest when it is incorporated in the holoenzyme. This observation suggests that the conformational changes in this unit on assembly of the enzyme are important in drug action [238]. The β-unit is modified in resistant bacteria by a single-step mutation leading to replacement of a single amino acid residue. The resistant enzyme-rifampicin complex has a greatly reduced stability [238] which accounts for the loss of response to the antibiotic. Rifampicin has been shown to enter the cells of both susceptible and resistant bacteria to the same extent [3].

The reaction of rifamycins with DNA and RNA polymerases from other sources is thought to be nonspecific in nature and is associated with increasing lipophilicity of the substituent groups, particularly in the 3-position [237, 238].

Absorption, distribution, metabolism

Almost all the available data come from studies with rifampicin, for which the more important physicochemical properties have been reported [239]. The molecule is zwitterionic, with an isoelectric point of 4.8. The ionization constants are solvent-dependent: in water $pK_{a1} = 1.7$ (proton loss from the 8-hydroxyl group) and $pK_{a2} = 7.9$ (proton gain at piperazine N-4). The 8-hydroxyl group is surprisingly acidic, but no explanation has been offered, although intramolecular hydrogen bonding may play an important part. The partition constant has been measured in a number of systems with log $P \approx 1.0$ except at pH values above 7.5; log $P = 1.19$ (*n*-octanol buffer, pH 7.4), 0.99 (benzene buffer, pH 5.5–7.0), 0.95 (benzene buffer, pH 7.5), 0.66 (benzene buffer, pH 8.0). More frequently, reverse-phase chromatography has been used to measure the relative lipophilicities of a variety of rifamycins.

A variety of dosage regimens have been used with rifampicin, e.g., 300, 450 and 600 mg daily or 900, 1200 mg weekly or monthly in a single dose either alone or in combination with other drugs [214, 236a, 245, 246]. In man, the drug is rapidly absorbed following oral administration and is widely distributed throughout the body [236a, 239]. It enters the skin and peripheral nerves [249] but the highest level is found in bile, liver and kidney-ureter [239]. Peak serum concentrations occur about 2 h after administration of the drug and exceed the MIC (0.3 μg/ml) for about 24 h after a dose of 600 mg (*Table 1.7*). The half-life ($t_{1/2}$) is 3 h, although the drug is about 75% bound to plasma proteins [236b, 239] and rifampicin (but not its major metabolite) is reabsorbed via the enterohepatic circulation [236a]. The drug is excreted in about equal amounts in the urine and faeces, although the reported recoveries of radiolabelled material vary considerably (48 to 94%) [236a, 239]. The drug crosses the placental barrier, and the blood-brain barrier when the meninges are inflamed. Rifampicin has therefore been used in the treatment of meningitis, but its value has been questioned [96, 236a].

The major metabolite is the more hydrophilic 25-desacetylrifampicin, which has about 25% of the activity of rifampicin [236a, 239, 250] and is rapidly excreted in the urine. Other metabolites are the quinone derivatives of rifampicin and 25-desacetylrifampicin, which are equiactive with the corresponding reduced compounds [250]. The oxidation potential of rifampicin has been determined polarographically, $E_{1/2} = +0.10$ V against the standard calomel

electrode, E = 0.43 V, and indicates that the oxidation may occur either biologically or by atmospheric oxidation [235, 239]. 3-Formylrifamycin (70) has been identified as an inactive metabolite [239].

Side-effects

Rifampicin is a very potent inducer of liver microsomal enzymes and modifies the therapeutic response to other drugs; in particular, dapsone in combination therapy and prednisone-prednisolone in the treatment of ENL are more rapidly metabolized [251, 252]. High levels of hydrazine, a potent carcinogen and mutagen, have been reported in the plasma of patients on combined isoniazid-rifampicin therapy, particularly those which are slow acetylators [252a]. A great variety of toxic symptoms involving important disturbances of the blood, gastrointestinal tract, liver and kidneys have been reported. Rifampicin should be regarded as a potential hepatotoxic agent [236a, 253, 254]. A specific toxic effect on liver enzymes could be the cause of the reported 70% fall in plasma 25-hydroxycholecalciferol, which occurred during a 2-week treatment period and may represent the earliest biochemical lesion in a drug-induced osteomalacia [255]. Rifampicin-dependent antibodies can be detected in many patients and are thought to be associated with some side-effects, particularly rifampicin 'flu' syndrome and renal failure which are most common with intermittent dosage regimens [236a, 256, 257]. Pseudomembranous colitis [258], a very serious condition, has been associated with rifampicin therapy, but the association has been disputed. Rifampicin also has pronounced immunosuppressant properties [96, 236a]. Five different rifampicins were examined for their antibacterial and immunosuppressant properties. There was no correlation between these two biological actions [258a]. The number of serious and even potentially fatal side-effects stresses the need for the most careful clinical supervision and monitoring of rifampicin therapy which was advocated at a recent meeting of ILEP [259].

SECOND-LINE DRUGS

Chemically these belong to three different groups, thioureas (thiambutosine, thiocarlide), thiosemicarbazones (thiacetazone) and thioamides (prothionamides, ethionamide), which encompass a great diversity of biological activities. They all emerged from extensive and long-established programmes of work aimed at the development of improved antitubercular drugs [260].

THIOUREAS: THIAMBUTOSINE, THIOCARLIDE

These two compounds (71c, d) have been selected from a large number of thiourea derivatives which have been prepared since 1941. They were introduced in leprosy therapy in 1956 [111, 136]. Their chemistry and biological activity have been comprehensively reviewed [3, 260]. Synthesis is by well-established routes. Although some N^1-aryl-N^3-alkylthioureas were active against *M. tuberculosis*, the greatest activity was found amongst N^1,N^3-diarylthioureas. The structure-activity relationships are summarized in *Figure 1.27*. The essential molecular features are the thiourea moiety, which must have a hydrogen atom on both the N-1 and N-3 atoms, and the 4-alkoxyphenyl ring (ring A).

It is now generally recognized that thiambutosine has only a bacteriostatic action against *M. leprae* [83, 89, 91, 195, 261] (see *Table 1.7*). It is very poorly and erratically absorbed from the gut, over 75% of the dose being excreted

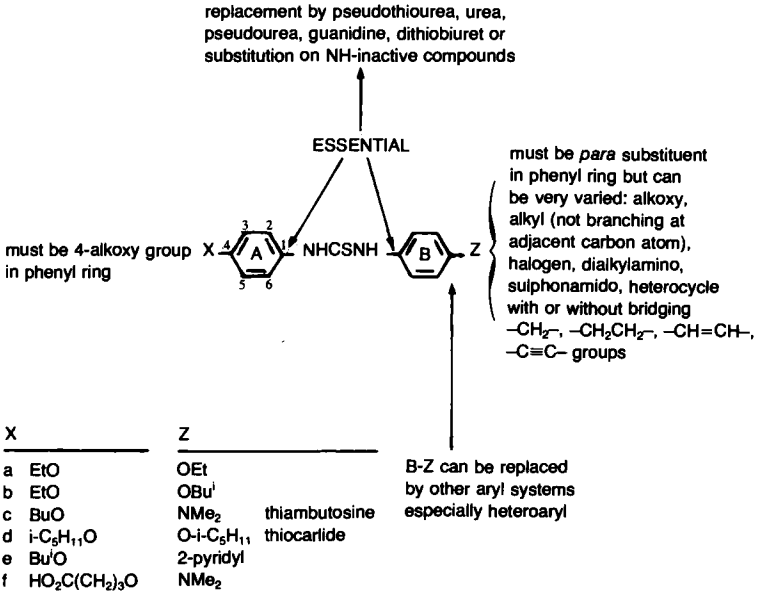


Figure 1.27. Summary of the major structure-activity relationships in diarylthiourea derivatives.

unchanged in the faeces and only 10% being absorbed [91, 262]. Thiocarlide is even more poorly absorbed [91]. Effective therapy requires high dosage (1500 mg) three times daily, which even then only gives serum levels in excess of the MIC for about 24 h (*Table 1.7*). The half-life ($t_{1/2}$) is about 7 h. Resistance develops rapidly (2–4 years) [3, 89, 132, 198, 260, 261]. An extensive 10-year study covering 1958–1968 [262] in which thiambutosine was used as the second-choice drug showed that 14% of patients developed resistance (16 months to 11 years after therapy, average 5.8 years). There is a high incidence of cross-resistance with thiacetazone, prothionamide and ethionamide [3, 198, 260, 261, 263] possibly of a multi-step type [263]. The mode of action of the thiourea derivatives (71a, c) probably involves inhibition of mycolic acid and general lipid synthesis which has been observed with (71d) in *M. tuberculosis* [3]. This is also in agreement with the postulated mechanism of action of drugs which are cross-resistant with (71c, d). Both thiambutosine and thiocarlide are generally well tolerated, but a number of side-effects have been reported, including an antithyroid action, skin eruptions, hepatitis and various blood disorders [132, 264]. Despite all these limitations, thiambutosine is a candidate drug for use in combination therapy [261, 262] and it is still being used as a second-choice drug [265]. It remains, however, the least promising of the second-line drugs.

Nonetheless, research in this area is still being pursued. Substituted diarylthioureas are being synthesized and tested against *M. tuberculosis* [266, 267] and a limited QSAR study [268] has confirmed that the bulk of the activity is associated with the 4-alkoxyphenyl ring (ring A) (*Figure 1.27*), the optimum size for the alkyl group being *n*-butyloxy, *n*-pentyloxy or *n*-hexyloxy. This analysis suggests that thiambutosine may well be the best compound in the series [266–268]. The tentative identification of (71f), a water-soluble, possibly active, urinary metabolite of thiambutosine [269], suggests that this compound might prove more active. A more fundamental change in which the sulphur atom of the thiourea moiety is replaced by various groups including cyanoimino (=N·CN) and nitromethylene (=CH·NO_2) gives compounds which show only slight activity against *M. tuberculosis* [270]. Recent studies on the antiviral activity of substituted diarylthioureas have also appeared [271].

THIOSEMICARBAZONES: THIACETAZONE

This compound (72a) has emerged from a large number of thiosemicarbazones which were synthesized following the discovery of moderate antimicrobial activity in sulphathiadiazole [3, 260]. It was shown in 1950 to be active in

leprosy [96, 136]. It is prepared by conventional methods. The structure-activity relationships in antitubercular and antileprotic thiosemicarbazones are summarized in *Figure 1.28*. The thiosemicarbazone moiety is essential and any variation in it, except for reduction, leads to inactive compounds. An aromatic ring is essential for activity but can vary greatly in its character. Thiacetazone has no significant bactericidal action [83, 89, 261, 272] and resistance to the drug develops rapidly, extensive cross-resistance with other second-line drugs also occurs [89, 198, 260–263]. The mechanism of action has not been established [3] but mycolic acid and lipid synthesis is probably involved, which agrees with the observed cross-resistance. Against *M. tuberculosis*, *p*-aminosalicylic acid [3] does not antagonize its effect and there is no cross-resistance with isoniazid, with which it has been used in combination therapy. Metal chelation may play a part in its action or efficient transport [273]. Although thiacetazone is almost completely absorbed following oral dosage [96, 264], with a half-life of 8–12 h, the serum levels exceed the MIC for only about 2 days (*Table 1.7*). It is very extensively metabolized before excretion

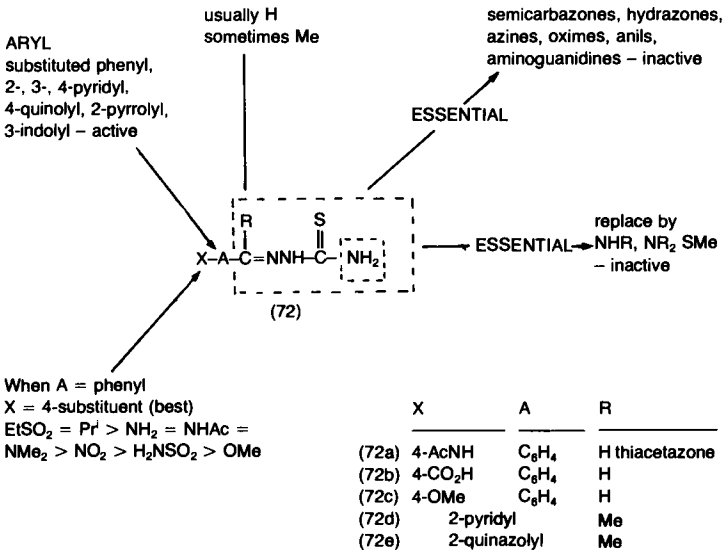
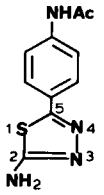


Figure 1.28. Summary of the structure-activity relationships in arylthiosemicarbazones.

in the urine. The major metabolites arise as a result of hydrolysis to thiosemicarbazide and the aldehyde, followed by further metabolism. The inactive thiadiazole (73) has also been identified in urine [260]. This observation contrasts with the reported bactericidal activity, against *M. leprae* in mice, of the imidazolylthiadiazole (74), which shows no activity against *M. tuberculosis* [87, 274]. The thiazoles (75, 76), analogous to the thiourea (71d), show only moderate *in vitro* activity against *M. tuberculosis* [260]. The implications of these contrasting reports for the development of new drugs with selective action against *M. leprae* remain to be investigated.



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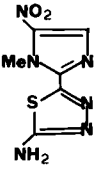

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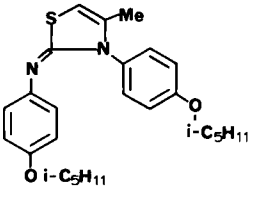

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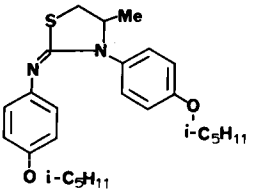
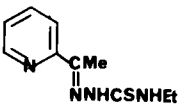


(76)

The major drawback to the use of thiacetazone is the frequency and wide range of toxic effects. The more serious ones include haemolytic anaemia, agranulocytosis, cerebral oedema, ototoxicity, extensive skin eruptions and hepatic dysfunction [96, 264, 275, 276]. Research in this area still continues. Thiosemicarbazone derivatives of 2-acetylpyridine (72d), despite earlier reports of low activity and high toxicity [260], have recently been extensively investigated. Their reported activity against malaria parasites led to a wide variety of compounds being screened against various mycobacteria *in vitro*, including *M. tuberculosis* [277]. The relationships between lipophilicity and MICs was found to be parabolic, with log $P_o \approx 4.0$, except in the case of *M. smegmatis* ATTC 607, where the log *P* relationship was biphasic [278]. It was concluded that the high concentration of lipid in the mycobacterial cell wall was a significant factor in the penetration of these compounds to attain active MICs. However, *in vitro* tests can often be misleading [187]. This study is being extended to include 2-acetylquinazoline thiosemicarbazones (72e). A selection of the 2-acetylpyridine thiosemicarbazones has now been tested against *M. leprae* in the mouse foot pad. Four compounds were found to be particularly active at feeding levels of 0.05% and reduced *M. leprae* multiplication to less than 30% of the control. The best compound, code-named PP, (77), was bactericidal, reducing multiplication to less than 5% of the control. The introduction of an ethyl substituent on N-4 contrasts with the earlier

structure-activity relationships [260]. Log P_0 was 2.3–2.5 for antileprotic activity, which is significantly less than that for *M. tuberculosis* and most other mycobacteria, but corresponds to that found for *M. smegmatis* 607 *in vitro* [278a]. This organism may therefore provide a useful preliminary screen for

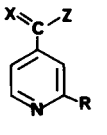


(77)

antileprotic compounds. However, the optimal log P_0 value in the mouse foot pad may be a reflection of pharmacokinetic factors; a log $P_0 \approx 2.0$ is optimal for drug transport through mammalian tissues [278b]. It was noticed that exposure of *M. leprae* to the drug led to the emergence of organisms which were no longer acid-fast [18, 36]. This observation, also seen with isoniazid against *M. tuberculosis*, suggests that these compounds inhibit mycolic acid synthesis [278]. 2-Acetylpyridine thiosemicarbazones with one or two alkyl substituents have also been found to be particularly active against *Neisseria gonorrhoeae*, *N. meningitis* [279] and *T. rhodensiense* [279a]. The effect of the replacement of the sulphur atom by various other groups is also being explored in this class of compounds [270]. The toxicity of this class of compounds is reduced by the introduction of a 6-substituent in the pyridine ring [279b] or surprisingly, by the replacement of the sulphur atom by selenium [279c].

THIOAMIDES: ETHIONAMIDE, PROTHIONAMIDE, PYRAZINAMIDE

These compounds arose from various drug development programmes which followed the observation of the tuberculostatic activity of isonicotinamide (78a) in 1948 [3, 260]. These studies led to the development of isonicotinic hydrazide (78j), a potent antitubercular drug which has no antileprotic activity, and the isothionicotinamides (78d, e). Initially, ethionamide (78d) was widely investigated but, because it is better tolerated [280] in man, prothionamide (78e) is now being widely studied in the treatment of leprosy. The chemistry of these compounds has been extensively reviewed [260]. They are synthesized by conventional methods and the main structure-activity relationships are summarized in *Figure 1.29*. The thioamide group is essential and must be attached directly to the pyridyl ring (the 4-thioamides are most active). Substitution of the amide nitrogen atom usually gives less active compounds. In contrast, substitution in the pyridyl ring gives more potent compounds when



(78)

	R	X	Z
a:	H	O	NH_2
b:	Et	O	NH_2
c:	Pr^n	O	NH_2
d:	Et	S	NH_2
e:	Pr^n	S	NH_2
f:	Et	S^+-O^-	NH_2
g:	Pr^n	S^+-O^-	NH_2
h:	Et	O	OH
i:	Pr^n	O	OH
j:	H	O	$NHNH_2$

alkyl (or benzyl) groups are attached to the 2-position, but further substitution leads to very much reduced activity. Both ethionamide and prothionamide have a pronounced bactericidal effect against *M. leprae*, which makes them, at present, the most favoured second-line drugs [83, 87, 89, 198, 261, 272]. The rate of the development of resistance and the extent of cross-resistance is

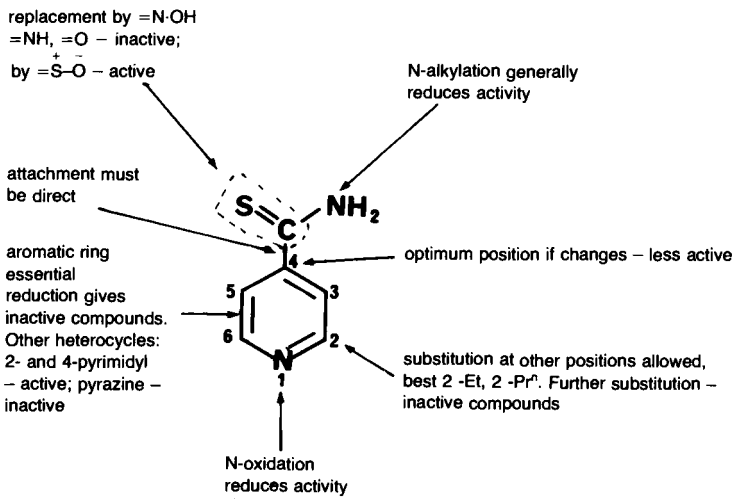
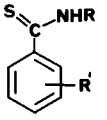


Figure 1.29. Summary of the structure-activity relationships in arylthioamides.

similar to that of the other second-line drugs [3, 96, 198, 260, 261, 263]. The mechanism of action has not yet been clarified. Both compounds appear to be similar to isoniazid (78j) in their action. They suppress mycolic acid synthesis and probably other lipid syntheses and may be transformed intracellularly into isonicotinic acids (78h, i) which are fully ionized and therefore trapped inside the cell where, after quaternization, they compete with nicotinic acid and are incorporated into false nicotinic acid-adenine nucleotides – the Kruger-Theimer hypothesis [3, 281]. The activity of (78d, e) against *M. leprae* may therefore represent a difference in transport, penetration and/or metabolism of these compounds when compared with isoniazid (78j), which is inactive against *M. leprae*. Recently, isoniazid, which is inactive against *M. lufu*, has been reported to be synergistic with the active prothionamide (78e) [228].



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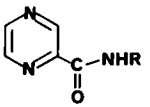


(80)

R = c-C_6H_{11}, C_6H_{13} a: R = H
R' = 2-OMe, 4-OMe b: R = morpholinomethyl

However, this understanding of the mechanism of action of isothionicotinamides does not account for the reported activity of thiobenzamides (79), which lack the basic nitrogen atom of (78d, e) but are 5–15-times more active than prothionamide against *M. smegmatis* [282]. In these compounds, the antimicrobial activity correlates with both the lipophilicity of the R group and the rate of the reaction of the thioamide group with methyl iodide as expressed in Equation (4):

$$\log \frac{1}{\text{CMI}} = -3.308 - 0.0636 \log k + 1.582 (\log k)^2 + 0.757 \pi_R \qquad (4)$$

CMI is the minimal inhibitory concentration, mol l^{-1}, k is the rate constant for the reaction of the thioamide group with methyl iodide and has an optimal value, $k_o = 1.05$.

Both ethionamide and prothionamide are rapidly absorbed from the gastrointestinal tract, giving serum levels, some 60-times greater than the MICs, which extend over 2 days. Ethionamide has a half-life of 2–4 h [236b, 261]. Both drugs are extensively metabolized; only 5% of the unchanged drug is

excreted in the urine. The major metabolites are the equipotent sulphoxides (78f, g) and the inactive carboxamides (78b, c) and carboxylic acids (78h, i) together with the corresponding 1,4-dihydropyridines [3, 96, 260, 261, 283] and an *N*-methylated product [260]. The very extensive and serious toxic effects of (78d, e) provide a real obstacle to their use in therapy; severe gastrointestinal, hepatotoxic and central nervous system disturbances are common [3, 96, 236b, 260, 261] and may arise from the liberation of hydrogen sulphide as a result of metabolism by the microflora of the gut.

Pyrazinamide (80a) and the morpholinomethyl derivative (80b) which serves as a prodrug, being metabolically transformed into (80a), have a unique activity against semi-dormant microbial forms [89, 91, 104]. However, they are reported to be inactive against *M. leprae* (87) although they possess bactericidal activity against *M. tuberculosis*. Their use and development is limited by severe and frequent toxic side-effects, particularly on the liver [3, 96, 236a, 260]. New research in this area involves the replacement of the sulphur atom by other groups [270].

COMBINATION THERAPY

The reasons for advocating combinations of drugs are as follows: (i) to prevent the emergence of resistant organisms, which is evidenced by relapse during therapy – the most common factor in drug resistance is prolonged monotherapy; (ii) to reduce as quickly as possible the number of viable bacilli, particularly in lepromatous leprosy patients, thereby reducing the spread of infection and allowing an early return to the community; (ii) to minimize toxic side-effects associated with high and prolonged drug dosage.

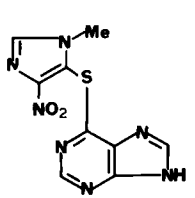
The idea of combination therapy is not new [111]. Today the drugs used are selected from the current first- and second-line drugs mentioned above. An assessment of their efficacy in combination therapy is, because of the nature of the disease, a long-term exercise requiring careful monitoring. Dapsone, because of its very high potency and cheap costs, remains the major chemotherapeutic agent. Fortunately, resistance develops very slowly, 5–24 years (average 15.8 years) [262], and primary or secondary resistance can often be overcome by increasing the dose of dapsone without precipitating toxic effects [284]. Dapsone in combination with rifampicin provides a rapid bactericidal action; various dosage regimens are under investigation, with rifampicin being given as an initial dose (1500 mg), daily (600 mg) or intermittently, weekly (900 mg) or monthly (1200–600 mg) [214, 228, 272]. Dapsone and prothionamide, another bactericidal drug, form another combination under

investigation [228]; intermittent therapy with prothionamide is not recommended and is probably ineffective if intervals are longer than 3 days [280, 285, 286]. A recent study with rifampicin plus prothionamide in dapsone-resistant lepromatous leprosy led to rapid clinical improvement over a short period, 18–24 months, when therapy was stopped. No relapses have occurred after 4 years without further therapy. This may indicate that future drug treatment of lepromatous leprosy need not be life-long [287]. Rifampicin plus clofazimine has also been advocated as a suitable treatment for dapsone-resistant leprosy [210, 228, 272].

Multi-drug regimens of dapsone plus rifampicin plus prothionamide and dapsone plus rifampicin plus clofazimine are also being investigated [228]. The need to monitor such therapy carefully has led to the development of an HPLC assay method for the simultaneous measurement of dapsone, rifampicin, clofazimine and their major metabolites [288]. An interesting review of therapy in leprosy in the light of experiences with tuberculosis chemotherapy has appeared [288a].

ANTI-INFLAMMATORY, IMMUNOSUPPRESSANT AND IMMUNOSTIMULANT DRUGS

The current use of these compounds in leprosy is to control or ameliorate the reactional states that occur as a result of the progress of the disease and/or antileprotic drug therapy (*Figures 1.11, 1.19*). The most potent drugs are prednisone and prednisolone, which are effective in the majority of cases. Azathioprine (81) is recommended for steroid refractory cases [211]. Clofazimine, as already mentioned, exerts a useful anti-inflammatory action



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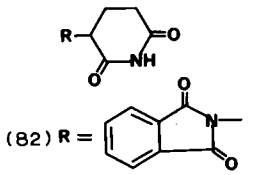
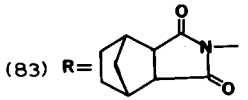
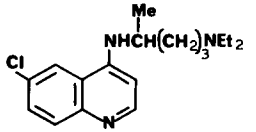




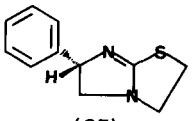


(84)

in ENL in addition to its antileprotic action [210–212]. Whilst both dapsone and/or its metabolites and rifampicin exert market effects on the immune response system in a variety of test systems, the meaning of these effects in treatment has yet to be evaluated [96, 172, 181–183, 236, 289, 290].

Thalidomide (82), despite its well-known toxic effects, particularly teratogenicity and polyneuritis, has emerged as a useful drug in the treatment of ENL [291, 292]. It may act by suppressing the synthesis of antibodies of the IgM class [293, 294]. A number of studies of thalidomide derivatives have been reported, but detailed structural details are scanty. One study reports no success in the search for non-teratogenic phthalimide derivatives and stresses the unique anti-inflammatory and immunosuppressant properties of thalidomide [295]. Another mentions the trials of nine compounds, but no details are given [296]. Taglutimide (83), which has only immunosuppressant properties and no teratogenic action, is currently undergoing clinical evaluation in ENL [228, 297]. Chloroquine (84), another immunosuppressant drug, has also been recommended to control light lepra reactions [265].

The immunosuppression associated with lepromatous leprosy has led to the testing of a number of immunostimulant drugs. Levamisole (tetramisole, 85) has been reported to have no effect in mice when given at the beginning or the middle of the infection [298]. In leprosy patients, levamisole has shown



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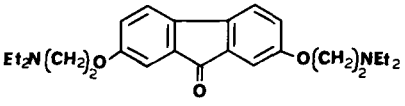


(86)

little or no useful therapeutic effect [298a, b]. Paradoxically, levamisole has been reported to reduce the dose of thalidomide (an immunosuppressant drug) required to control ENL [299]. The immunopharmacology of levamisole has recently been reviewed and attention drawn to the paradox of its immunosuppressant and immunostimulant properties. The overall effect of the drug depends on dosage, time of administration and host genetic background [300–301]. Tilorone (86), an interferon inducer with a broad spectrum of antiviral activity, suppresses cell-mediated immunity but stimulates the humoral response [301]. It inhibits multiplication of *M. leprae* in mouse foot pad more effectively than poly (I-C), a known interferon inducer, which has been reported to be inactive [87]. The direct antimicrobial action of tilorone is thought to outweigh its immunostimulant action [302, 302a].

Immunostimulant polysaccharides, particularly β-D-1,3-glucan, have also been reported to suppress multiplication of *M. leprae* in the mouse foot pad [303].

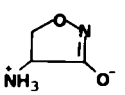
OTHER DRUGS INVESTIGATED FOR ANTILEPROTIC ACTIVITY

A wide variety of drugs has been examined for antileprotic activity, and folk remedies abound. However, it is only comparatively recently that reliable experimental methods have become available for their evaluation. In this section, only those compounds which have been the subject of more recent study will be reported.

ANTIBIOTICS

A range of compounds which affect a variety of biochemical systems in micro-organisms have been examined [87]. Drugs affecting the cell membrane peptidoglycan which are active against *M. leprae* in mice are cycloserine (87), which has a weak bacteriostatic action at high doses, and cephaloridine, which has a bactericidal effect [87]. Cycloserine is reported to be as active as dapsone in man [304]. Currently, derivatives of cycloserine are under investigation, but no details are yet available [305]. The development of cephalosporins and penicillins, which are generally inactive against mycobacteria, has not been pursued.

Streptomycin (88) was the most active of the drugs affecting ribosome function and thereby protein synthesis. It has a pronounced bacteriostatic effect in mice. Gentamicin, a related aminoglycoside, was active only at much larger doses. Earlier, streptomycin was reported to be as active as dapsone in man [304] and recently it has been shown to have a bactericidal effect greater than dapsone but less than prothionamide. It is particularly effective against



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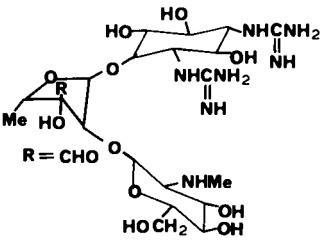
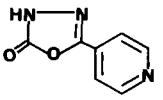


(88)

extracellular bacilli. Its use has been advocated as a second-line drug [306]. However, the known toxicity of the compound, the need for regular injections, and the rapid emergence of resistance in man, are all important factors militating against its use in this way. Tetracycline and lincomycin, which have similar sites of action, are both inactive. Clindamycin and the basic peptides capreomycin and viomycin, all of which have a similar site of action, do, however, have a bacteriostatic action. Streptovaricin, which resembles rifampicin, is bactericidal [87].

OTHER ANTITUBERCULAR DRUGS

Isoniazid (78j) and *p*-aminosalicylic acid both showed some activity against *M. leprae* in mice, but in man their activity was either temporary (isoniazid) or ambiguous (*p*-aminosalicylic acid) [87, 304]. Ditophal, a prodrug for ethyl mercaptan, was inactive in mice, but caused a temporary improvement in man followed by a deterioration which is probably a result of the very rapid development of resistance [87, 111, 304]. Ethambutol is inactive [87, 304]. A recent study with *M. smegmatis* indicates that its antimycobacterial action is due to a competitive inhibition of mycolic acid transfer from mycolyl acetyl trehalose which accumulates in treated cells [307]. The oxadiazole (89), reported as a lead compound in an earlier review, was found to be bacteriostatic [87, 111]. Macrocylon, a polymeric surfactant related to the antitubercular 'Tritons', has been the subject of conflicting reports [111]; it has not been investigated further. There is no obvious reason why antitubercular drugs should show such varied activity against *M. leprae*. These observations only confirm that *M. leprae* differs significantly from *M. tuberculosis* in its permeability to drugs and/or the structure of the biochemical target sites.



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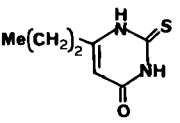
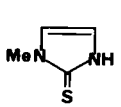


(90)

ANTITHYROID DRUGS

These compounds can be regarded as cyclic analogues of thioureas. Their activity against *M. leprae* in man was reported following treatment with propylthiouracil (90) of a case of lepromatous leprosy with thyrotoxicosis. Both

conditions improved [136]. Furthermore, the massive rise in thyroxine levels which take place in the third trimester of pregnancy are associated with a downgrading of the state of the leprosy patient [61c, d, 308]. The question which arises is whether the antileprotic action is an indirect effect arising from the antithyroid action of the drug, or a direct antileprotic effect. Methimazole (91), another antithyroid drug, was more effective than (90) and was shown to have a bacteriostatic action [87]. Recently, the activities of these two compounds and the antithyroid thiazolidinone (92) were evaluated against a number of mycobacteria, including *M. leprae*. The antimicrobial action is separate from the antithyroid activity, since the latter, but not the former, effect is blocked by the administration of thyroid substance. However, all three compounds were less active than thiambutosine [309].



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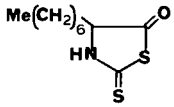
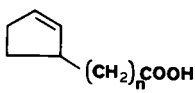


(92)

NATURAL PRODUCTS

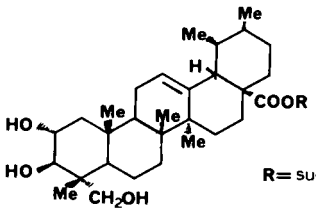
Chaulmoogra oil, a mixture principally of the triacylglycerols of hydnocarpic (93) and chaulmoogric (94) acids, has been widely used in the treatment of leprosy from the earliest times [260, 310]. It is little used today except where modern drugs are not available or are too expensive. Its efficacy has been the subject of much hope, debate and uncertainty [310], so much so that the identification of a definite bacteriostatic action of the sodium salt of the acid (94) was described as surprising in 1971 [87]. Recently, it has been suggested that chaulmoogric acid may exert its action by inhibiting biotin-dependent enzyme systems which play a key role in fatty acid synthesis. However, evidence has now been presented that it is incorporated into both the phospholipids (15%) and the triacylglycerols (27%) of *M. vaccae* and *M. intracellulare*. The changes in both the structure and function of the cell membrane may be responsible for the inhibition of the growth of these organisms [311].



(93) n=10
(94) n=12

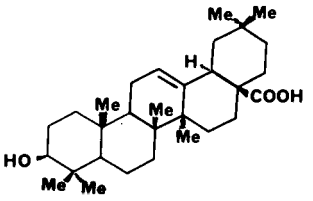
Chaulmoogric acid derivatives are currently being investigated, but no details are available [305]. The possibilities, arising from these observations, for the development of new drugs are considered in the next section.

The traditional Indian plant 'mandukaparni' (not identified but probably *Centella asiatica* [312]), has been used from 600 B.C. as a folk remedy for leprosy. An initial report of the use of the dry, powdered plant (root, leaves, stems), administered as a pill to lepromatous leprosy patients, appeared in 1974 [313]. All the patients showed considerable clinical improvement after 8 months. The active principle was identified as the terpenoid glycoside, asiaticoside (95), which is claimed in a later study to give spectacular results in lepromatous leprosy [314]. It is thought to act by inhibiting the synthesis of hyaluronic acid which is described as an essential nutrient, associated with carbohydrate metabolism in *M. leprae* [313, 314]. The bacilli which are formed in its presence are claimed to have a fragile cell wall which is more susceptible to destruction by host cells and/or drugs [314]. Many more extensive clinical and experimental studies are required to confirm (or disprove) this interesting and exciting development.



R= sugar residues

(95)



(96)

Achyranthes aspera, another traditional plant of India with antileprotic properties, contains a related glycoside, oleanolic acid (96). A decoction of the plant is claimed to be synergistic with dapsone and to be an effective treatment for lepra reactions [96]. *Lasiosiphon kraussianus* (krausii) is another tropical plant reported to be effective against leprosy [315]. The Chinese herb lei-gong-teng (*Trigerygium wilfordii* Hook F) and its extracts have been used successfully to treat both type I and type II reactions [315a].

MISCELLANEOUS COMPOUNDS

The ubiquitous ascorbic acid, in large doses, 0.15–0.45% w/w of the diet, inhibits the growth of *M. leprae*. It is a nonspecific inhibitor of bacterial glucuronidase, which is necessary for hyaluronic acid metabolism [316, 317].

Zinc sulphate (400 mg/day) is reported to be effective in the treatment of leprosy [318]; doses of 220 mg three times daily have been used to assist the healing of wounds [319].

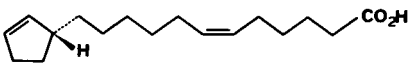
Iodide-iodine, once associated with the treatment of leprosy, has been recommended for heroic therapy in the young strong patient (!) [320], but does not suppress the multiplication of *M. leprae* in the mouse foot pad [309]. However, potassium iodide is reported to be useful in the treatment of ENL [320a]. A new drug, 2-isopropylaminopyrimidine phosphate, which increases muscle reinnervation, has been used to treat peripheral neuritis in leprosy patients [320b].

POSSIBILITIES FOR NEW DRUGS

In the light of the current knowledge about leprosy and the causative agent, *M. leprae*, three new areas of research, which could lead to selectively active novel drugs, are outlined.

DERIVATIVES OF CHAULMOOGRIC AND HYDNOCARPIC ACIDS

These acids (93, 94), which are the *R*-(+)-isomers [321], are obtained from chaulmoogra oil, which in turn is expressed from the seeds of *Hydnocarpus wightiana* or *Taraktogenos kurzii*. The oil (a mixture of triacylglycerols) and the ethyl esters or the sodium salts of the acids were widely used to treat leprosy [132, 321] until the advent of the sulphones. Long after the use of the oil in leprosy therapy had lapsed, chaulmoogric acid was found to exert a definite bacteriostatic action against *M. leprae* [87]. The total fatty acids of the oil are reported to be more bactericidal than any of its single fractions [322]. It would be interesting to know the basis of their synergy and whether hydnocarpic acid (93) and gorlic acid (97) (the latter constituting a high proportion (21%) [323, 323a] of the acids in the natural oil) are active in the same way. It seems likely from very recent work that chaulmoogric acid is incorporated into the phospholipids and triacylglycerol fraction of the cell membrane of mycobacteria [311]. Chaulmoogric acid, therefore, is likely to be a parasite-specific compound which is processed by the normal cell-wall-synthesizing enzymes of the



(97)

mycobacterium. These enzymes do not occur in the host. In contemporary terms, it is a prime candidate for the development of active-site-directed irreversible enzyme inhibitors [133, 324] which could be tested against a number of mycobacteria but particularly *M. leprae* using both *in vitro* and *in vivo* test systems.

Early workers in this area carried out extensive chemical studies aimed at the synthesis of compounds (93) and (94), and prepared a wide range of derivatives [321, 325, 325a], some of which were tested for biological activity [325a]. However, specific tests for antileprotic activity were not available to them and the claims made must be discounted or confirmed by modern testing methods. *Figure 1.30* summarizes the major structural variations which could

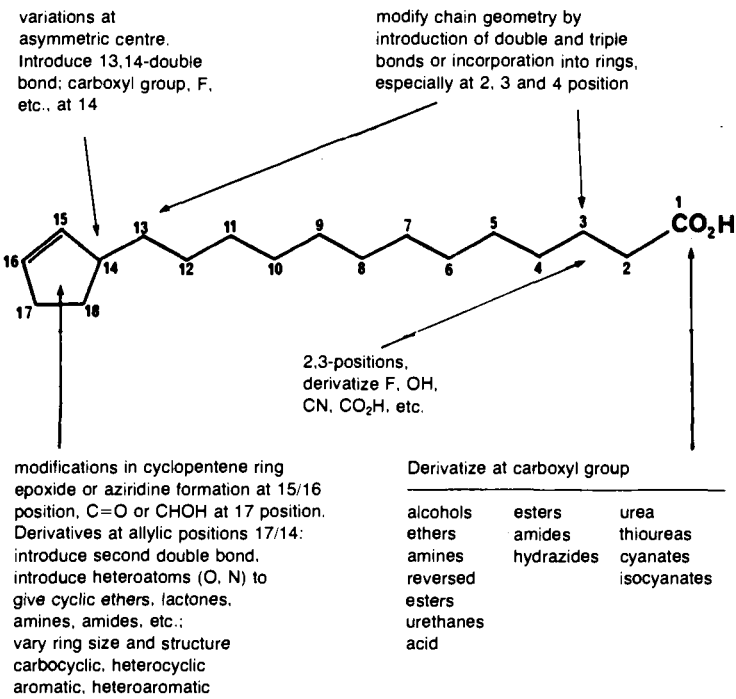
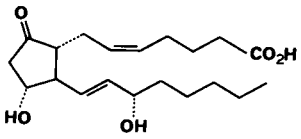
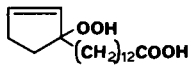


Figure 1.30. Proposed molecular variations in chaulmoogric, hydnocarpic and gorlic acids leading to novel antimycobacterial drugs.

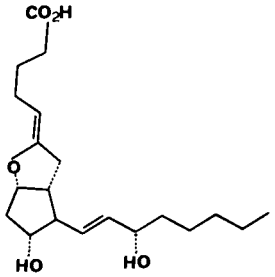
be made to obtain suitable compounds for biological evaluation [326]. An unexpected link between chaulmoogric and hydnocarpic acids and prostaglandins may have been identified during the preparation of this review. The early comments about the action of chaulmoogric oil and/or its constituent fatty acids draw attention to the stimulation of phagocytosis [322, 327] by these compounds and to modification of the patient's response to the disease [65].



(98)



(100)



(99)

Both observations are consistent with an improvement of the host's cell-mediated immunity (CMI). Recently, prostaglandins E_1, E_2 (98) and I_2 (99) have been shown to act as negative-feedback regulators of CMI. Indomethacin (an inhibitor of prostaglandin synthetase) and tranylcypromine (an inhibitor of PGI_2 synthetase) both blocked this effect. Thromboxane A_2, in contrast, augmented the CMI [328]. It is entirely possible that compounds (93), (94) and (97) and the racemic hydroperoxide (100) which is readily formed [329] could function as either inhibitors of the enzymes of the arachidonic acid cascade or block the action of prostaglandins at their effector sites. Such action would improve the CMI. If this link is established, then the application of these compounds in other diseases involving the immune system becomes obvious and exciting. The peroxide of chaulmoogric acid is reported to be active against Rous sarcoma [329], and chaulmoogra oil is also a traditional remedy for rheumatism [330].

α,ε-*meso*-DIAMINOPIMELIC ACID DERIVATIVES

2,6- (or α,ε)-*meso*-Diaminopimelic acid (101), which is not found in mammals, occurs most frequently as the *meso*-(*R,S*) form and is an integral cross-linking component of the peptidoglycan cell wall of many micro-organisms [32]. It is found in all mycobacterial cell walls. It is surprising that no detailed structure-

activity study of this compound has been carried out, although occasional structural analogues have been mentioned [331] and some do block its uptake by bacteria. Here again is a prime candidate for the development of parasite-selective active-site-directed irreversible enzyme inhibitors analogous to the penicillins [326a]. Some possibilities are indicated in *Figure 1.31*.

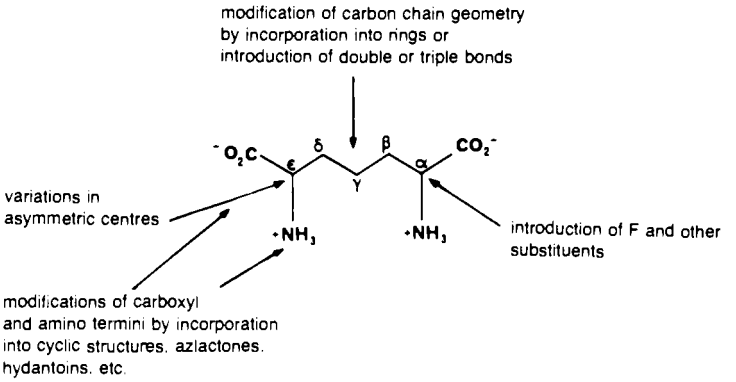


Figure 1.31. Proposed molecular variations in α,ε-meso-*diaminopimelic acid leading to novel antimycobacterial drugs.*

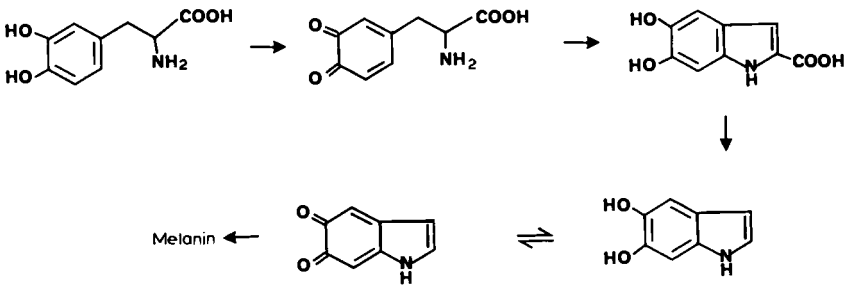


Figure 1.32. Summary of the main steps in the synthesis of melanin from DOPA.

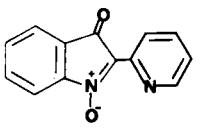
ANALOGUES OF DOPA AND 5,6-DIHYDROXYINDOLE

The development of the two groups of compounds (93, 94, 97, 101) described above should provide new specific antimycobacterial drugs. However, the identification of a putative unique and specific diphenoloxidase system (*Figure 1.32*) in *M. leprae* [40] affords another major opportunity for the design of specific antileprotic compounds. The identification of this enzyme system continues to be disputed [41, 41a] and it has been clearly demonstrated in only one laboratory. Nonetheless, there is evidence of its importance in the metabolism of *M. leprae*.

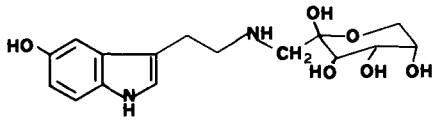
(i) Radiolabelled L-DOPA is incorporated into *M. leprae*. This observation provides the basis of an *in vitro* system for testing organisms for dapsone resistance and new compounds for antileprotic activity [24, 27, 28, 86].

(ii) The enzyme has been shown to have much in common with other tyrosinase and DOPA-oxidase systems [332]. It is a copper-dependent enzyme system which utilizes a variety of DOPA-related substrates to synthesize melanin (*Figure 1.32*). Important features of the system are: the presence of a decarboxylase enzyme as part of the enzyme complex [333]; exogenous reducing agents do not reduce the quinones once they are formed [334]; and it is strongly inhibited by the copper-chelating diethyl dithiocarbamate (102) [333-337]; other metal-chelating compounds cause much smaller or no inhibition [335, 336]. The substrate stereospecificity is low and the electronic characteristics of the substrates are very wide [333, 335, 336], in agreement with the occurrence of the enzyme in a phylogenetically primitive organism. It is certainly very different in these ways from the host enzyme, which is very specific in both the stereochemical and electronic characteristics of its substrate [334]. The function of the enzyme in *M. leprae* is unknown, but it may play an important role in terminal respiratory process or in some detoxification mechanisms [335].

(102)



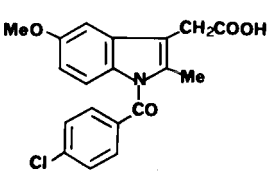
(103)



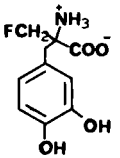
(104)

(iii) The suppression of melanin formation by *M. leprae* is consistent with the presence of this enzyme [338]. Lepromatous leprosy patients have excessive levels of diphenoloxidase activity in both skin and serum compared with tuberculoid leprosy patients and controls [339].

(iv) Initial studies with a number of candidate compounds drawn from analogues of DOPA used as β-agonists (kindly supplied by Glaxo Ltd), and a variety of indole derivatives showed that the 2-(2-pyridyl)isatogen (103), which has strong oxidizing properties and can be described as a meta-quinone [340], inhibits DOPA uptake by *M. leprae* [341]. More recently, the serotonin derivative (104) has also been shown to block DOPA uptake in *M. leprae* and to inhibit growth in the mouse foot pad [342]. The uptake of 5-hydroxytryptamine by blood platelets in leprosy patients is reduced. In severe cases the uptake is increased by compound (104) [342a]. A limited clinical study with this compound has yielded promising results [342b].



(105)



(106)

In the light of all this information, it is again possible to make further suggestions about the future development of completely novel antileprotic drugs which would be highly selective in their action against this enzyme in the bacillus. A number of established compounds such as indomethacin (105), an anti-inflammatory drug, and (±)-α-monofluoromethyl-DOPA (106), a DOPA-decarboxylase inhibitor which was designed as an enzyme-activated irreversible inhibitor [343], might well prove to be inhibitors of DOPA uptake by *M. leprae*, even though they are used for very different purposes [344]. The possibilities for drug development from DOPA (107) and 5,6-dihydroxyindoles (108), analogues of compounds in the biochemical pathway, are outlined in *Figures 1.33* and *1.34*.

Mankind's heroic struggle with leprosy has many different aspects. The heavy burden of economic, social and cultural restrictions and taboos upon leprosy sufferers [345] has not been mentioned here but is still of great importance today. The skilled surgery and compassionate devotion in serving and rehabilitating the millions scarred by the disease is another remarkable story. We trust that this account of the chemotherapy of leprosy gives hope for a future in which the application of our growing knowledge can give rise to new remedies for this ancient disease.

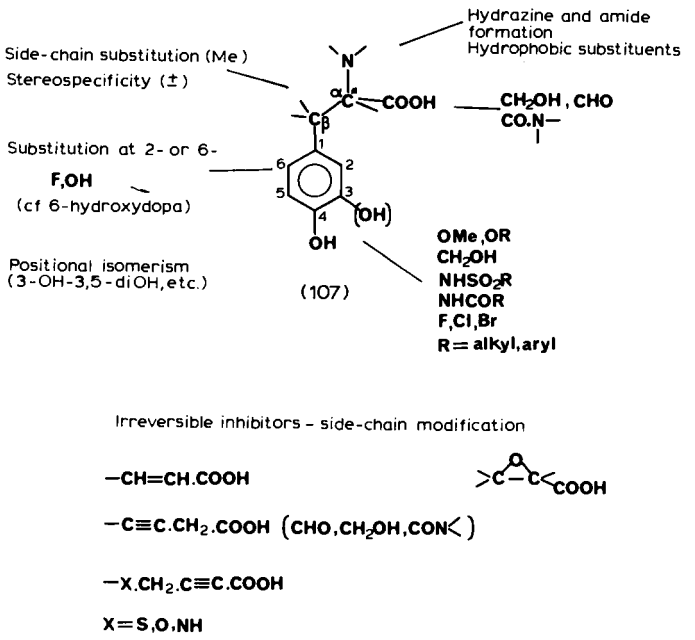


Figure 1.33. Proposed molecular variations in DOPA leading to specific antileprotic drugs.

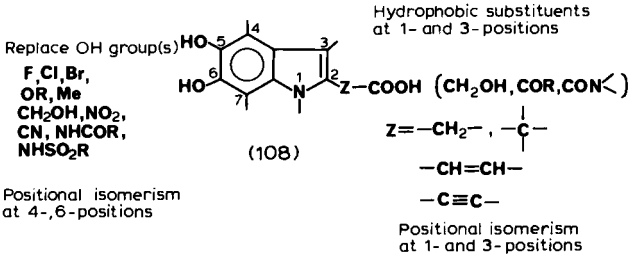


Figure 1.34. Proposed molecular variations in 5,6-dihydroxyindole leading to new specific antileprotic drugs.

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REFERENCES

1. R.G. Cochrane in: Leprosy in Theory and Practice, 2nd Edn., eds. R.G. Cochrane and T.F. Davey (Wright, Bristol, 1964), p. 1.
2. Stanley G. Browne, Documenta Geigy Acta Clinica (J.R. Geigy S.A., Basle, 1970).
3. P. Sensi and G. Gialdroni-Grassi in: Burger's Medicinal Chemistry, 4th Edn., ed. M.E. Wolff (Wiley, New York) Part II, (1979) 297.
3a. O. Closs and M. Harboe, Lepr. Rev., 52 (1981) Suppl. 1.
4. 3rd Annual Report (1 July 1978–30 June 1979) UNDP-World Bank-W.H.O. Special Programme for Research and Training in Tropical Diseases. Lepr. Rev., 52 (1981) 349.
4a. T. Land, Times Higher Educational Supplement, May 28th (1982) 10.
4b. J.H. Maugh, Science, 215 (1982) 1083.
4c. T. Godal, Prog. Allergy, 25 (1978) 211.
4d. K. Saha and S.C. Lahiri, Lepr. Rev., 52 (1981) 315.
5. R. Berkow (ed.), Merck Manual, (Merck and Co. Ltd., Rahway, NJ) 13th Edn. (1977) p. 126.
6. C.C. Gillespie (ed.), Dictionary of Scientific Biography, (Charles Scribener & Sons, New York) Vol. VI (1972) p. 101–103.
7. R.J.W. Rees and M.F.R. Waters, Int. J. Lepr., 30 (1962) 266.
8. Y.T. Chang, Int. J. Lepr., 45 (1977) 235.
9. Dharmendra, Lepr. India, 49 (1977) 10.
10. T. Warndorff, Int. J. Lepr., 48 (1980) 441.
10a. G. Boerrigter and J.M. Pounighaus, Lepr. Rev., 52 (1981) 283, D.S. Ridley, Lepr. Rev., 52 (1981) 284.
11. W.F. Kirchheimer, Lepr. India, 49 (1977) 44.
11a. G.P. Walsh, W.M. Meyers, C.H. Binford, P.J. Gerone, R.H. Wolf and J.R. Leininger, Lepr. Rev., 52 (1981) suppl. 77–83.
12. J.R. Leininger, K.J. Donham and M.J. Rubins, Vet. Pathol., 15 (1978) 339.
13. J.R. Leininger, K.J. Donham and W.M. Meyers, Int. Lepr., 48 (1980) 414.
14. J. Convit, J. Reticuloendothel. Soc., 24 (1978) 605.
15. W.F. Kirchheimer, Lepr. India, 51 (1979) 60.
16. W.F. Kirchheimer and E.E. Storrs, Int. J. Lepr., 39 (1971) 693.
17. E.E. Storrs, G.P. Walsh, H.P. Burchfield and C.H. Bingford, Science, 183 (1973) 851.

18. W.H. Jopling, Handbook of Leprosy (Heinemann, London, 1971) p. 9.
19. L. Kato, Int. J. Lepr., 45 (1977) 175; Abstr. from Lepr. Rev., 49 (1978) 328.
19a. Y. Matsuo, Int. J. Lepr., 48 (1980) 498.
19b. S.N. Caro, Q.X., Wu, Q. Liu and B. Jiang, Int. J. Lepr., 48 (1980) 47.
20. O.K. Skinsnes, E. Matsuo, P.H. Chang and B. Anderson, Int. J. Lepr., 43 (1975) 193.
21. G.T. McCormick and R.M. Sanchez, Int. J. Lepr., 47 (1979) 499.
22. N. Nakamura, J.H. Hanks, A.M. Dople and H.B. Funks, Int. J. Lepr., 48 (1980) 455.
23. S.R. Pattyns and F. Portracts, Int. J. Lepr., 48 (1980) 455.
24. E.J. Ambrose, N.H. Antia and S.R. Khanolkar, Nature (London), 249 (1974) 854.
25. G.P. Talwar, A.D. Krishman and P.D. Gupta, Infect. Immun., 9 (1974) 187.
26. G.P. Talwar and I. Nath, Indian Science Congress, Hyderabad, 3–7 Jan. (1979).
27. S.R. Khanolkar, E.J. Ambrose, R.G. Chulawala and C.V. Bapat, Lepr. Rev., 49 (1978) 178.
28. E.J. Ambrose, S.R. Khandkhar and R.G. Chulawala, Lepr. India, 50 (1978) 131.
28a. M. Sathish and I. Nath, Int. J. Lepr., 49 (1981) 187.
29. K.V. Desikan and Sreevasta (1979) in: 11th Biennial Conference of the Indian Association of Leprologists, Madras, 5–8 April, 1979. Reported in Lepr. Rev., 50 (1979) 243.
30. L. Barksdale and Kwang-Shin Kim, Bacteriol. Rev., 41 (1977) 217.
31a. P. Draper, Leprosy: Proceedings of the XIth International Leprosy Congress, Mexico City, 13–18 Nov., 1978, eds. F. Latapi, A. Saul. O. Rodriguez, M. Malacara and S.G. Browne (Excerpta Medica, Amsterdam, 1980) p. 97.
31b. T. Kusaka, Y. Fukurishi, N. Akimori, G.P. Walsh, W.M. Meyer and C.H. Binford, Int. J. Lepr., 48 (1980) 494.
32. E. Work and D.L. Dewey, J. Gen. Microbiol., 9 (1953) 394.
33. G.T. McCormick and R.M. Sanchez, Int. J. Lepr., 47 (1979) 495.
34. H.J. Nguyen, D.D. Trach, N. von Man, T.H. Ngoan, L. Dunia, M.A. Ludosky-Diawara and E.L. Benedetti, J. Bacteriol., 138 (1979) 552.
35. O. Closs, R.N. Mshana and M. Harboe, Scand. J. Immunol., 9 (1979) 297.
35a. T.P. Gillis, M. Abe, W.E. Bullock, O. Rojas-Espinosa, E. Garcia-Ortigoza, P. Draper, W. Kirchheimer and T.M. Buchanan, Int. J. Lepr., 49 (1981) 287.
36. P.J. Brennan and W.W. Barrow, Int. J. Lepr., 48 (1980) 382.
37. D. Sundari and D.E.S. Steward-Tull, Int. J. Lepr., 47 (1979) 483.
38. M. Ishaque, L. Kato and O.K. Skinshes, Int. J. Lepr., 45 (1977) 114.
39. M. Ishaque and L. Kato, Int. J. Lepr., 45 (1977) 120.
40. K. Prabhakaran, Microbios, 5 (1972) 273.
41. L. Kato, M. Ishaque and C. Adoppol, Int. J. Lepr., 44 (1976) 431.
41a. Se Jong Kim, M. Ishaque and L. Kato, Proceedings of XIth International Leprosy Congress (1978) p. 121.
41b. K.J. Shatty, N.H. Antia and P.R. Krishnaswamy, Int. J. Lepr., 49 (1981) 49.
41c. D. Gregory and P.R. Wheeler, J. Gen. Microbiol., 121 (1980) 457.
42. S.T. Pattyn, Lepr. India, 49 (1977) 526.
43. J.K. Webb, C.A. Mimms and J.L. Turk, Lepr. Rev., 51 (1980) 51.
44. M.F.R. Waters, R.J.W. Rees, A.C. McDougall and A.B.G. Laing, Proceedings of the XIth International Leprosy Congress (1978) p. 289.

44a. K. Toman. Int. J. Lepr., 49 (1981) 205.
45. A.B.A. Karat, Lepr. India, 50 (1978) 405.
45a. J.M. Grange, Mycobacterial Diseases (Edward Arnold, London, 1980).
46. M. Heywood, Courtesy of the Leprosy Mission.
47. T.F. Davey, Lepr. Rev., 49 (1978) 269.
48. 11th Biennial Conference of the Indian Association of Leprologists. Madras, 5–9 April, 1979; Reported in Lepr. Rev., 50 (1979) 243.
49. D.S. Ridley and W.H. Jopling, Int. J. Lepr., 34 (1966) 255.
50. D.S. Ridley and M.F.R. Waters, Lepr. Rev., 40 (1969) 143.
50a. L. Lenzini, P. Rottoli and L. Rolloli, Clin. Exp. Immunol., 27 (1977) 230.
51. D.S. Ridley, in Ref. 1, pp. 620–622.
52. W.H. Jopling, in Ref. 18, p. 3.
53. W.H. Jopling, in Ref. 18, p. 34.
54. S.W.A. Kupfer, in Ref. 1, p. 183.
54a. V. Mehra and B.R. Bloom, Infect. Immun., 23 (1979) 787.
55. L.F. Badger, in Ref. 1, pp. 70, 95.
56. S.G. Spickett, in Ref. 1, p. 107.
57. K.F. Schaller and W. Kuls, Ethiopia: A Geomedical Monograph (Springer-Verlag, Berlin, 1972) quoted in Lepr. Rev., 50 (1979) 260.
57a. R. Rolston, M. Matthews, P.M. Taylor and J.S. Koshy, Int. J. Lepr., 49 (1981) 31.
57b. G.H. Ree, F. Martin, K. Myles and J. Peluso, Lepr. Rev., 52 (1981) 171.
57c. S. Shilo, Y. Livshin, J. Sheskin and J.M. Spitz, Lepr. Rev., 52 (1981) 127.
58. W.H. Jopling, in Ref. 18, p. 42.
59. R.H. Dash, E. Samuel, S. Kaur, B.N. Datta and G.K. Rastogi, Horm. Metab. Res., 10 (1978) 362.
60. A.S. Cologlu, Lepr. Rev., 50 (1979) 213.
60a. M. Elliott, Lepr. Rev., 52 (1981) 355–61.
61. G.L. Stoner, Lepr. Rev., 52 (1981) 1.
61a. S. Chehl, M.J. Morales and R.C. Hasting, Lepr. Rev., 53 (1982) 74.
61b. G.L. Stoner, Lepr. Rev., 53 (1982) 75.
61c. M.E. Duncan, R. Melson, J.H. Pearson and D.S. Ridley, Lepr. Rev., 52 (1981) 245.
61d. M.E. Duncan, J.H. Pearson and R.J.W. Rees, Lepr. Rev., 52 (1981) 263.
62. J.H. Schwab, Bacteriol. Rev., 39 (1975) 121.
63. W.F. Kirchheimer, R.M. Sanchez and E.J. Shannon, Int. J. Lepr., 46 (1978) 353.
64. Anon., in Ref. 4, p. 128.
65. T.F. Davey, Lepr. Rev., 46 (1975) 5.
66. C.C. Shepard and Y.T. Chang, Int. J. Lepr., 32 (1964) 260.
67. E. Freerksen and M. Rosenfeld, Arzneim.-Forsch., 22 (1972) 1235.
68. L. Kato, E. Mankiewicz and I. de Thokoly, Experientia, 34 (1978) 1322.
69. W.H. Feldman, in Ref. 1, p. 56.
70. G. Middlebrook, in Ref. 1, p. 152.
71. Lepr. India, 50 (1978) ed. Dharmendra (The whole volume is devoted to vaccine development).
72. J.L. Stafford, M.J. Shield, and G.W.W. Rook, in Ref. 31a, p. 102.
73. C.C. Shepard, J. Exp. Med., 112 (1960) 445.
74. C.C. Shepard and Y.T. Chang, Proc. Soc. Exp. Biol. N.Y., 109 (1962) 636.
75. P.J. Patel and M.J. Lefford, Infect. Immun., 19 (1978) 87.

76. UNDP-World Bank-W.H.O.-Special Programme for Research and Training in Tropical Diseases Newsletter No. 15, (Feb. 1981) 10.
77. S.G. Browne, in Ref. 2, p. 40.
78. R.J.W. Rees, Nature (London), 211 (1966) 657.
79. R.J.W. Rees, M.F. Waters, A.G. Weddell and E. Palmer, Nature (London), 215 (1967) 599.
80. R.J.W. Rees and A.G. Weddell, Ann. N.Y. Acad. Sci., 154 (1968) 214.
81. R.J.W. Rees, A.G. Weddell and J.M. Pearson, Br. Med. J., 3 (1969) 216.
82. C.C. Shepard, Int. J. Lepr., 39 (1971) 340; 48 (1980) 492.
83. M.J. Colston, G.R.F. Hilson and D.K. Banerjee, Lepr. Rev., 49 (1978) 7.
84. Workshop on Experimental Leprosy in Ref. 31a, pp. 359–264.
85. J. Lew, Int. J. Lepr., 48 (1980) 463.
86. E.J. Ambrose, S.R. Khanolkar, R. Chulawala, N.H. Antia and K.K. Kotecha, in Ref. 31a, p. 229.
87. C.C. Shepard, Int. J. Lepr., 39 (1971) 340.
88. S.R. Pattyn, Lepr. Rev., 43 (1972) 126.
89. G.A. Ellard, Lepr. Rev., 45 (1974) 31.
90. E.F. Elslager, Prog. Drug Res., 18 (1974) 99.
91. G.A. Ellard, Lepr. Rev., 46 (1975) 41.
92. Bull. W.H.O, 54 (1976) 235.
93. S.G. Browne, The Diagnosis and Management of Early Leprosy (Leprosy Mission, London, 1975).
94. W.H. Jopling, in Ref. 18, p. 49.
95a. Martindale Extra Pharmacopoeia (Pharmaceutical Society Press) 27th Ed. (1977) p. 1497.
95b. V. Seshadri, A.K. Bhatta and S. Rangaswami, Indian J. Chem. Sect. B 20 (1981) 773.
96. G.L. Goodman and A. Gilman, The Pharmacological Basis of Therapeutics (Macmillan, New York) 6th Edn. (1980) p. 1200.
97. R.J.W. Rees and J.H.S. Pettit, Lancet, ii (1964) 673.
98. R.R. Jacobson in Ref. 31a, p. 306.
99. J.M.H. Pearson, R.J.W. Rees and M.F.R. Waters, Lancet, ii (1975) 69.
100. J.M.H. Pearson, G.S. Haile and R.J.W. Rees, Lepr. Rev., 48 (1977) 129.
101. J.T. de las Aguas, in Ref. 31a, p. 300.
102. J.M.H. Pearson, G.S. Haile, R.St.C. Barnetson and R.J.W. Rees, Lepr. Rev., 50 (1979) 183.
103. V. Balraj, K. Jesudasan, C.J.G. Chacko, M. Christian, P.M. Taylor, E.P. Fritschi and C.K. Job, Int. J. Lepr., 48 (1980) 397.
104. G.A. Ellard, P.J. Gammon and J.M. Harris, Lepr. Rev., 45 (1974) 224.
104a. G.A. Ellard, Lepr. Rev., 52 (1981) 201.
104b. H. Huikeshoven, Int. J. Lepr., 49 (1981) 228.
104c. G.A. Ellard, J.M.H. Pearson and G.S. Haile, Lepr. Rev., 52 (1981) 237.
105. K.J. Hagan, S.E. Smith, Kin Ma Gyi, Maung Maung Lwin, Yi Yi Myaing and Kin Maw OO, Lepr. Rev., 50 (1979) 201.
106. R.J.W. Rees, M.F.R. Waters and A.B.G. Laing, Lepr. Rev., 49 (1978) 127.
107. B.K. Girdhar, Sreevatsa and K.V. Desikan, Lepr. India, 50 (1978) 325.
108. 3rd Annu. Report W.H.O, 1979, p. 133.

109. L. Levy, Antimicrob. Agents Chemother., 14 (1978) 792.
110. M.F.R. Waters, R.J.W. Rees and G.A. Ellard, Int. J. Lepr., 36 (1968) 651.
111. S.R.M. Bushby, in Ref. 1, p. 345.
112. R.G. Cochrane, in Ref. 1, p. 376.
113. S.G. Browne, Leprosy – New Hope and Continuing Challenge – Revised Edition (1977) (The Leprosy Mission, London) p. 52.
114. F.A. Berti, B. Rieckemann, C. Perego and H.W. Rzeppa, Mem. Inst. Butantan, 21 (1948) 111; Chem. Abstr. 44 (1950) 3935.
115. F.A. Berti, L.M. Ziti, C. Perego, H.W. Rzeppa, B.H.G. Reickmann and O.P. Rapp, Rev. Brasil. Leprol., 20 (1952) 104; Chem. Abstr. 50 (1956) 11520.
116. W.H. Linnell and J.B. Stenlake, J. Pharm. Pharmacol., 2 (1950) 736.
117. W.H. Linnell and J.B. Stenlake, J. Pharm. Pharmacol., 2 (1950) 937.
118. N.P. Buu-Hoi, N.D. Xuong and. N.B. Tien, J. Org. Chem., 21 (1956) 415.
119. A.P. Cheung and P. Lim, J. Pharm. Sci., 66 (1977) 1723.
120. C.E. Orzeeh, N.G. Nash and R.D. Daley, in: Analytical Profiles of Drug Substances, ed. K. Florey, (Academic Press, New York) Vol. 5 (1976) p. 87.
121. J. March, Advanced Organic Chemistry (McGraw-Hill, New York) 2nd Ed. (1977) p. 1188. G. Ronsisville, Farmaco, Ed. Sci., 36 (1981) 885.
122. C.A. Mannan, G.J. Kool and B.J. Kho, J. Pharm. Sci., 66 (1977) 1618.
123. J. Lowe, Lancet, 258 (1950) 145.
124. H. Ross and F. Genar, Int. J. Lepr., 19 (1951) 153.
125. H. Floch and N. Rist, Rev. Brasil. Leprol., 18 (1952) 111; Chem. Abstr., 47 (1953) 7659.
126. T. Ozawa, C.C. Shepard and A.B.A. Karat, Am. J. Trop. Med. Hyg., 20 (1971) 274; Chem. Abstr., 74 (1972) 86236.
127. Y. Kurono and K. Ikeda, Chem. Pharm. Bull., 21 (1973) 1198.
128. Y. Kurono, K. Ikeda and K. Uekama, Chem. Pharm. Bull., 22 (1974) 1261.
129. J.B. Stenlake, Foundations of Molecular Pharmacology (Athlone Press, 1979) Vol. 2, p. 267.
130. W.J. Colwell, G. Chan, V.H. Brown, J.I. DeGraw, J.H. Peters and N.E. Morrison, J. Med. Chem., 17 (1974) 142.
131. R. Bachner, G. Ismail, J. Allen and L. Boxer, Infection (Suppl. 1) 6 (1978) S 129.
132. Ref. 95 p. 1504.
133. B.R. Baker, The Design of Active-Site Directed Irreversible Enzyme Inhibitors (Wiley, New York, 1967).
134. K.R. Chatterjee, Dharmendra and R. Bose, Bull. Calcutta Sch. Trop. Med., 2 (1954) 56; Chem. Abstr., 49 (1955) 12700.
135. E.F. Elslager, Z.B. Gavrilis, A.A. Phillips and D.F. Worth, J. Med. Chem., 12 (1969) 357.
136. T.F. Davey, in Ref. 1, p. 391.
137. E.F. Elslager, A.A. Phillips and D.F. Worth, J. Med. Chem., 12 (1969) 363.
138. E.F. Elslager, D.B. Capps and D.F. Worth, J. Med. Chem., 12 (1969) 597.
139. V.S. Reznik, N.G. Pashkurov, A.A. Muslinkin, E.N. Zhurkina, N.M. Goloshchapov and U.K. Stekeovskii, Br. Pat. 1, 396, 667, 4 June (1975); Chem. Abstr., 83 (1975) 209401.
140. N.M. Goloshchapov, V.K. Steklovskii, V.S. Reznik, N.G. Pashkurov, A.A. Muslinkin and E.N. Borison, Otkrytiya Izobret. Prom. Obratsy Tovarnye Znaki 52 (1975) 14; Chem. Abstr., 83 (1975) 65483.

141. W.H. Jopling, Lepr. Rev., 52 (1981) 104.
142. D.H. Hutson, Foreign Compound Metabolism in Mammals, ed. D.E. Hathaway, Vol. 3, (1975) (Chemical Society, London) p. 466.
143. J.A. Montgomery, T.P. Johnston and Y.F. Shealy, in: Medicinal Chemistry, ed. A. Burger, Part I, 3rd Edn. (Wiley, New York, 1970) p. 680.
144. N.M. Goloshchapov, A.A. Yushchenko, Zn. G. Umerov, V.A. Evastratova, T.S. Gnetynk, B.E. Ivanov, V.S. Rezik, N.G. Pashkurov, S.S. Liverman and S.A. Sharova, Otkrytiya Izobret. Prom. Obratsy Tovarnye Znaki 55 (1978) 243; Chem. Abstr., 90 (1979) 97725.
145. N.M. Goloshchapov, E.F. Lovretskaya, L.J. Chamorovskaya, V.V. Chugunov, B.E. Ivanov, V.S. Reznik, N.G. Pashkurov, N.N. Buslaeva, G.F. Zaika and Yn.S. Butov, Otkrytiya Izobret. Prom. Obratsy Tovarnye Znaki (1979) 107; Chem. Abstr., 92 (1980) 140719.
146. R.A. Ahmad and H.J. Rogers, Br. J. Clin. Pharmacol., 10 (1980) 519.
147. C.M. Carpenter, Ann. N.Y. Acad. Sci., 54 (1951) 101.
148. Merck Index, ed. M. Windholz, 9th Ed. (Merck & Co. Inc., Rahway, NJ, 1976) 8763, p. 1162.
149. H. Ross, Int. J. Lepr., 15 (1947) 236.
150. H. Ross, Int. J. Lepr., 18 (1950) 333.
151. R.G. Cochrane, Practitioner, 188 (1962) 67.
152. H. Brodhage, Lepr. Rev., 29 (1958) 148.
153. E. Grunberg and R.J. Schneider, Ann. N.Y. Acad. Sci., 54 (1951) 107.
154. A. German, T.B. Loc and Y. Painvin, Fr. M 1121 (1962) Chem. Abstr., 58 (1963) 3277.
155. W.A. Hadler and L.M. Ziti, Arzneim.-Forsch., 6 (1956) 531; Chem. Abstr., 51 (1957) 614.
156. E.L. Jackson, J. Am. Chem. Soc., 68 (1946) 1438.
157. M. Hjelm and C.-H. de Vardier, Biochem. Pharmacol., 14 (1965) 119.
158. P.A. Kramer, B.E. Glader and T.-K. Lai, Biochem. Pharmacol., 21 (1972) 1265.
159. Z.H. Israili, S.A. Cucinell, J. Vaught, E. Davis, J.M. Lesser and P.G. Dayton, J. Pharmacol. Exp. Ther., 187 (1973) 138.
160. B. Loev, F. Dowalo and V.J. Theodorides, J. Med. Chem., 16 (1973) 161.
161. V.S. Reznik, N.G. Pashkurov, A.A. Muslinkin, N.M. Smirnov and N.M. Golashchapov, Fr. Demande 2,271,210 (1975); Chem. Abstr., 85 (1976) 5681.
162. D.F. Worth, E.F. Elslager and A.A. Philips, J. Med. Chem., 12 (1969) 591.
163. H. Uota and J. Tanizaki, Japan. Pat. 1554 (1951); Chem. Abstr., 47 (1953) 4910.
164. N.P. Buu-Hoi, Tran-Van-Bang and D.N. Xwong, Bull. Acad. Natl. Med. Paris, 146 (1962) 78.
165. J.B. Stenlake and W.H. Linnell, J. Pharm. Pharmacol., 2 (1950) 736.
166. W.A. Hadler, C.M. Carvalho and A.C. Mauri, Rev. Brasil. Leprol., 20 (1952) 104; Chem. Abstr., 50 (1956) 11520.
167. N. Mukerjee, S. Kundu and R. Bose, Bull. Calcutta Sch. Trop. Med., 9 (1961) 14; Chem. Abstr., 55 (1961) 17894.
168. W.A. Hadler and L.M. Ziti, Int. J. Lepr., 29 (1961) 191.
169. C.C. Shepard, Proc. Soc. Exp. Biol. Med., 124 (1967) 93835.
170. N.E. Morrison, Int. J. Lepr., 39 (1971) 34.
171. A.R.D. Adams and M.F.R. Waters, Br. Med. J., ii (1966) 872.

172. A.C. McDougall, Clin. Exp. Dermatol., 4 (1979) 139.
173. R. Gelber and L. Levy, in Ref. 31a, p. 228.
174. C.C. Shepard, Int. J. Lepr., 37 (1969) 389.
175. S.R. Pattyn, Int. J. Lepr., 40 (1972) 320.
176. J.I. DeGraw, V.H. Brown, W.T. Colwell and N.E. Morrison, J. Med. Chem., 17 (1974) 144.
177. R. Gelber and L. Levy, Int. J. Lepr., 44 (1976) 124.
178. R. Gelber and L. Levy, Int. J. Lepr., 46 (1978) 111.
179. Anon., in Ref. 31a, p. 365.
180. R.J. Cenedella and J.J. Jarrell, Am. Trop. Med. Hyg., 19 (1970) 592.
180a. J. Delville and P.J. Jacques, Biochem. Soc. Trans., 6 (1978) 394.
181. D.M. Thompson, Proc. R. Soc. Med., 68 (1975) 273.
182. M. Gidoh, S. Tsutsumi, T. Funazu, S. Koide and M. Narita, Int. J. Lepr., 48 (1980) 462.
183. R. Anderson and E.M.S. Gatner, Lepr. Rev., 52 (1981) 19, R. Anderson, E.M.S. Gatner, C.E. van Rensburg, G. Grabow, F.M.G.H. Inkamp, S.K. Kok and A. van Rensberg, Antimicrob. Agents Chemother., 19 (1981) 495.
184. P.K. Das, P.R. Keatser, K.W. Pondman, H. Huikeshoven, J.E. Landheer, D.L. Leiker and R.J.W. Rees, Lancet, i (1980) 1309.
185. A. Albert, Selective Toxicity (Chapman and Hall, London) 6th Edn. (1979) p. 543.
186. M.S. Tute, Adv. Drug Res., 6 (1971) 1.
187. J.T. Penniston, L. Beckett, D.L. Bentley, and C. Haasch, Mol. Pharmacol., 5 (1969) 333.
188. M.S. Tute in: Physical Chemical Properties of Drugs, eds. S.H. Yalkowsky, A.A. Sinkula and S.C. Valvani (Dekker, New York, 1980) p. 152; D. Bawden and M.S. Tute, Eur. J. Med. Chem., 16 (1981) 299.
188a. J.K. Seydell, M. Richter and E. Wempe, Int. J. Lepr., 48 (1980) 18; J.K. Seydell and K.-J. Schaper in: Enzyme Inhibitors as Drugs, ed. M. Sandler, (MacMillan, London 1980) p. 53.
188b. M. Bergel, Int. J. Lepr. 49 (1981) 89.
188c. J.K. Seydell, Int. J. Lepr., 49 (1981) 90.
189. K.R. Chatterjee and R.K. Podder, Nature (London), 180 (1957) 854.
190. Anon., in Ref. 31a, p. 377.
191. G.A. Ellard, Br. J. Pharmacol., 26 (1966) 212.
192. H. Uehleke and S. Tabarelli, Naunyn-Schmiedeberg's Arch. Pharmakol., 278 (1973) 55.
192a. D.J. Back, H.S. Purba and C. Staiger, Br. J. Pharmacol., 72 (1982) 109P.
192b. V. Sritharan, V.P. Bharadwaj, K. Venkatesan and B.K. Girdhar, Int. J. Lepr., 49 (1981) 307.
192c. M. Homeida, A. Babikr and J.K. Daneshmend, Br. Med. J., ii (1980) 1180.
192d. K.K. Murthy and K.K.R. Babu, Lepr. India, 52 (1980) 443.
193. B. Naafs and H.W. Wheate, Lepr. Rev., 49 (1978) 153.
194. M. Bergel, in Ref. 31a, p. 231.
195. L. Gricicute and L. Tomatis, Int. J. Cancer, 25 (1980) 123.
196. J.H. Peters, G.R. Gordon, W. Tanaka and V.F. Simmon, in Ref. 31a, p. 230.
196a. Anon., Lancet, ii (1981) 184.
197. H. Arnold, E. Helmert, T. Mobus, R. Prigge, H. Rauen and T. Wagner-Jauregg, Chem. Ber., 75B (1942) 369.

198. M.J. Colston, G.R.F. Hilson, G.A. Ellard, P.J. Gammon and R.J.W. Rees, Lepr. Rev., 49 (1978) 101.
199. G. Tarabini, Proc. 5th Int. Congr. Chemother. (Vienna) 2, (1967) 909; Chem. Abstr., 70 (1969) 27556.
200. M.L. Gaind and A.S. Soli, U.S. Armed Forces Med. J., 20 (1964) 12.
201. J. Languillon and J. Clary, Bull. Soc. Pathol. Exotique, 57 (1964) 531; Chem. Abstr., 62 (1965) 16855.
202. F. Wilkinson, E. Santabaya and J. Gago, Abstr. 5th Int. Congr. Chemother., Vienna (1967) 733.
203. V.C. Barry, Sci. Proc. Dublin Soc., Series A, 16 (1969) 153.
204. Y.T. Chang, Antimicrob. Agents Chemother., (1962) 294.
205. S.G. Browne and L.M. Hogerzeil, Lepr. Rev., 33 (1962) 6.
206. L. Levy, Am. J. Trop. Med. Hyg., 23 (1974) 1097.
207. C.C. Shepard, L.L. Walker, R. van Landingham and M.A. Redus, Proc. Soc. Exp. Biol. Med., 137 (1971) 728.
208. M. Lechat, in Ref. 31a, p. 3.
209. C.C. Shepard, Proc. Soc. Exp. Biol. Med., 132 (1969) 120.
210. S.J. Yawalkar and W. Vischner, Lepr. Rev., 50 (1979) 135.
211. R.St.C. Barneston, in Ref. 31a, p. 311.
212. F.M.J.H. Inkamp, Lepr. Rev., 52 (1981) 135.
221a. J.M.H. Pearson, Lepr. Rev., 52 (1981) 293.
213. R.C. Hasting, R.R. Jacobson and J.R. Trautman, Int. J. Lepr., 44 (1976) 287.
214. Workshop Report on Experimental Chemotherapy, in Ref. 31a, p. 374.
215. L. Levy, Lepr. Rev., 52 (1981) 23.
216. V.C. Barry, J.G. Belton, M.L. Conalty, J.M. Denneny, D.W. Edward, J.F. O'Sullivan, D. Twomey and F. Winder, Nature (London), 179 (1957) 1013.
217. N.E. Morrison and G.M. Marley, Int. J. Lepr., 44 (1976) 133.
218. N.E. Morrison and G.M. Marley, Int. J. Lepr., 44 (1976) 475.
219. N.E. Morrison and G.M. Marley, Int. J. Lepr., 45 (1977) 188.
220. N.E. Morrison and G.M. Marley, Int. J. Lepr., 46 (1978) 109.
221. P.M. Rhodes and D. Wilkie, Biochem. Pharmacol., 22 (1973) 1047.
222. C. de Duve, in: Activation of Macrophages, ed. W.H. Wagner (Elsevier, New York, 1974) pp. 79–83.
223. C.V. Reich and E. de la Cruz, Int. J. Lepr., 47 (1979) 612.
224. N.E. Morrison, Int. J. Lepr., 47 (1979) 613.
225. D.K. Bannerjee, G.A. Ellard, P.J. Gammon and M.F.R. Waters, Am. J. Trop. Med. Hyg., 23 (1974) 1110.
226. L. Ohma and I. Wahlberg, Lancet, ii (1975) 933.
227. W.H. Jopling, Lepr. Rev., 47 (1976) 1.
227a. A.C. McDougall and R.L. Jones, J. Neurol. Neurosurg. Psychiatr., 44 (1981) 116.
228. W.H.O. Report on the Third Meeting of the Scientific Working Group on the Chemotherapy of Leprosy (October 1980) p. 7.
228a. E.M.S. Gatner, R. Anderson, C.E. van Rensburg and F.M.J.H. Inkamp, Lepr. Rev., 53 (1982) 85; F.M.J.H. Inkamp, R. Anderson and E.M.S. Gatner, Lepr. Rev., 53 (1982) 148.
229. H. Wheate, Lepr. Rev., 52 (1981) 190.
230. R.R. Jacobson, in Ref. 31a, p. 307.

231. E. de Bergeyck, P.G. Janssens and A. de Muynck, Lepr. Rev., 51 (1980) 221.
232. A. Bryceson, Lepr. Rev., 50 (1979) 258.
233. G.H. Mason, R.B. Ellis-Pegler and J.F. Arthur, Lepr. Rev., 48 (1977) 175.
234. G. Ramu and C.G.S. Iyer, Lepr. India, 48 (1976) 722.
234a. S.G. Browne, D.J. Harman, H. Wandby and A.C. McDougall, Int. J. Lepr., 49 (1981) 167.
235. E.J. Corey and D.A. Clark, Tetrahedron Lett., 21 (1980), 2045; H. Nagaska, W. Rutsch, G. Schmid, H. Iio, M.R. Johnson and Y. Kishi, J. Am. Chem. Soc., 102 (1980) 7962; H. Iio, H. Nagaska and Y. Kishi, J. Am. Chem. Soc., 102 (1980) 7965; H. Nagaoka and Y. Kishi, Tetrahedron, 37 (1981) 3873.
236. Anon., in Ref. 95a, p. 1176.
236a. Anon., in Ref. 95a, p. 1596.
237. G. Lancini and W. Zanichelli, in: Structure-Activity Relationships among the Semisynthetic Antibiotics, ed. D. Perlman (Academic Press, New York, 1977) pp. 531–600.
238. W. Wehrli and M. Stachelin, in: Antibiotics III, Mechanism of Action of Antimicrobial and Antitumour Agents, eds. J.W. Corcoran and F.E. Hahn (Springer-Verlag, Berlin, 1975) pp. 252–268.
239. G.C. Gallo and P. Radaelli, in: Analytical Profiles of Drug Substances, ed. K. Florey, Vol. 5. (1976) pp. 467–513.
240. D.V.A. Opromolla, L. de Souza Lima and G. Caprara, Lepr. Rev., 36 (1965) 123.
241. R.J.W. Rees, J.M.H. Pearson and M.F.R. Waters, Br. Med. J., i (1970) 89.
242. M.F.R. Waters, R.J.W. Rees, J.M.H. Pearson, A.B.G. Laing, H.S. Helmy and R.H. Gelber, Br. Med. J., i (1978) 133.
243. R.R. Jacobson and R.C. Hastings, Lancet, ii (1976) 1304.
244. Anon. in Ref. 228, p. 2.
245. J. Languillon, S.J. Yawalkar and A.C. McDougall, in Ref. 31a, p. 319, Int. J. Lepr., 47 (1979) 37, S. Paul and E.J. Rose, Int. J. Lepr., 49 (1981) 342.
246. A.B.G. Laing, M.F.R. Waters and R.J.W. Rees, in Ref. 31a, p. 322.
247. S.R. Pattyn, J. Bourland, J. Warnsdorff, A. Cap and E.J. Saerens, in Ref. 31a, p. 321; Lepr. Rev., 53 (1982) 9.
248. S.R. Pattyn and E.J. Saerens, Ann. Soc. Belge Med. Trop., 57 (1977) 169.
249. A.C. McDougall, J.A. Rose and D.G. Grahame-Smith, Experientia, 31 (1975) 1068.
250. C.R. Goucher, J.H. Peters, G.R. Gordon, J.F. Murray, Jr., W. Ichikawa, T.M. Welch and R.H. Gelber, Int. J. Lepr., 46 (1978) 113.
251. G. Acocella and R. Conti, Tubercle, 61 (1981) 171.
252. W.H. Jopling and J.H.S. Pettit, Int. J. Lepr., 47 (1979) 610.
252a. T.W. Beever, L.A. Blair and M.J. Brodie, Br. J. Clin. Pharmacol., 13 (1982) 599 P.
253. R.Y. Poupon, D. Meynield, J. Petit, P. Gustot and F. Darnis, Ann. Med. Interne, 130 (1979) 371.
254. D.G. Girling and K.L. Hitze, W.H.O. Bulletin, 57 (1979) 45.
255. M.J. Brodie, A.R. Boobis, C.J. Dollery, C.J. Hillyard, D.J. Brown, I. MacIntyre and B.K. Park, Clin. Pharmacol. Ther., 27 (1980) 810; Int. J. Lepr., 48 (1980) 461; Clin. Pharmacol. Ther., 28 (1981) 363.
256. K.U. Min, S.M. Park and S.J. Hue, Int. J. Lepr., 48 (1980) 223.
257. E. Rossi and G. Montagna, Int. J. Lepr., 48 (1980) 86.
258. G. Fournier, J. Orgiazzi, B. Lenoir and M. Dechavanne, Lancet, i (1980) 101.

258a. J.E. Kasik and M. Monick, Antimicrob. Agents Chemother., 19 (1981) 134.
259. ILEP Special Meeting on Drug Therapy in Leprosy, Lepr. Rev., 52 (1981) 191.
260. A.R. Lewis and R.G. Shepard, in Ref. 143, Ch. 19.
261. M.J. Colston, G.A. Ellard and P. Gammon, Lepr. Rev., 49 (1978) 115.
262. M.F.R. Waters, J.M.H. Pearson and R.J.W. Rees, Int. J. Lepr., 44 (1976) 152.
263. S.R. Pattyn and M.J. Colston, Lepr. Rev., 49 (1978) 324.
264. Anon., in Ref. 95a, p. 1603.
265. M. Harachap, Lepr. Rev., 52 (1981) 155.
266. K.V. Kale and S.N. Kulkarni, J. Indian Chem. Soc., 54 (1977) 1104.
267. K.V. Kale and S.N. Kulkarni, Indian J. Chem., 17B (1979) 530.
268. M. Hooper and S.N. Kulkarni, Br. J. Pharmacol., 77 (1982) 574P.
269. G.A. Ellard, Lepr. Rev. 32 (1961) 233 and 249.
270. M.S. Tute, G.A. Ellard, D. Self and M. Hooper, unpublished data.
271. A.S. Galabov, B.S. Galabov and N.A. Neykova, J. Med. Chem., 23 (1980) 1048.
272. G.A. Ellard, in Ref. 31a, p. 215; Lepr. Rev., 51 (1980) 199.
273. A. Albert, in Ref. 185, p. 435.
274. L. Levy, Proc. Soc. Exptl. Biol. Med., 153 (1976) 34.
275. G.C. Ferguson, A.J. Nunn, W. Fox, A.B. Miller, D.K. Robinson and R. Tall, Tubercle, 52 (1971) 166.
276. M.Z. Mani, M.V. Rajan, M. Matthew and C.M. Singh, Int. J. Lepr., 48 (1980) 344; G. de L. White, Int. J. Lepr., 48 (1980) 225.
277. N.E. Morrison, D.L. Klayman and F.M. Collins, Int. J. Lepr., 47 (1979) 670.
278. N.E. Morrison, D.L. Klayman and F.M. Collins, Int. J. Lepr., 48 (1980) 497.
278a. N.E. Morrison and F.M. Collins, Int. J. Lepr., 49 (1981) 180.
278b. C. Hansch, E.J. Lien and F. Helmer, Arch. Biochem. Biophys. 128 (1968) 319.
279. A.S. Dobek, D.L. Klayman, E.T. Dickson Jr., J.P. Scovill and E.C. Framont, Antimicrob. Agents Chemother., 18 (1980) 27.
279a. R.A. Casero, D.L. Klayman, G.E. Childs, J.P. Scovill and R.E. Deshardins, Antimicrob. Agents Chemother., 19 (1981) 317.
279b. N.E. Morrison, in Ref. 188c.
279c. D.L. Klayman, J.P. Scovill, J.F. Bartosevich and C.J. Mason, Eur. J. Med. Chem.-Chim. Ther., 16 (1981) 317.
280. S.R. Pattyn, Lepr. Rev., 49 (1978) 199.
281. J.K. Seydel, K.-J. Schaper, E. Wempe and H.P. Cordes, J. Med. Chem., 19 (1976) 483.
282. R.C. Moreen, P. Loisean, J. Bernard, F. Sebastian and R. Levy, Eur. J. Med. Chem., 14 (1979) 317.
283. J.H. Peters, J.T. Murray, G.G.R. Tatsukawa and Y. Matsuo, Int. J. Lepr., 47 (1979) 682; 48 (1980) 302.
284. R.R. Jacobson and R.C. Hastings, Int. J. Lepr., 48 (1980) 498.
285. M.J. Colston, G.R.F. Hilson and R.D. Lancaster, Am. J. Trop. Med. Hyg., 29 (1980) 103.
286. M.J. Colston, G.R.F. Hilson and R.D. Lancaster, in Ref. 31a, p. 232.
287. M. Rosenfeld, E. Freerksen, E. Bonnici and G. Depasquale, in Ref. 31a, p. 316.
288. S. Tsutsumi and M. Gidoh, Int. J. Lepr., 48 (1980) 496.
288a. G. Acocella, Int. J. Lepr., 49 (1981) 331.
289. S. Tsutsumi, S. Godoh, M. Nasita, S. Koide and T. Tunzau, Int. J. Lepr., 46 (1978) 113.

290. G. Ramu, U. Sengupta and K.V. Desikan, Lepr. Rev., 51 (1980) 207.
291. As Ref. 95a, p. 1818.
292. M.F.R. Waters, A.B.G. Laing, A. Ambikapathy, Br. Med. J., i (1979) 792.
293. R. Melsom and M.E. Duncan, Int. J. Lepr., 47 (1980) 472.
294. E.J. Shannon, R.O. Miranda and R.C. Hasting, in Ref. 31a, p. 167.
295. R.C. Hasting, M.J. Morales, S.E. Belk and E.J. Shannon, Int. J. Lepr., 48 (1979) 672.
296. J. Sheskin, in Ref. 31a, p. 299.
297. H. Koch, Drugs of Today, 16 (1980) 388.
298. C.C. Shepard, R. van Landingham and L.L. Walker, Infect. Immun., 16 (1977) 564.
298a. R. Sher, A.A. Wadee, M. Joffe, S.H. Kok, F.M.J.H. Inkamp and I.W. Simpson, Int. J. Lepr., 49 (1981) 159.
298b. S. Kim and Y.W. Cinn, Scienta Lepro, 3 (1980) 65; Int. J. Lepr., 49 (1981) 361.
299. J.E. Cardama, J.C. Gatti, L.M. Balina, J.C. Ocampo and M. Gabrielli, in Ref. 31a, p. 326.
300. G. Renoux, Drugs, 19 (1980) 89.
300a. G. Renoux, Trends Pharmacol. Sci., 2 (1981) 248.
301. F.K. Hess and K.R. Freter in Ref. 3, p. 696.
302. L. Levy, F. Aizer, H. Ng and T.M. Welch, Lepr. Rev., 49 (1978) 215.
302a. R.H. Levin and W.L. Albrecht, Prog. Med. Chem., 18 (1981) 135.
303. J. Delville and P.T. Jacques, Adv. Exp. Med. Biol., 121A, (1980) 269; Chem. Abstr., 92 (1980) 174517.
304. C.C. Shepard and Y.T. Chang, Int. J. Lepr., 32 (1964) 260.
305. Anon., in Ref. 108, p. 138.
306. S.T. Pattyn and E. Saerens, Lepr. Rev., 49 (1978) 275.
307. K. Takayama, E.L. Armstrong, K.A. Kunugi and J.O. Kilburn Int. J. Lepr., 48 (1980) 86.
308. W. Jopling, Int. J. Lepr., 48 (1980) 211.
309. L. Levy and J.A. Anandan, Proc. Soc. Exp. Biol. Med., 158 (1978) 582.
310. R.G. Cochrane, in Ref. 1, Ch. 21.
311. C.R. Goucher, M.C. Cabot, K.A. Borchardt and S. Cruz, 79th Annual Meeting of the American Society of Microbiology May (1979); M.C. Cabot and C.R. Goucher, Lipids, 16 (1981) 146.
312. Anon., in Ref. 148, p. 859.
313. S. Chowdhury and S. Ghosh, Bull. Calcutta Sch. Trop. Med., 22 (1974) 4.
314. S. Chaudhry in Ref. 31a, p. 233.
315. R.P. Tubery, Fr. M. 7333 24 Nov. (1969) Chem. Abstr., 75 (1971) 132993.
315a. Anon., Acta Acad. Med. Sinicae, 1 (1979) 70; Int. J. Lepr., 49 (1981) 124.
316. O.K. Skinsnes, Int. J. Lepr., 44 (1976) 375.
317. R.C. Hastings, V. Richard, Jr. S.A. Christy and M.J. Morales, Int. J. Lepr., 44 (1976) 427.
318. T. Astumi, K. Okamura, Y. Yamamoto and R. Kojima, Japan. Kokai 7,869,837; Chem. Abstr., 89 (1978) 123331.
319. Anon., in Ref. 95a, p. 222.
320. E. Benincasa, Int. J. Lepr., 45 (1977) 299.
320a. T. Horio, S. Imamura, K. Danno and S. Ofuji, Arch. Dermatol., 117 (1981) 29.
320b. M. Neumann, Drugs of the Future, VII (1982) 315.

321. Merck Index, ed. M. Windholz, 9th Edn. (Merck & Co. Inc., Rahway, NJ, 1976) 1998–2000, p. 256.
322. S.K. Chaudhri and S. Ghosh, Ind. J. Dermatol., 18 (1973) 55.
323. R.A. Yurina, M.J. Goryaev and A.D. Dembitskii, Izv. Akad. Nauk. Kaz. SSR, Ser. Kim., 19 (1969) 27.
323a. A. Sen Gupta, S.C. Muta and A.P. Waghrey, J. Sci. Food Agr., 14 (1963) 457; Chem. Abstr., 59 (1963) 11767c; I. Zeman and I. Pokorny, J. Chromatogr., 10 (1963) 14; Chem. Abstr., 59 (1963) 15504.
324. Enzyme-Activated Irreversible Inhibitors, eds. N. Seiler, M.J. Jung and J. Koch-Weser (Elsevier/North-Holland, New York, 1978).
325. N.P. Buu-Hoi, M. Sy and N.D. Xuong, C. R. Acad Sci. 240 (1955); Chem. Abstr., 50 (1956) 7100 and preceding papers, D.G.M. Diaper and J.C. Smith, Biochem. J., 42 (1948) 581; Chem. Abstr., 43 (1949) 1730. T. Kariyone and H. Ageta, Kakugaku Zasshi, 77 (1957) 764; Chem. Abstr., 51 (1957) 17775. H.P. Kaugmann and M.M. Despande, Arch. Pharm., 291 (1958) 576. S.V. Tirodkor, M.S.R. Nair and H.H. Mathur, Indian J. Chem., 6 (1968) 184; Chem. Abstr., 69 (1968) 76701 and preceding papers. Gj. Stefanovic, I. Pegkovic-Tadic and B. Jakovlevic-Simonovic, Tetrahedron Lett., 51 (1966) 6315.
325a. K. Burschkies and C. Scholten, Naturwissenschaften, 31 (1943) 591; Chem. Abstr., 32 (1944) 5001 and previous papers. R. Prigge, Naturwissenschaften, 32 (1944) 83; Chem. Abstr., 42 (1948) 5995h. Th. Wagner-Jauregg, Z. Ges. Extl. Med., 113 (1944) 505; Chem. Abstr., 44 (1950) 757; S.G. Quinza and P.R. Arno, Oleagineux (1956) 619; Chem. Abstr., 51 (1957) 7651. T. Tyoki, Japanese Pat. 3369 (1955), Chem. Abstr., 51 (1957) 14212.
326. M. Hooper, unpublished data.
327. L. Kato and B. Gozsy, Int. J. Lepr., 23 (1955) 406, 413.
328. K.H. Leung and E. Mihich, Nature (London), 288 (1980) 597.
329. P. Baranger and M.K. Filer, Giorn. Ital. Chemioterap., 3 (1956) 384; Chem. Abstr., 52 (1958) 11288; A.G. Davies and J.E. Packer, Chem. Ind., (1960) 1165; J. Chem. Soc., (1961) 4390.
330. T.E. Wallis, Textbook of Pharmacognosy (Churchill, London) 5th Edn. (1967) p. 210.
331. E. Work, Methods in Microbiology (Academic Press, New York) Vol. 6A (1971) p. 407.
332. W.H. Vanneste and A. Zuberbuhler, in: Molecular Mechanisms of Oxygen Activation, ed. O. Hayashi (Academic Press, New York, 1974) Ch. 9.
333. K. Prabhakaran, J. Bacteriol., 107 (1971) 787.
334. K. Prabhakaran, Lepr. Rev., 44 (1973) 112.
335. K. Prabhakaran, E.B. Harris and W.F. Kirchheimer, J. Bacteriol., 100 (1969) 935.
336. K. Prabhakaran, E.B. Harris and W.F. Kirchheimer, Microbios, 5 (1972) 273.
337. K. Prabhakaran, in Ref. 31a, p. 120.
338. K. Prabhakaran, Lepr. India, 49 (1977) 462.
339. K. Reza, S. Talib, and S.K. Imam, Br. Med. J., ii (1979) 900.
340. S.P. Hiremath and M. Hooper, Adv. Heterocycl. Chem., 22 (1978) 86.
341. M. Hooper and P.R. Mahadevan, unpublished data.
342. P. Jayaraman, P.R. Mahadevan, M. Mester and L. Mester. Biochem. Pharmacol., 29 (1980) 2526.

342a. L. Szabados, J.-M. Launay, M. Mester, F. Cottnot, J. Pennee and L. Mester, Int. J. Lepr., 49 (1981) 42.
342b. R.J.W. Rees, Proc. THELEP *ad hoc* Subcommittee on Drug Development, Geneva, 29 Mar. 1982.
343. P. Bey, in Ref. 324, p. 27.
344. J.R. Fozard, M. Spedding, M.G. Palfreymen, J. Wagner, J. Mohring and J. Koch-Wester, J. Cardiovasc. Pharmacol., 2 (1980) 229.
345. Report of the Meeting on Social and Economic Aspects of Leprosy, 1–4 Dec. (1981) UNDP-World Bank-W.H.O. Special Programme for Research and Training in Tropical Diseases.

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2 The Design, Synthesis and Development of a New Class of Potent Antineoplastic Anthraquinones

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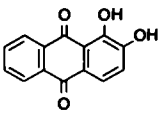
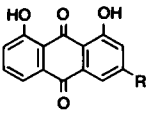
INTRODUCTION

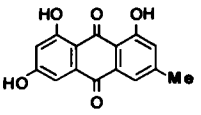
Among numerous quinone derivatives, the anthraquinones, which are most widely distributed in nature and have been found in bacteria, moulds, higher fungi and lichens, flowering plants and insects [1], occupy a prominent role in the dyestuff industry. The technique of dyeing with madder (*Rubia tinctorum* L., Rubiaceae, Shee-Tsao) probably originated in the Far East and came to Europe in ancient times via India, Persia, Egypt, Greece and Rome [2, 3]. More than 100 years ago, the active principle of the dyestuff constituent of madder was found to be alizarin (1,2-dihydroxyanthraquinone or 1,2-dihydroxy-9,10-anthracenedione,(1)) [4, 5] which was subsequently synthesized [6–9]. Since then, with the proper combination of auxochromic substituents, a variety of dyes with colour shades ranging from red, orange, blue, to purple were manufactured and resulted in thousands of patented anthraquinone formulations [10–16]. The bright colour shades, the thermal stability of anthraquinone dyes relative to the benzoquinone and the naphthaquinone dyes, plus the practice of using the reduced, soluble leuco intermediates for vat dyeing, have affirmed the importance of anthraquinone derivatives in the dyestuff industry. The pioneering work on the synthesis of hydroxylated anthraquinones also laid a solid foundation to the chemistry and structural modification studies of anthraquinones to this date.



(1)



(2a) R = Me
(b) R = CH_2OH
(c) R = CO_2H

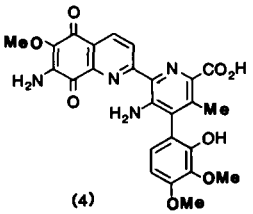


(3)

On the medicinal front, it is known that the use of rhubarb (*Rheum officinale* Baill. *Polygonaceae*, Da-Huang; the active ingredient consists of a mixture of anthraquinones including chrysophanic acid (2a), aloe-emodin (2b), rhein (2c) and emodin (3)) as a laxative has been known for well over 1000 years [17, 18]. These cathartic drugs are still being listed in modern Pharmacopeia. Some 2,6-bis(aminoalkoxy)anthraquinones were reported to possess broad-spectrum antiviral activity against encephalomyocarditis (EMC) virus given either orally or subcutaneously [19]. The anticancer activity of the tetracyclic anthracycline antibiotics adriamycin (doxorubicin), daunomycin (daunorubicin) and carminomycin are well known [1, 20–29]. Newer anthracycline antibiotics such as the aclacinomycins [30, 31], nogalamycin [32–34], the cinerubins [35, 36], the roseorubicins [37], the rubeomycins [38], the bohemic acid complex [39, 40] and others [41] are also reported to possess interesting anticancer activity. Certain simpler synthetic hydroxylated anthraquinone derivatives were claimed to possess low yet reproducible antitumour properties [42–44]. The present review describes the development of a class of relatively simple anthraquinones which possess outstanding antineoplastic activity.

HYPOTHESES

In 1963, the structure of the anticancer antibiotic streptonigrin [45–47] was elucidated as (4) [48]. In the same publication, these investigators noticed the presence of a common structural unit (5) among several very differently consti-



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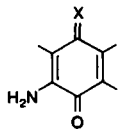
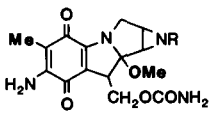
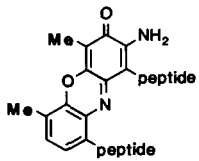


(5) X = O, -N



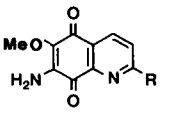
(6a) R = H
(b) R = Me



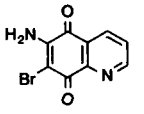
(7)

tuted anticancer antibiotic molecules including streptonigrin, mitomycin C (6a) [49–52], profiromycin (6b) [53, 54] and the actinomycins (7) [55–59]. These authors thus suggested that an intimate relationship may exist between the *ortho*-amino-substituted quinonoid unit, (5), and the marked anticancer activity of those otherwise structurally different antibiotics.

This interesting postulation prompted us to synthesize and study compounds possessing the recommended *o*-aminoquinonoid unit. Initially, synthesis of 7-amino-6-methoxy-5,8-quinolinediones (8a, 8b), which contain the bicyclic moiety of streptonigrin, and a structurally related 7-bromo-6-amino-5,8-quinolinedione (9), was conducted [60, 61]. In addition, some substituted aminobenzoquinones (10) [62] and alkylaminonaphthoquinones (11) [63] were also synthesized and evaluated biologically at the U.S. National Cancer Institute. However, none of these compounds showed any anticancer activity.



(8a) R = H
(b) R = Me



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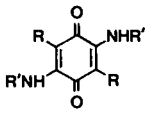
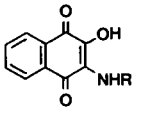
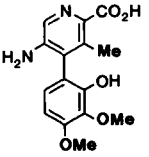


(10) R = Me, Ph
R' = H, alkyl



(11) R = H, alkyl

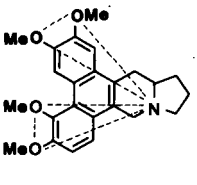


(12)

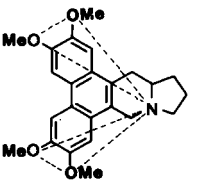
It therefore appeared that the concept of the anticancer activity of the *o*-aminoquinone unit may not be entirely applicable as such and perhaps other factors need to be considered. To satisfy our curiosity, 5-amino-4-(3,4-dimethoxy-2-hydroxy)phenyl-3-methyl-2-pyridinecarboxylic acid (12), the remaining bicyclic portion of streptonigrin, and other structurally related com-

pounds were also synthesized [64, 65], but were again found to be inactive.

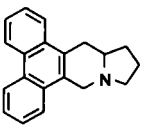
While puzzled by the aforementioned facts, we were attracted by the information that a number of tylophora alkaloids, including tylocrebrine (13) and tylophorine (14), were reported to possess antileukaemic activity against L1210 lymphoid leukaemia in mice [66, 67]. Tylocrebrine and tylophorine differ only in the position of one methoxy group. In order to understand further the importance of these methoxy groups and to uncover any possible minimum structural requirements for biological activity of compounds of this type, the nucleus 9,11,12,13,13a,14-hexahydrodibenzo[*f,h*]pyrrolo[1,2-*b*]isoquinoline (15) [68] was resynthesized and evaluated. The latter compound does not retain the original activity exhibited by the methoxy derivatives [69].



(13)

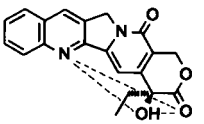


(14)



(15)

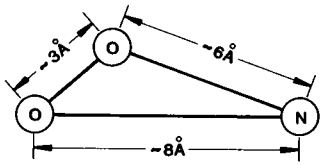
The importance of the oxygen-containing functions of the tylophora alkaloids to anticancer activity is therefore evident. At about this time, several reports on the structure-activity relationship study of the anticancer alkaloid camptothecin (16) [70–73] were published. The hydroxy and carbonyl groups presented in the lactone ring portion of camptothecin play an important role in the activity displayed by this alkaloid, since modification or replacement of these functions resulted in deprivation of its oncolytic property [70, 73].



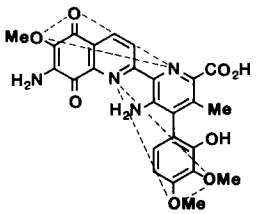
(16)

Using the Briegleb-Stuart [74, 75], the CPK [76, 77] and the Dreiding [78] molecular models, a comparative study of the structure of these compounds was conducted and a common atomic arrangement among these differently constituted molecules was noticed [79]. It consists of three electronegative atoms (each containing at least one lone pair of electrons) – one nitrogen and

two oxygen atoms – which form a triangular pattern and are separated from one another at appropriate interatomic distances shown as follows:



It is obvious that no such triangular pattern could be drawn in the case of compound (15) or the dehydroxylated camptothecin. When this pattern is applied to the structure of streptonigrin, two triangles of similar size could be visualized (see (17)); each involves more than the two separated moieties (8a or 12) of streptonigrin, which may explain the biological inactivity of these two compounds.



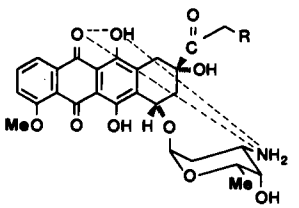
(17)

The observed triangular pattern was used to examine the structure of other natural and synthetic compounds possessing anticancer activity. Initially, measurements of interatomic distances from the molecular models of various structures constructed on the basis of available information of their spatial conformation were carried out. This led to the postulation of the presence of the triangular pharmacophore in more than twenty antineoplastic compounds [79]. Later, it was found that several of the originally proposed compounds should not be incorporated into the original list and, on the other hand, a few newer antineoplastic compounds were reported to possess the designated structural feature in accord with the original observation. The latter groups include a number of alkoxyisoquinoline derivatives such as coralyne [80–84], nitidine [84–89], fagaronine [90–94], and ungeremine [95–99]. A detailed discussion on the assessment of the N-O-O triangulation hypothesis was recently published [100].

Originally, the triangular feature was postulated as a contributing factor in the binding to one of the pertinent receptor sites in certain biopolymers

involved in leukaemia genesis [79]. It was subsequently suggested that compounds with this triangular pattern may simply share a common transport system into neoplastic cells, where each compound can then exert its biological action against the target cells [101].

The anthracycline antibiotics, adriamycin (doxorubicin, 18a) and daunomycin (daunorubicin, 18b) are among the most effective antitumour agents used clinically in recent years [1, 20, 29]. However, these drugs are rather toxic. In addition to producing stomatitis, alopecia, nausea, vomiting, mucositis and bone marrow depression, both antibiotics can cause severe cardiac toxicity. The latter is characterized by a delayed and insidious cardiomyopathy with ECG abnormalities, cyanosis, dyspnea and, in some cases, irreversible congestive heart failure. It therefore creates a very serious problem, especially in the long-term treatment of cancer patients. The toxicity could often be fatal if the accumulated dose of these drugs exceeded a limited amount [102–104]. Although several studies claim that preadministration of ascorbate (vitamin C), α-tocopherol (vitamin E), disodium edetate, 1,2-di(3,5-dioxopiperazin-1-yl)propane, *cis*-diamminedichloroplatinum, selenium, or ubiquinone-10 (coenzyme Q_{10}) may relieve the cardiotoxicity [103, 105–111], the problem remains a grave concern in cancer chemotherapy, specially since the usefulness of some free-radical scavengers such as α-tocopherol as protective agents for cardiotoxicity has been questioned [112, 113].



(18a) R = OH
(b) R = H

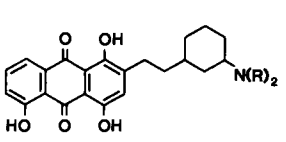
The nitrogen component of the proposed triangular pattern [79] for adriamycin (18a), daunomycin (18b) and related anthracycline antibiotics is located in the amino sugar daunosamine, whereas the two designated oxygen units are situated in the aglycone portion of these molecules. If separated, neither the aglycones nor daunosamine retained anticancer activity [79]. Based on the N-O-O triangulation concept, it was proposed to remove the daunosamine from these molecules and replace the amino function, which contains the pertinent nitrogen atom for completing the triangulation feature,

with an appropriate amino group at a proper spatial distance from the oxygen atoms on the aglycone moiety [114]. Another reason for the proposed removal of the amino sugar is that it may be responsible for the unwanted cardiac action and that in the case of the cardiotonic digitalis, the aglycones are more transient and less potent in their myocardial actions, whereas the glycosides enhance cell penetrability, allowing digitalis to be readily taken up by cardiac tissue [114].

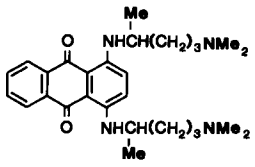
SELECTION OF PROPER STRUCTURES FOR SYNTHESIS

Although a large number of partial and total syntheses were reported for adriamycin, daunomycin and related anthracycline antibiotics, the overall yields of these syntheses are usually exceedingly low because of the vast number of synthetic steps involved. Consequently, for most biological and pharmacological studies, and for clinical evaluation and treatment, the drugs required still relied on naturally produced antibiotics as the main, if not the sole, source even though the yields isolated from the culture broth are generally less than 0.1%. In order to design anticancer compounds for practical use and to simplify the synthesis, it was decided to eliminate the non-planar portion from the tetracyclic aglycone as well as the daunosamine portion (with the exception of the nitrogen constituent), since neither was essential in the participation of the proposed N-O-O triangular pharmacophore. This circumvented the problem of the usual synthetic complications resulting from the stereochemistry of the aglycone nonplanar ring, the stereochemistry of daunosamine and related sugars, and the coupling procedure of these units. In other words, properly selected amino-substituted hydroxyanthraquinones and their derivatives should be a reasonable choice for our synthesis.

A literature search of anthraquinones of this type, which were reported to have close biological connotations, revealed that many anthraquinones and hydroxyanthraquinones containing the aminoalkylamino substituents, such as



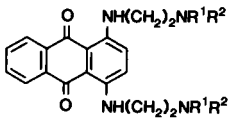
(19a) R = H
(b) R = Me



(20)

(19) and (20), readily interact with DNA [115–117]. These compounds were also designed as model compounds of anthracycline antibiotics.

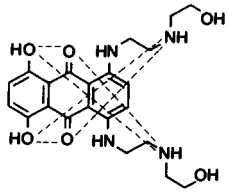
None of these compounds, however, was reported to possess antineoplastic activity. A measurement of interatomic distances from their atomic models indicated that the distances between the nitrogen atoms on the side-chains to the oxygen atoms were much greater than those hypothesized for the triangular pattern. Based on our calculation, the most appropriate location of the terminal nitrogen atom for compounds of type 20 would be two carbons away from the proximal nitrogen atom. Consequently, initial structural modification of compound (20) was proposed as the general structure (21).



(21)

One of these compounds which was originally synthesized as a blue dye for both natural and synthetic textiles is 1,4-bis{2-[(2-hydroxyethyl)-amino]ethyl}amino-9,10-anthracenedione (21a), R^1 = H, R^2 = $(CH_2)_2OH$; NSC-196473) [118]. This compound was also claimed to show 'presumptive activity against leukaemia in mice' [118]. The interesting observation prompted us to synthesize several other compounds of this type [119]. Activity exhibited by these compounds was very encouraging [119–121]. Their structure-activity relationship will be discussed in the next section.

In order to complete the original N-O-O triangular feature, two hydroxyl substituents were introduced into the parent structure and resulted in the creation of 1,4-dihydroxy-5,8-bis{2[(2-hydroxyethyl)amino]ethyl}amino-9,10-anthracenedione (22, NSC-279836, DHAQ), which possesses outstanding antineoplastic activity in experimental animal systems [119–121]. As shown, DHAQ contains two proposed triangular pharmacophores.



(22)

A brief account of the development of DHAQ and related aminoanthraquinones has been reported [122]. Both NSC-196473 (21, R^1 = H, R^2 = $(CH_2)_2OH$) [118] and DHAQ were originally synthesized as free bases. Subsequently, salts of both compounds were prepared [123–125]. Since the literature use of these compounds and their salts was quite liberal and sometimes only NSC numbers were referred to, the list (*Table 2.1*) with NSC numbers is given for a quick categorization. The NSC numbers are issued by the National Cancer Institute of the U.S. National Institutes of Health sequentially; thus, they also provide a chronological history of development. Throughout this review, for the sake of simplicity, compound (22) and its salts will be collectively mentioned as DHAQ, whereas NSC-196473 and its salts will be called AQ.

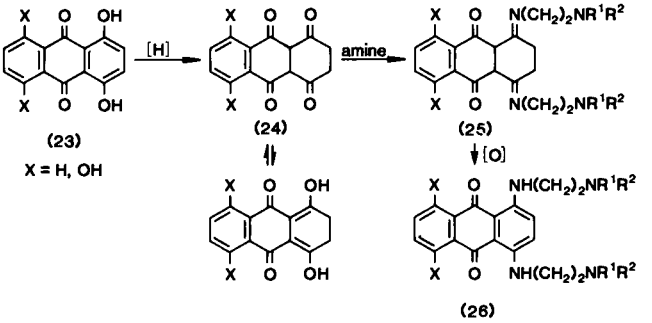
Table 2.1. NAMES OF SOME ANTHRAQUINONES

Parent compound	*Free base or salt*	*NSC No.*	*Other abbreviations or trival names*	*Reference*
AQ	Free base	196473		118
			HAQ	129
			bisalkylAAD	121
	diacetate	287513	ANT	123
			HAQ	132
			DAD	133
			ametantrone	135
DHAQ	free base	279836		119
			dihydroxybisalkylAAD	121
			DIOHA	126
			anthracenedione	131
	diacetate	299195	DHAQ diacetate	134
	dihydrochloride	301739	DHAQ	130
			CL 232315	124
			DHAD	127
			mitoxantrone	128

SYNTHESIS

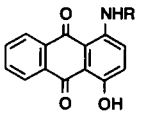
Preparation of various 1,4-bis(substituted alkylamino)-9,10-anthracenediones has been described in the literature of synthetic dyes and pigments. Compounds such as (21) and DHAQ (22) can be readily prepared by the general

procedure [119, 136–141] involving the condensation of leucoquinizarins (24) with an excess amount of the appropriate amines at 50–55°C, followed by air oxidation of the dihydro intermediates (25) to the desired products, (26). Leucoquinizarins (24) are obtained by chemical reduction of the corresponding 1,4-dihydroxy-9,10-anthracenediones (23) with either metal-acid (such as tin or stannous chloride and hydrochloric acid) [142-144] or sodium hydrosulphite [145]. The structures of leucoquinizarins and kinetic studies on the amination reactions were reported recently [146, 147].



In contrast to the synthesis of adriamycin and related anthracycline antibiotics, synthesis of these anthraquinone derivatives can be accomplished in large quantities in less than three steps from the commercially available starting materials. In fact, even the leucoquinizarins (24) are available from industry: for example, both leucoquinizarin (23, X = H) and 5,8-dihydroxyleucoquinizarin (23, X = OH) were made available to us from Bayer, AG of West Germany. Since the intermediate Schiff bases (25) need not be isolated but can be oxidized directly by air from the reaction mixture, the entire synthetic production can be conducted in one reaction vessel.

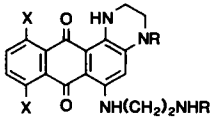
Although several compounds of type 26 (X = H) were reported to have been prepared by the condensation of the unreduced quinizarin with a large excess of amines at higher reaction temperature [125, 148], the yields and purity of products are usually not satisfactory because dealkylation and other side-reactions often take place at elevated reaction temperature. In most cases, only one hydroxyl group of quinizarin is replaced and the other hydroxyl group remains intact [116, 147, 149]. Preparation of these monoaminomonohydroxyanthraquinones (27) can best be conducted in the presence of a solvent, such as butanol [116, 149]. Amination of quinizarin in the presence of metal salts gives a number of β-aminated derivatives and (27), but no 1,4-diaminated product [150].



(27)

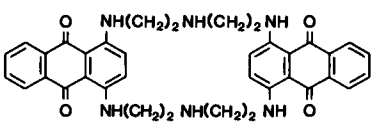
The reaction sequence from (24) to (26) can be monitored either by thin-layer chromatography or by ultraviolet absorption spectra, since the intermediate Schiff bases (25) (colour: orange to green) have a characteristic λ_{max} between 460 and 500 nm in ethanol, whereas the final products (26) (colour: blue) have λ_{max} between 525 and 660 nm. In most cases, the completion of oxidation can even be judged by visual inspection. Oxygen in air is generally adequate for carrying out the oxidation of (25) to (26) [119, 137]. Aminated leuco compounds have also been oxidized by chloroanil [125], oleum [151]. alkaline solution [152, 153], an excess of amine [154], or hot nitrobenzene [125, 155, 156]. The latter, however, often causes dealkylation of the aminated products [147].

The reaction temperature (50–55°C) during amination ((24) to (25)) is quite important, especially when the terminal amino group is either primary (R^1, R^2 = H) or secondary (as in the case of preparing DHAQ) rather than tertiary. At higher temperature, a side-reaction takes place, involving the cyclization of one of the side-chains to form (28) [119, 137]. Contrary to a recent claim [125], cyclization of this type occurs when the quinizarins are still at the reduced stage (i.e., the leuco form) [137].

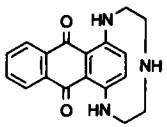


(28)

In addition to the expected (26; X = H, R^1, R^2 = H) and the cyclized (28; X, R = H) products, when ethylenediamine is condensed with leucoquinizarin, a third compound melting at 308–310°C has also been isolated [119]. The structure of this compound, judged by elemental analysis, mass spectrometric determination, and spectroscopic measurements, is a dimeric aminoanthraquinone (29) formed by the elimination of two equivalents of ammonia. The monomeric compound (30), proposed earlier [119], is incorrect.



(29)



(30)

ANTINEOPLASTIC ACTIVITY OF 1,4-BIS(SUBSTITUTED AMINOALKYLAMINO)ANTHRAQUINONES AND RELATED COMPOUNDS IN EXPERIMENTAL ANIMALS

Because of the ease of chemical synthesis, compounds of this type are more amenable to structural variation than are the anthracycline antibiotics. Consequently, a number of compounds were synthesized to conduct a structure-activity relationship study [119]. Prior to the discussion of inhibitory activity, some background information on the test system used may be helpful.

THE NATIONAL CANCER INSTITUTE SCREENING PROGRAM

In 1955, the National Cancer Institute (NCI) of the National Institutes of Health (NIH) of the United States established the Cancer Chemotherapy National Service Center (CCNSC) as the focal point for its anticancer drug development efforts [157], including screening of synthetic and natural products and evaluation of efficacy and safety of compounds found to be 'active' against animal tumours. The task is currently under the jurisdiction of the Development Therapeutic Program of the Division of Cancer Treatment, NCI. More than 350,000 synthetic compounds have been submitted from all over the world and evaluated under this programme.

Currently the tumour system selected for initial screening of synthetic agents is lymphocytic leukaemia P388. The original tumour line was induced in 1955 in a DBA/2 mouse by painting the skin with 3-methylcholanthrene (MCA). P388 is now induced by an ascite fluid implanted intraperitoneally (i.p.) into BDF_1 or CDF_1 mice. The agent to be tested is administered i.p. 24 h after implantation of the tumour. The most common schedule is i.p. daily for 9 days. The survival time of six treated animals with leukaemia is then compared with that of the untreated (controlled) leukaemic mice, and the results are expressed as a percentage of mean survival time of tested versus controlled animals (*T/C*) [158, 159]. An initial *T/C* value equal to or greater than 120% is considered necessary for activity. For an agent to be recognized

as of significant value so as to be considered by the NCI decision network committee (DN2), one of the criteria is that the *T/C* value should be equal or surpass 175% [159–161].

Other test systems commonly used at NCI for drug evaluation include: (a) melanotic melanoma (B16 melanocarcinoma). Its acceptable lower limit for positive control compound is $T/C \geq 140\%$, *T/C* for passing DN2: $\geq 150\%$; (b) lymphoid leukaemia L1210, acceptable *T/C* values are 125% and, for passing DN2, $\geq 150\%$; (c) colon 26 (C6), *T/C* values are $\geq 130\%$ and, for passing DN2, $\geq 140\%$ [158, 159], among others.

ACTIVITY OF 1,4-BIS{2-[(2-HYDROXYETHYL)AMINO]ETHYL}AMINO-9,10-ANTHRACENEDIONE (21a; NSC-196473, AQ)

This compound exhibits exceptional activity against all the aforementioned four tumour systems, as shown in *Table 2.2*.

Table 2.2. ANTITUMOUR ACTIVITY OF AQ (21a)

Tumour system	*Dose (mg/kg)*	T/C *(%)*
P388 leukaemia	25	517
	16	416
	6.25	189
	3.2	239
B16 melanoma	8	280
	4	248
	2	186
L1210 leukaemia	16	250
	8	220
	4	157
Colon 26	8	286
	4	215
	2	139

ACTIVITY OF THREE COMPOUNDS STRUCTURALLY CLOSELY RELATED TO AQ (21a)

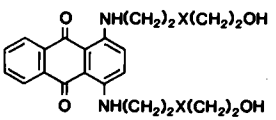
Stepwise shortening of the side-chain substituent R^2 from (21a) resulted in compounds retaining the original activity, but at a somewhat lower level [120] (*Table 2.3*).

Table 2.3. ANTITUMOUR ACTIVITY OF ANALOGUES OF AQ (21a)

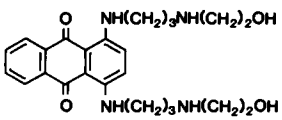
	P388		*B16*		*L1210*		*C6*	
Compound	*Dose*	T/C	*Dose*	T/C	*Dose*	T/C	*Dose*	T/C
(21b, R^1 = Et, R^2 = H)	16	166	16	158	16	130	16	169
NSC-276740	8	174	8	157	8	128	8	166
	4	157	4	127	4	122	4	145
(21c, R^1 = Me, R^2 = H)	12.5	200	8	231	8	185	8	225
NSC-291923	4	175	4	230	4	150	4	215
	2	183	2	150	2	146	2	219
					1	134		
(21d, R^1 = R^2 = H)	16	243	8	218	25	272	16	227
NSC-281246	8	203	4	210	12.5	227	8	213
	4	194			6	156	4	175
	2	185						

OTHER STRUCTURAL MODIFICATIONS OF AQ (21a)

The importance of the nitrogen atom in the centre of the side-chain can be demonstrated as follows. Replacement of this NH linkage by either a sulphur atom (31a) or a methylene unit (31b) resulted in compounds with no activity against leukaemia P388. Compound (32), which contains an additional methylene unit between the two nitrogen atoms on the side-chain of (21a), possesses only marginal activity against P388 (*T/C* values of 133, 130 and 117% at doses of 100, 50 and 25 mg/kg, respectively) [119].

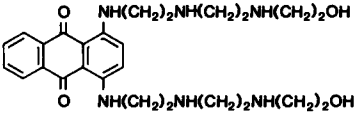


(31a) X = S
(b) X = CH_2



(32)

Insertion of an addition ethylamino unit into the side-chain of (21a), as in (33), drastically reduces the activity below the marginal level, suggesting that additional basic centres may not be desirable [119].



(33)

ACTIVITY OF DHAQ AND COMPARISON WITH THAT OF ADRIAMYCIN AND DAUNOMYCIN

DHAQ (22), which possess two complete N-O-O triangle patterns, was found to exhibit extensive activity against all four experimental tumour systems. A comparison of its antineoplastic activity with that of the two established anti-cancer drugs adriamycin and daunomycin [120] is given in *Table 2.4.*

Table 2.4. COMPARISON OF ANTITUMOUR ACTIVITIES

	P388		*B16*		*L1210*		*C6*	
Compound	*Dose*	T/C	*Dose*	T/C	*Dose*	T/C	*Dose*	T/C
Adriamycin hydrochloride	2	254	6	189	4	291	10.5	205
	1	250	3	412	2	251	8.1	148
	0.5	177	1.5	412	1	129	6.3	197
			0.75	210	0.5	134		
			0.5	158				
Daunomycin hydrochloride	4	105	1	285	2	145	4	128
	2	224	0.5	285	1	126	2	124
	1	234	0.25	188	0.5	120		
	0.5	191	0.13	142				
	0.25	194						
DHAQ	2	280	8	303	18	308	6	305
	1	450	2	317	10.8	261	3	324
	0.5	351	1	222	4	230	1	365
	0.25	280	0.5	262	2	223	0.5	365
					1	274	0.25	359
					0.25	196		

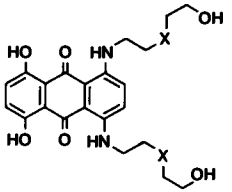
The activity of DHAQ compared favourably with that of the two anthracycline antibiotics. DHAQ, which is comparatively less toxic at higher doses and more active at lower doses than adriamycin and daunomycin, also showed some inhibitory activity against P388 leukaemia strains that had become resistant to adriamycin and daunomycin [120]. The non-ring-hydroxylated compounds (21a)–(21d), however, are completely cross-resistant to the P388 sublines resistant to the two antibiotics. The biological activity profiles of these active aminoanthraquinones and of the anthracycline antibiotics are therefore similar. DHAQ has retained some antileukaemic activity against the resistant strains. Perhaps this may be due to a lipophilicity change caused by ring hydroxylation, which may modify the cellular transport mechanism.

The following question may be posed: If the N-O-O triangulation pattern were indeed of significance to antineoplastic activity, why is it that compounds such as (21a)–(21d), which do not contain all three electronegative atoms at the proper position for the desired triangle formation, still have good inhibitory activity? The answer may be found in the following considerations. Since the dosages required for the unhydroxylated anthraquinones (21a)–(21d) are at least 10-times higher than that required for DHAQ, the possibility of a portion of the chemicals undergoing *in vivo* hydroxylation of (21a)–(21d) to 'complete' the N-O-O triangulation cannot be disregarded. In fact, chemical hydroxylation of 1,4-diamino-9,10-anthracenedione diborate with an oxidizing agent such as sodium persulphate yielded the corresponding 5,8-dihydroxyl derivative [15], which further indicates that positions 5 and 8 of 1,4-diamino-9,10-anthracenedione are electronically the preferred locations for hydroxylation.

ACTIVITY OF SEVERAL DHAQ ANALOGUES

Although the carbon isostere of (21a), compound (31b), was found to be without activity against leukaemia P388 [119], the absence of ring-hydroxylated functions on (31b) may create some doubt that the inactivity of (31b) could be due to the lack of two electronegative elements from the three essential components to complete the N-O-O triangular feature. Consequently, the carbon isotere of DHAQ (22), compound (34a) (NSC-321458), was synthesized. This compound also was found to be without activity against leukaemia P388.

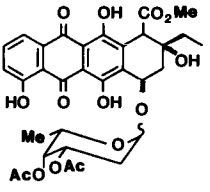
In several recent publications, it is reported that in certain synthetic analogues of the anthracycline antibiotics, when the amino function of the amino sugar is replaced by a hydroxyl or an acetoxyl group, they still possess some activity against P388 or L1210 leukaemia. These compounds include 2′-deoxy-di-*O*-acetyl-D-ribofuranosyl-ε-rhodomycinone (35) [162], 7-*O*-(2,6-dideoxy-α-L-lyxohexopyranosyl)daunomycin (36a), 3′-deamino-3′-hydroxy-



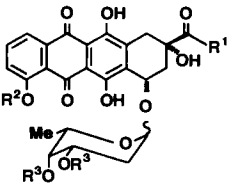
(34a) X = CH_2
(b) X = O

daunomycin and its diacetyl derivative (36b) [163], the corresponding 3′-deamino-3′-hydroxyadriamycin analogue (36c) and its diacetyl derivative (36d) [164], and 2′-deoxy-L-fucopyranosylcarminomycinone (36e) [165]. However, the dosages required for activity are generally larger than those for the established antineoplastic agents adriamycin, daunomycin or DHAQ. Activity displayed by these oxygenated analogues may not always be potent. In addition, most other glycosides synthesized by these investigators failed to show activity against P388 or L1210 leukaemia [162–165]. Nevertheless, the information suggests that the significance of the nitrogen atom in both the side-chains of DHAQ (22) be re-examined with respect to the corresponding oxygen atom.

Compound (34b) was therefore synthesized [166]. This compound failed to show activity against P388 leukaemia even at a dosage as high as 200 mg/kg. This information, coupled with the other negative results of compounds (31a), (31b) and (34a), indicated that the oxygen atom, the sulphur atom, or the carbon atom, cannot replace the special nitrogen atom for antineoplastic activ-



(35)



(36a) $R^1 = R^3 = H$, $R^2 = Me$
(b) $R^1 = H$, $R^2 = Me$, $R^3 = Ac$
(c) $R^1 = OH$, $R^2 = Me$, $R^3 = H$
(d) $R^1 = OH$, $R^2 = Me$, $R^3 = Ac$
(e) $R^1 = R^2 = R^3 = H$

ity in the aminoanthraquinone series. The relevancy of the nitrogen atom to the originally proposed N-O-O triangulation pharmacophore is therefore reaffirmed.

For achieving antineoplastic activity, this pertinent nitrogen atom does not necessarily need to be in the form of secondary or primary amines. A compound such as 1,4-dihydroxy-5,8-bis[2-(dimethylamino)ethyl]amino-9,10-anthracenedione (26, X = OH, $R^1 = R^2$ = Me; NSC-332361) containing an tertiary amino function also displays activities shown in *Table 2.5*. Its activity is, however, slightly lower than that of DHAQ.

A comparison of the structures of DHAQ and adriamycin revealed another interesting fact: although the two side-chains of DHAQ are identical, these chains could assume different structural conformations. One of the terminal hydroxyl groups may occupy the same spatial position as the hydroxyl function on the side-chain of adriamycin (18a), whereas the other terminal hydroxyl group of DHAQ may occupy the same position as the hydroxyl function on the amino sugar daunosamine. In addition, in the latter case, both the hydroxyl groups and the amino functions are separated by exactly two carbon atoms.

Excessive steric hindrance at the vicinity of the essential nitrogen atom has a detrimental effect on the antineoplastic activity. The *N,N'*-dibenzylated derivative of DHAQ (26, X = OH, $R^1 = CH_2Ph$, $R^2 = CH_2CH_2OH$; NSC-322778) for example, only exhibits activity against P388 leukaemia at high dosage (*T/C*: 194 and 166 at 320 and 160 mg/kg, respectively). Dosages higher than those of the parent compounds are also required for inhibitory action against P388 leukaemia in the *N*-benzylated derivatives of the anthracycline antibiotics [167].

Table 2.5. ACTIVITY OF NSC-332361

Tumour system	*Dose (mg/kg)*	T/C *(%)*
P388 leukaemia	25	304
	12.5	226
	6.25	182
	3.12	166
B16 melanoma	6.25	151
L1210 leukaemia	25	171
	12.5	233
	6.25	218

1-HYDROXY-4-(SUBSTITUTED AMINO)ANTHRAQUINONES

Compounds of type (27), wherein the substituent, R, contains the proper amino function separated from the ring-proximal nitrogen by two carbon atoms, still possess antineoplastic activity (*Table 2.6*) [149]. As expected, higher dosages are required for achieving inhibitory action. Again, the side-chain $NHCH_2CH_2NHCH_2CH_2OH$ seems to be a better choice than $NHCH_2CH_2NMe_2$.

Table 2.6. EFFECT OF VARIATION IN THE SIDE-CHAIN OF COMPOUND (27)

	P388		*B16*		*L1210*		*C6*	
Compound	*Dose*	T/C	*Dose*	T/C	*Dose*	T/C	*Dose*	T/C
(27, $R=CH_2CH_2NHCH_2CH_2OH$)	50	261	80	186	80	156	16	263
NSC-299187	25	198	40	163	40	146	8	245
	12.5	179	20	162	20	125	4	212
	6.25	164	10	124			2	152
(27, $R=CH_2CH_2NMe_2$)	160	153	200	156	160	139	not tested	
NSC-300579	80	135	100	145				
			40	135				

COMPARISON OF THE ANTINEOPLASTIC ACTIVITY OF DHAQ WITH SEVERAL STANDARD ANTICANCER DRUGS

As mentioned previously (see section on activity of DHAQ), the activity of DHAQ compared favourably with those of adriamycin and daunomycin [120]. Subsequent studies indicated that DHAQ is more active than adriamycin [168], cyclophosphamide, 5-fluorouracil, methotrexate, vincristine, cytosine arabinoside, or thio-TEPA in the treatment of P388 and L1210 leukaemias [169]. DHAQ is active by the following routes of administration: intraperitoneal, subcutaneous and intravenous [169]. DHAQ, like adriamycin, is not active when administered by the oral route.

Therapeutic synergism in experimental animals was observed when DHAQ or AQ was combined with other clinically useful agents including *cis*-platinum, 5-fluorouracil, vincristine [126, 170] in transplantable animal tumour studies. Combination therapy is particularly useful when there is no overlap in toxicity so that the maximum tolerated dose of each agent can be used.

IN VITRO BIOLOGICAL ACTIVITY

CHINESE HAMSTER CELLS

A study of the antiproliferative activity of adriamycin, DHAQ, AQ and several related aminoanthraquinones on Chinese hamster ovary (CHO) cells [171] revealed the following results: both DHAQ and adriamycin inhibited cell proliferation and shifted the cell population toward larger cells. Adriamycin, which possesses one N-O-O triangular pharmacophore, inhibited cell replication at a concentration of 50 nM (5×10^{-8} M), whereas DHAQ, which possesses two N-O-O sets, exhibited inhibitory activity at concentrations as low as 10 nM. Inhibitory activity of the dimethylamino analogue of DHAQ (26, X = OH, $R^1 = R^2$ = Me; NSC-332361) and of the monohydroxylated analogue of DHAQ (1-hydroxy-5,8-bis{2-[(2-hydroxyethyl)amino]ethyl}amino-9,10-anthracenedione, NSC-326663; MHAQ) are at the proximate level as that of adriamycin. These data are comparable with the *in vivo* results. On the other hand, AQ was ineffective even at the highest concentration (1000 nM) tested. This again emphasizes the importance of the N-O-O triangulation structural feature, since no ring-hydroxylation could be realized in this *in vivo* experiment [171, 172].

The effect of DHAQ and adriamycin on the survival of synchronous CHO cells also revealed similar results, i.e., it takes a 5–10-fold greater concentration of adriamycin than that of DHAQ to achieve the same level of cell killing [173]. There are also differences in the cell-cycle response: DHAQ produces the greatest cytotoxicity to cells in the G_1 phase and blocks cell-cycle progression at the G_2 phase, whereas adriamycin is more effective in the S phase [173–176]. In other words, although the biological activity profiles of DHAQ and adriamycin are similar, their cytotoxic effects toward cell cycle population are not the same and are actually complementary to each other. Cell-cycle kinetics study of AQ revealed that, although the drug-induced cycle block is specific to the G_2 phase, the drug-induced kill is not cycle phase-specific [123].

Both DHAQ and adriamycin induce sister-chromatic exchanges (SCE) and chromosomal aberrations in CHO cells. DHAQ has a stronger genotoxic effect (at 1 nM concentration) than either adriamycin or the other anthraquinone derivatives studied [134, 177]. The DNA-damaging action of these compounds correlates well with their antiproliferative effect on cells [134–171], and this action is detectable at concentrations significantly below that which causes inhibition of cell proliferation.

HUMAN TUMOUR CELLS

Both DHAQ and AQ were studied on cell survival and cell cycle progression in cultured Friend leukaemia cells and the results are in agreement with those studies with Chinese hamster cells [123, 175, 178]. The inhibitory effects of DHAQ on dividing and non-dividing (i.e., G_0 phase) human diploid fibroblasts and colon carcinoma cells were explored. It was found that DHAQ can kill both dividing and non-dividing cells equally with high efficiency [179, 180]. On the other hand, adriamycin is more effective against dividing cells of both strains and cytosine arabinoside is without effect against the plateau-phase fibroblasts. DHAQ, therefore, could be useful against slow-growing tumours with low growth fractions.

All five human breast tumour xenocraft lines were found to be sensitive to DHAQ, suggesting that DHAQ may be useful in the treatment of human breast cancer [181].

The use of a soft agar clonogenic assay for human tumour stem cells [182–184] has been studied by many investigators in recent years. Although still at its development stage, the assay may be applicable in the prediction and correlation of clinical response. DHAQ was reported to possess excellent antitumour activity in this assay against adenocarcinoma of the lung, small cell lung cancer, melanoma and biliary tree cancer, and good antitumour activity against breast cancer, ovarian cancer, non-Hodgkin's lymphoma, head and neck cancer, squamous cell lung cancer, soft tissue sarcoma, gastric cancer, and hepatomas [185–187]. DHAQ is inactive against colon cancer in the human tumour cloning system [186].

MODE AND MECHANISMS OF ANTINEOPLASTIC ACTION

Direct interaction or intercalation with DNA has long been assumed as a common mechanism of action among a number of antineoplastic agents containing planar polycyclic ring structures, including the anthracycline antibiotics [24, 188]. However, this popular hypothesis still cannot be accepted at its face value, since the DNAs studied by different investigators are generally not specific for neoplastic cells. Many other chemotherapeutic agents useful against bacteria, malaria and schistosomiasis, etc., are said to owe their biological action to DNA intercalation. One of the questions often asked is, 'If binding to DNA produces effective chemotherapeutic agents, then what is the basis of their selective toxicity?'

Studies on the interaction of amino-substituted anthraquinones with DNA

were initiated prior to the synthesis of DHAQ [115–117]. As mentioned previously, although a number of these anthraquinones were found to bind to DNA as characterized by spectroscopic measurements, no antineoplastic activity from these compounds was noted [189]. Intercalating to DNA by DHAQ was demonstrated by studies involving the production of protein-associated DNA single-strand breaks in mouse L1210 cells [127], causing an upward shift in the calf thymus DNA melting temperature [121], displacement of fluorescence polarization of the binding of acridine orange to calf thymus DNA, *Micrococcus luteus* DNA and synthetic DNA polymers [131], and suppression of G_2 phase progression, binding to nuclear chromatin, as well as from spectrophotometric titration [190, 191]. Nevertheless, since all the aminoanthraquinones evaluated, regardless of their activity as antitumour agents in vivo, were found to bind strongly to DNA [121, 191], factors other than DNA binding must account for the antineoplastic activity of compounds of this group. A recent report that adriamycin could exert its cytotoxic action without entering the cells but solely by interaction at the cell surface [192] further affirmed the fact that DNA intercalation cannot serve as the ultimate answer for antineoplastic action. The observed drug-DNA intercalation could just be a late subterminal event (after the DNAs in eukaryotic cells had been stripped of all the surrounding histones and other proteins) rather than the prime mode of action.

DHAQ, which strongly inhibits the synthesis of DNA and moderately inhibits RNA and protein synthesis, induces both single- and double-strand breaks on simian virus 40 chromosomal DNA. Since this drug inhibits both cellular and viral chromosome replication, the primary target of DHAQ is believed to be at the chromosome level [193].

Adriamycin, DHAQ and related aminoalkylaminoanthraquinones which demonstrated anticancer activity in experimental animals were found to bind to certain macromolecular lipids extracted from L1210 leukaemia cells [194, 195]. These lipids, which have molecular weights between 10,000 and 25,000, did not bind in significant quantities those aminoalkylaminoanthraquinones which are inactive as antineoplastic agents [194, 195]. These isolated macromolecular lipids may furnish information on binding sites for antineoplastic agents *in vivo*.

In a study on the development of resistance to DHAQ in human colon carcinoma cells, it was found that resistance could be due to an alteration of the cell membrane or cytoplasm, which resulted in decreased uptake of this drug [196]. Perhaps detailed investigation of the cell membrane of normal and neoplastic cells could not only uncover clues to the mechanism of action of DHAQ, but may assist in further understanding of the biochemistry of cancer cells as well.

PHARMACOLOGICAL AND TOXICOLOGICAL STUDIES

PHARMACOLOGY

Since metabolic activation of free-radical semiquinone intermediates with subsequent formation of superoxide has been demonstrated for many quinone-containing antineoplastic agents [197], both DHAQ and AQ were subjected to metabolic activation by NADPH-cytochrome *P*-450 reductase [132]. This enzyme has been established as the flavoprotein which most effectively catalyzes the one-electron transfer from NADPH to oxidized quinone, forming the semiquinone radical which then reacts with molecular oxygen to form superoxide, with concomitant regeneration of the oxidized quinone [198, 199].

Although adriamycin and daunomycin are readily metabolized by *P*-450 reductase, both DHAQ and AQ are bioactivated at a substantially slower rate [132]. AQ actually inhibits *P*-450 reductase activity and microsomal oxidative drug metabolism [132].

A number of methods using the high-performance liquid chromatography (HPLC) technique and analyzed by reversed-phase partition chromatography have been utilized to assess the purity of various aminoanthraquinones and to detect levels of DHAQ from plasma, urine and other tissues in experimental animals and patients [137, 200–202]. In beagle dogs, DHAQ disappears rapidly from plasma after intravenous injection and is detected shortly in bile and urine. After 24 h, DHAQ can be found in kidneys, liver, spleen and stomach, but not in brain, spinal cord and bone marrow. There is extensive tissue binding by DHAQ. This drug is not metabolized to any active form and appears to be excreted unchanged [203–206]. The pharmacological disposition of AQ is similar to that of DHAQ. There is a high plasma clearance and AQ is excreted in the urine unchanged. AQ can be found distributed in the liver, kidneys, pancreas, small intestine and stomach [129].

Clinical pharmacology studies revealed that DHAQ is cleared from the plasma essentially biphasically with a long half-life (37.4 h) during the terminal phase, which is compatible with its low urinary excretion. Contrary to the results from experimental animal studies, there is indication that DHAQ is extensively metabolized [207]. DHAQ readily penetrates tissues, is highly bound to plasma protein, is retained by nucleated blood cells, and makes a slow exit [206–210]. Consequently, the persistence of DHAQ would have significant clinical implications.

TOXICITY IN EXPERIMENTAL ANIMALS

In preliminary testing in rats, it was found that DHAQ caused chronic cardiotoxicity in rats but at an accumulated dose higher than that required for adriamycin [120]. Subsequent study using Sprague-Dawley rats disclosed that AQ was 10-fold less toxic than adriamycin and caused only minor electrocardiogram (ECG) changes, whereas DHAQ was somewhat more toxic than adriamycin, caused marked leukopenia and induced ECG changes and moderate elevation of serum glutamic-oxaloacetate transaminase, lactic dehydrogenase, and creatine phosphokinase. Both aminoanthraquinones induced marked alterations of mitochondrial structure in the heart, but no dilation of the sarcoplasmic reticulum or distortion of the contractile elements [211]. The investigators concluded that the cardiotoxic effects of the anthracenediones are of a less specific nature than those caused by adriamycin.

In studies involving a combination of DHAQ and radiation therapy against L1210 mice and Walker 256 carcinoma rats, wherein therapeutic benefits greater than that produced by either agent alone were reported [212, 213], a delayed (greater than 200 days) toxicity in rats was observed [214]. This long-term toxicity is not detectable by short-duration toxicity screening. Appropriate precautions should therefore be considered in the design of clinical trials.

Toxicity assessment of DHAQ in beagle dogs indicated that myelosuppression [215], gastrointestinal toxicity, and fluctuating decreases in white blood cell counts [216] were observed. Adriamycin-associated cardiotoxicity such as ECG changes and progressive cardiomyopathy was not observed in dogs treated with DHAQ [216]. No evidence of either morphologic or clinical cardiotoxicity was seen in dogs treated with the maximum tolerated dose (0.25 mg/kg i.v. once every 3 weeks) of the dihydrochloride salt of DHAQ [217].

CLINICAL TRIALS

At an average starting intravenous dose of 12 mg/m^2* every three weeks, myelosuppression (in the form of leucopenia, granulocytopenia, neutropenia and, to a lesser degree, thrombocytopenia) appears to be the dose-limiting toxicity of the dihydrochloride salt of DHAQ in children and adults [128, 215, 218–235] but it is usually of short duration and is rapidly reversible. Non-

* For large animals, the dose of drug is expressed more effectively as milligrams per square metre of body surface. This convention is also being used for cancer patients.

haematologic side-effects such as nausea, vomiting and anorexia are uncommon and insignificant [128, 215, 220, 225, 227, 234]. Blue or green discoloration of the urine occurred in some cases [218–220, 222, 225] but not in others [221, 230].

The cardiotoxicity of adriamycin, which was one of the major reasons for the development of DHAQ [114, 119, 120, 122], has been extensively studied. In most clinical trials there was no report of DHAQ cardiotoxicity [128, 221, 230, 232, 234, 236, 237]. Patients who developed congestive heart failure were usually those who had received prior adriamycin therapy [231, 238–241]. Some patients treated with DHAQ were found to have ECG changes or acute arrhythmias, but it is believed that this should not pose a major problem [233, 240]. It is also important that care be taken to distinguish drug-induced arrhythmias from the pre-existing but unrecognized or undetected arrhythmias in cancer patients.

The possible mode of action of the cardiotoxicity of adriamycin and related antibiotics has been studied by many investigators. A number of hypotheses, including free radical formation, interaction between adriamycin and cardiolipin (a phospholipid of the inner mitochondria membrane) [242], and cardiac guanylate cyclase inhibition [243], were postulated. None of these postulations correlates well with actual clinical results. For example, adriamycin and all of the aminoanthraquinones, including DHAQ, were found to be inhibitory to cardiac guanylate cyclase, but aclacinomycin A, another anthracycline antibiotic, was considered as non-inhibitory [243]. However, cardiac evaluation in patients revealed that both DHAQ and aclacinomycin showed slight dilation of tubular structures and mitochondria and occasional myofibrillary loss but no patient developed congestive heart failure [244]. These studies suggest that DHAQ, unlike adriamycin, is at most only mildly cardiotoxic (see p. 118).

For the dihydrochloride salt of DHAQ, the most common dosage used in Phase I clinical trials was 5 mg/m^2 weekly for a total of 6 weeks [229] or 3–4 mg/m^2 daily × 5 at 4-week intervals [231]. The recommended dosage for Phase II trials ranges from 6 mg/m^2 weekly × 3 [215, 235], 3–4 mg/m^2 daily × 3 at 3-week intervals [220, 225], 12 mg/m^2 as a single dose repeated every 3–4 weeks [218], to 4–4.2 mg/m^2 daily × 5 at 4-week intervals [221, 226]. For poor-risk patients, dosages were recommended to be reduced by 25–60% [221, 225, 226].

Phase I clinical trials for AQ have also been conducted. In general, a higher dosage than for DHAQ was used in clinical trials, which is in accord with the experimental animal studies [120]. At a starting dose of 10 mg/m^2 of the diacetate salt of AQ and dose levels escalating up to 180 mg/m^2, the following was observed: leucopenia was dose-related, predictable, rapidly reversible and

dose-limiting. Thrombocytopenia was only encountered in very few courses. Recoverable severe neuropenia was also seen. There was no evidence of cumulative myelosuppression with repeated courses. Non-haematological toxic effects were negligible. Reversible blue skin discoloration and dark blue urine were observed [133, 245, 246]. As in the case for DHAQ, AQ appears to be well tolerated and easy to handle. A dose schedule of 135 mg/m^2 daily for 2–3 weeks for Phase II clinical studies of AQ in solid tumours was recommended [245].

Preliminary Phase II clinical trials for DHAQ revealed the following results: DHAQ has significant activity against refractory metastatic breast cancer [226, 229–231, 246—248]. Responses have been observed in Hodgkin's disease [230, 235], non-Hodgkin's lymphoma, melanoma, renal cell carcinoma, and cylinderoma of the oral cavity [229, 230, 249], acute lymphoblastic leukaemia, non-lymphocytic leukaemia, blastic chronic myelogenous leukaemia [233, 250], and a minor tumour response against fibrosarcoma [225].

DHAQ has shown activity against the model Madison 109 lung carcinoma [251]. This drug was found to have some activity against oat cell carcinoma of the lung [234] and may have a role in the treatment of patients with advanced non-oat cell cancer [252].

GENERAL REMARKS

In the search for synthetic compounds for use as chemotherapeutic agents, the following steps are generally followed: (a) locate promising areas for the synthetic effort; (b) rationalize specific target compounds; (c) devise efficient and practical synthetic methods; (d) synthesize compounds in limited quantity for the purpose of identification and characterization; (e) prepare compounds in adequate quantity for biological evaluation; (f) test the compounds in proper biological systems, and (g) analyze and interpret biological data resulting from the tests. When compounds so designed are found to possess activity, the results will be compared with known agents and, if improvements such as minimizing toxicity and/or increasing the activity or specificity are needed, the above procedure is repeated beginning with step (b). In other words, this is a constant process in a drug development laboratory.

In the unceasing search for chemotherapeutic agents, too often step (e) could not be realized because the synthetic routes devised in step (c) were not practical. In our opinion, more than 10–12 synthetic steps from available starting chemicals cannot be considered as practical unless each of the steps involved can give rise to products in extremely high yields.

Following the aforementioned scheme, the sequence of design, synthesis and development of DHAQ and related anthraquinones is presented. Since these compounds, which do not contain asymmetric carbon atoms, are relatively simple in chemical structure and easy to synthesize, studies of compounds of this type will continue in connection with the mode and mechanism of action, short- and long-term toxicity, and their possible use, either alone or in combination with other agents or modalities, for the treatment of cancer.

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REFERENCES

1. R.H. Thomson, Naturally Occurring Quinones (Academic Press, London, 1971) p. 1.
2. L.F. Fieser, J. Chem. Educ., 7 (1930) 2609.
3. G. Schaefer, CIBA Rev., 4 (1941) 1407.
4. C. Graebe, Ann. Chem., 146 (1868) 1.
5. C. Graebe and C. Liebermann, Ann. Chem., Suppl. 7 (1870) 257.
6. H. Caro, C. Graebe and C. Lierbermann, Br. Pat. 1936/69 (1869).
7. W.H. Perkin, Br. Pat. 1948/69 (1869).
8. H. Caro, C. Graebe and C.Liebermann, Ber., 3 (1870) 359.
9. W.H. Perkin, Monit. Sci. (1879) 1011.
10. R.N. Shreve, Dyes Classied by Intermediates (Chemical Catalog Company, Inc., New York, 1922).
11. H.A. Lubs, The Chemistry of Synthetic Dyes and Pigments (Reinhold, New York, 1955).
12. J.K. Skelly, Chem. Ind., (1965) 1525.
13. E.J. Coates, J. Soc. Dyers Colour., 83 (1967) 95.
14. K. Venkataraman, The Chemistry of Synthetic Dyes (Academic Press, New York) Vol. 2 (1952); Vol. 4 (1970-1971).
15. G.W. Greenhalgh, Endeavour, 35 (1976) 134.
16. A.T. Peters and B.A. Tenny, J. Soc. Dyers Colour., 93 (1977) 373, 378.
17. E. Steinegger and R. Hänsel, Lehrbuch der allgemeinen Pharmakognosie (Springer, Berlin, 1963).
18. M.H. Zenk and E. Leistner, Lloydia, 31 (1968) 275.
19. A.D. Sill, E.R. Andrews, F.W. Sweet, J.W. Hoffman, P.L. Tiernan, J.M. Grisar, R.W. Fleming, and G.D. Mayer, J. Med. Chem., 17 (1974) 965.
20. H. Brockmann, Fortschr. Chem. Org. Naturst., 21 (1963) 121.

21. C.C. Cheng and K.Y. Zee-Cheng, J. Pharm. Sci., 61 (1972) 485.
22. A. DiMarco, F. Arcamone. and F. Zunino, in: Antibiotics, Vol. 3, Mechanism of Action of Antimicrobial and Antitumor Agents, eds. J.W. Corcoran and F.E. Hahn (Springer-Verlag, New York) (1975) p. 101.
23. F. Arcamone, Lloydia, 40 (1977) 45.
24. A. DiMarco, in: Antineoplastic and Immunosuppressive Agents. II, eds. A.C. Sartorelli and D.G. Johns (Springer-Verlag, Berlin) (1975) pp. 593—614.
25. S.K. Carter, J. Nat. Cancer Inst., 55 (1975) 1265.
26. W.A. Remers, The Chemistry of Antitumor Antibiotics (John Wiley & Sons, New York) Vol. 1 (1979) pp. 63–132.
27. L.E. Goldberg, N.G. Shepelevtseva, I.P. Belova and T.P. Vertogradova, Antibiotiki, 29 (1976) 469.
28. S.T. Crooke, J. Med., 8 (1977) 295.
29. S. Hori, M. Shirai, H. Shinchi, T. Oki, T. Inui, S. Tsukagoshi, M. Ishizuka, T. Takeuchi and H. Umezawa, Gann, 68 (1977) 685.
30. J.R. Brown, Prog. Med. Chem. 15 (1978) 125.
31. T. Oki, T. Takeuchi, S. Oka and H. Umezawa, in: Recent Results in Cancer Research, eds. G. Mathé and F.M. Muggia (Springer-Verlag, Berlin) Vol. 74 (1980) p. 207.
32. P.F. Wiley, F.A. MacKellar, E.L. Caron and R.B. Kelley, Tetrahedron Lett., (1968) 663.
33. B.K. Bhuyan and C.G. Smith, in Ref. 24, p. 623.
34. P.F. Wiley, R.B. Kelly, E.L. Caron, V.H. Wiley, J.H. Johnson, F.A. MacKellar and S.A. Mizsak, J. Am. Chem. Soc., 99 (1977) 542.
35. R.K. Johnson, A.A. Overjera and A. Goldin, Cancer Treat. Rep., 60 (1976) 99.
36. T.W. Doyle, in: Anthracyclines: Current Status and New Developments, eds. S.T. Crooke and S.D. Reich (Academic Press, New York, 1980) p. 27.
37. Y. Matsuzawa, A. Yoshimoto, T. Oki, T. Inui, T. Takeuchi and H. Umezawa, J. Antibiot., 32 (1979) 420.
38. Y. Ogawa, H. Sugi, N. Fugikawa and H. Mori, J. Antibiot., 34 (1981) 938.
39. D.E. Nettleton, Jr., D.M. Balitz, T.W. Doyle, W.T. Bradner, D.L. Johnson, F.A. O'Herron, R.H. Schreiber, A.B. Coon, J.E. Moseley, and R.T. Myllymaki, J. Nat. Prod. 43 (1980) 242.
40. T.W. Doyle, D.E. Nettleton, R.E. Grulich, D.M. Balitz, D.L. Johnson, and A.L. Vulcano, J. Am. Chem. Soc., 101 (1979) 7041.
41. T. Nishimura, K. Muto and N. Tanaka, J. Antibiot., 31 (1978) 493.
42. S.M. Kupchan and A. Karim, Lloydia, 39 (1976) 223.
43. J. Fuska, A. Fusková and Z. Vaněk, Neoplasma, 23 (1976) 227.
44. V.K. Tandon, R. Singh, J.M. Khanna and N. Anand, Indian J. Chem., 15B (1977) 839.
45. K.V. Rao, K. Biemann and W.P. Cullen, Antibiot. Annu., (1959–1960) 950.
46. W.I. Wilson, C. Labra and E. Barrist, Antibiot. Chemother., 11 (1961) 147.
47. J.J. Oleson, L.A. Calderella, K.J. Mjos, A.R. Reith, R.S. Thie and I. Topim, Antibiot. Chemother., 11 (1961) 158.
48. K.V. Rao, K. Biemann and R.B. Woodward, J. Am. Chem. Soc., 85 (1963) 2532.
49. J.S. Webb, D.B. Cosulich, J.H. Mowat, J.B. Patrick, R.W. Broschard, W.E. Meyer, R.P. Williams, C.F. Wolf, F. Fulmor, C. Pidacks and J.E. Lancaster, J. Am. Chem. Soc., 84 (1962) 3185.

50. C.L. Stevens, K.G. Taylor, M.E. Munk, W.S. Marshall, K. Noll, G.D. Shah, L.G. Shah and K. Uzu, J. Med. Chem., 8 (1965) 1.
51. K. Sigiura, Cancer Res., 19 (1959) 438.
52. T. Hata, C. Hossenlopp and H. Takita, Cancer Chemother. Rep., 13 (1961) 67.
53. S. Wakaki, H. Marumo, K. Tamioka, G. Shimizu, E. Kato, H. Kamada, S. Kudo and Y. Fujimoto, Antibiot. Chemother., 8 (1958) 228.
54. S. Wakaki, Cancer Chemother. Rep., 13 (1961) 79.
55. S.A. Waksman and H.B. Woodruff, Proc. Soc. Exp., Biol. Med., 45 (1940) 609.
56. S.A. Waksman, Actinomycin (Interscience, New York, 1968).
57. L.D. Hamilton, W. Fuller and E. Reich, Nature (London), 198 (1963) 538.
58. C.T.C. Tan, R.B. Golbey, C.L. Yap, N. Wollner, C.A. Hackethal, L.M. Murphy, H.W. Dargeon and J.H. Burchenal, Ann. N.Y. Acad. Sci., 89 (1960) 426.
59. R.B. Livingston and S.K. Carter, Single Agents in Cancer Chemotherapy (IFI/ Plenum, New York, 1970).
60. T.K. Liao, W.H. Nyberg and C.C. Cheng, Angew. Chem. Int. Edn. Engl., 6 (1967) 82.
61. T.K. Liao, W.H. Nyberg and C.C. Cheng, J. Heterocycl. Chem., 13 (1976) 1063.
62. K.Y. Zee-Cheng and C.C. Cheng, J. Med. Chem., 13 (1970) 264.
63. E.G. Podrebarac and C.C. Cheng, J. Org. Chem., 35 (1970) 281.
64. T.K. Liao, P.J. Wittek and C.C. Cheng, J. Heterocycl. Chem., 13 (1976) 1283.
65. P.J. Wittek, T.K. Liao and C.C. Cheng, J. Org. Chem., 44 (1979) 870.
66. E. Gellert and R. Rudzats, J. Med. Chem., 7 (1964) 361.
67. T.R. Govindachari, in: The Alkaloids, ed. R.H.F. Manske (Academic Press, New York) Vol. 9, (1967) p. 517.
68. T.R. Govindachari, M.V. Lakshmikantham, K. Nagarajan and B.R. Pai, Tetrahedron, 4 (1958) 311.
69. K.Y. Zee-Cheng and C.C. Cheng, J. Med. Chem., 12 (1969) 157.
70. M.E. Wall, M.C. Wani, C.E. Cook, K.H. Palmer, A.T. McPahil and G.A. Sim, J. Am. Chem. Soc., 88 (1966) 3888.
71. A.T. McPahil and G.A. Sim, J. Chem. Soc. (B), (1968) 923.
72. R.E. Perdue, Jr., M.E. Wall, J.L. Hartwell and B.J. Abbott, Lloydia, 31 (1968) 229.
73. J.L. Hartwell and B.J. Abbott, Adv. Pharmacol. Chemother., 7 (1969) 117.
74. G. Briegleb, Angew. Chem., 62 (1950) 264.
75. A. Stuart, Die Struktur des Freien Molekuels (Springer-Verlag, Berlin, 1952).
76. L. Pauling and R.B. Corey, Proc. Natl. Acad. Sci U.S.A., 37 (1951) 729; 39 (1953) 253.
77. W.L. Koltun, Biopolymers, 3 (1965) 665.
78. L.F. Fieser, J. Chem. Educ., 40 (1963) 457; 42 (1965) 408.
79. K.Y. Zee-Cheng and C.C. Cheng, J. Pharm. Sci., 59 (1970) 1630.
80. K.Y. Zee-Cheng and C.C. Cheng, J. Pharm. Sci., 61 (1972) 969.
81. K.Y. Zee-Cheng and C.C. Cheng, J. Pharm. Sci., 62 (1973) 1572.
82. K.Y. Zee-Cheng, K.D. Paull and C.C. Cheng, J. Med. Chem., 17 (1974) 347.
83. M.J. Cho, A.J. Repta, C.C. Cheng, K.Y. Zee-Cheng, T. Higuchi and I.H. Pitman, J. Pharm. Sci., 64 (1975) 1825.
84. C.C. Cheng, R.R. Engle, J.R. Hudson, R.B. Ing, H.B. Wood, Jun., S.J. Yan and R.K.Y. Zee-Cheng, J. Pharm. Sci., 66 (1977) 1781.
85. K.Y. Zee-Cheng and C.C. Cheng, J. Heterocycl. Chem., 10 (1973) 85.
86. K.Y. Zee-Cheng and C.C. Cheng, J. Heterocycl. Chem., 10 (1973) 867.

87. K.Y. Zee-Cheng and C.C. Cheng, J. Med. Chem., 18 (1975) 66.
88. M. Cushman and L. Cheng, J. Org. Chem., 43 (1978) 286.
89. Z.X. Huang and Z.H. Li, Acta Chim. Sin., 38 (1980) 535.
90. W.M. Messmer, M. Tin-Wa, H.H.S. Fong, C. Bevelle, N.R. Farnworth, D.J. Abraham and J. Trojanek, J. Pharm. Sci., 61 (1972) 1858.
91. M. Tin-Wa, C.L. Bell, C. Bevelle, H.H.S. Fong and N.R. Farnworth, J. Pharm. Sci., 63 (1974) 1476.
92. J.P. Gillespie, L.G. Amoros and F.R. Stermitz, J. Org. Chem., 39 (1974) 3239.
93. F.R. Stermitz, J.P. Gillespie, L.G. Amoros, R. Romero, T.A. Stermitz, K.A. Larson, S. Earl and J.E. Ogg, J. Med. Chem., 18 (1975) 708.
94. M.L. Sethi, Can. J. Pharm. Sci., 16 (1981) 29.
95. M. Normatov, K.A. Abduazimov and S.Y. Yunusov, Uzbeksk. Khim. Zh., 9 (1965) 25; Chem. Abstr., 63 (1965) 7061.
96. T. Onaka, Y. Kanda and M. Natsume, Tetrahedron Lett. (1974) 1179.
97. T.Y. Owen, S.Y. Wang, S.Y. Chang, F.L. Lu, C.L. Yang and B. Hsu, K'o Hsueh T'ung Pao, 21 (1976) 285.
98. R.K.Y. Zee-Cheng and C.C. Cheng, J. Med. Chem., 21 (1978) 199.
99. Q.C. Pan, C.C. Pan, X.J. Chen, Z.C. Liu, Z.M. Meng and Q.L. She, Yao Hsueh Hsueh Pao, 14 (1979) 705.
100. C.C. Cheng and R.K.Y. Zee-Cheng, Heterocycles, 15 (1981) 1275.
101. R.H. Adamson, in: Recent Development in Cancer Chemotherapy (Karger, Basel, 1973) p. 402.
102. D.H. Huffman, R.S. Benjamin and N.R. Bachur, Clin. Pharmacol. Exp. Ther. 13 (1972) 895.
103. Adriamycin: New Drug Seminar, eds. N.R. Bachur, R.S. Benjamin, and T.C. Hall, San Francisco, CA, 1975; Cancer Chemother. Rep. Part 3, 6 (1975) 83.
104. E. Cadman, in: Cancer, Chemother., ed. F.E. Becker (Plenum Press, N.Y.) Vol. 5 (1977) p. 59.
105. R.J. Woodman, I. Kline and J.M. Venditti, Proc. Am. Assoc. Cancer Res., 13 (1972) 31.
106. C.E. Myers, W. McGuire and R. Young, Cancer Treat. Rep., 60 (1976) 961.
107. A.B. Combs, D. Acosta and K. Folkers, ICRS Med. Sci. Libr. Compend., 4 (1976) 403.
108. R.C. Mills, L.H. Maurer, R.J. Forcier, W.R. Grace, G.P. Burke, D.D. Karp, R.C. Smith, O.R. McIntyre and C. Bean, Cancer Treat. Rep., 61 (1977) 477.
109. C.E. Myers, W.P. McGuire, R.H. Liss, I. Ifrim, K. Grotzinger and R.C. Young, Science, 197 (1977) 165.
110. Y.M. Wang, F.F. Madanat, J.C. Kimball, C.A. Gleiser, M.K. Ali, M.W. Kaufman and J.V. Eys, Cancer Res., 40 (1980) 1022.
111. K. Fujita, K. Shinpo, K. Yamada, T. Sato, H. Niimi, M. Shamoto, T. Nagatsu, T. Takeuchi and H. Umezawa, Cancer Res., 42 (1982) 309.
112. J.G.S. Breed, A.N.E. Zimmerman, J.A.M.A. Dormans and H.M. Pinedo, Cancer Res., 40 (1980) 2033.
113. J.F. van Vleet, V.J. Ferrans and W.E. Weirich, Am. J. Pathol., 99 (1980) 13.
114. R.H. Adamson, Cancer Chemother. Rep., 58 (1974) 293.
115. W. Müller, R. Flügel and C. Stein, Ann. Chem., 754 (1971) 15.
116. J.C. Double and J.R. Brown, J. Pharm. Pharmacol., 27 (1975) 502.
117. J.C. Double and J.R. Brown, J. Pharm. Pharmacol., 28 (1976) 28.

118. R.C. Hoare, U.S. Pat. 4,051,155 (1977); Chem. Abstr., 88 (1978) 24251.
119. R.K.Y. Zee-Cheng and C.C. Cheng, J. Med. Chem., 21 (1978) 291.
120. C.C. Cheng, G. Zbinden and R.K.Y. Zee-Cheng, J. Pharm. Sci., 68 (1979) 393.
121. R.K. Johnson, R.K.Y. Zee-Cheng, W.W. Lee, E.M. Acton, D.W. Henry and C.C. Cheng, Cancer Treat. Rep., 63 (1979) 425.
122. C.C. Cheng, R.K.Y. Zee-Cheng, V.L. Narayanan, R.B. Ing and K.D. Paull, Trends Pharmacol. Sci., 2 (1981) 223.
123. D.P. Evenson, Z. Darzynkiewicz, L. Staiano-Coico, F. Traganos and M.R. Melamed, Cancer Res., 39 (1979) 2574.
124. R.E. Wallace, K.C. Murdock, R.B. Angier and F.E. Durr, Cancer Res., 39 (1979) 1570.
125. K.C. Murdock, R.G. Child, P.F. Fabio, R.B. Angier, R.E. Wallace, F.E. Curr and R.V. Citarella, J. Med. Chem., 22 (1979) 1024.
126. W.R. Laster, Jr., M.H. Witt and F.M. Schabel, Jr., Proc. Am. Assoc. Cancer Res., 21 (1980) 271 No. 1086.
127. L.F. Cohen, D.L. Glaubiger, H.E. Kann and K.W. Kohn, Proc. Am. Assoc. Cancer Res., 21 (1980) 277 No. 1111.
128. D.S. Alberts, K.S. Griffith, G.E. Goodman, T.S. Herman and E. Murray, Cancer Chemother. Pharmacol., 5 (1980) 11.
129. K. Lu and T.L. Loo, Cancer Res., 40 (1980) 1427.
130. F. Ostroy and R.A. Gams, J. Liq. Chromatogr., 3 (1980) 637.
131. C.L. Richardson, J. Roboz and J.F. Holland, Res. Commun. Chem. Pathol. Pharmacol., 27 (1980) 497.
132. E.D. Kharasch and R.F. Novak, Biochem. Pharmacol., 30 (1981) 2881.
133. M. Piccart, R. Abele, E. Cumps, P. Dodion, D. Dupont, Y. Kenis and M. Rozencweig, Proc. Am. Assoc. Cancer Res., 22 (1981) 192 No. 761.
134. W.W. Au, M.A. Butler, T.S. Matney and T.L. Loo, Cancer Res., 41 (1981) 376.
135. C.E. McManus, J.G. Kuhn, T.M. Ludden, D.M. Loesch, D.D. von Hoff, D. Gordon, C.A. Coltman, Jr., A.J. Grillo-Lopez and J. Bender, Proc. Am. Soc. Clin. Oncol., 1 (1982) 18, No. C-70.
136. F.B. Stilmer and M.A. Perkins, in: The Chemistry of Synthetic Dyes and Pigments, ed. H.A. Lubs (Reinhold, New York, 1955) p. 362.
137. C.W. Greenhalgh and N. Hughes, J. Chem. Soc. (C), (1968) 1284.
138. O. Bayer, in Houben-Weyl's Methoden der Organischen Chemie (Georg Thieme, Stuttgart) Vol VII/3c (1979), p. 90.
139. J. Houben and W. Fisher, Das Anthracen und die Anthrachinone (Georg Thieme, Leipzig, 1929) pp. 427-428.
140. M. Simon, J. Am. Chem. Soc., 85 (1963) 1974.
141. M. Ishikawa and M. Okazaki, Kogyo Kagaku Zasshi, 67 (1964) 138.
142. C. Liebermann, Ann. Chem., 212 (1882) 14.
143. K.H. Meyer and A. Sander, Ann. Chem., 420 (1920) 113.
144. K. Zahn and P. Ochwat, Ann. Chem., 462 (1928) 72.
145. I.G. Farben industrie A.-G., Br. Pat. 447,107 (1936); Chem. Abstr., 30 (1936) 6953.
146. M. Kikuchi, T. Yamagishi and M. Hida, Dyes Pigm., 2 (1981) 14.
147. M. Kikuchi, T. Yamagishi and M. Hida, Bull. Chem. Soc. Japan, 55 (1982) 1209.
148. M. Matsuoka, Y. Makino, K. Yoshida and T. Kitao, Chem. Lett. (1979) 219.
149. R.K.Y. Zee-Cheng, E.G. Podrebarac, C.S. Menon and C.C. Cheng, J. Med. Chem., 22 (1979) 501.

150. K. Yoshida, M. Matsuoka, T. Ueyama, Y. Yamashita and T. Kitao, Chem. Lett., (1978) 765.
151. M.I. Krasnosel'skaya, L.D. Leichenko, N.A. Parfenov, V.I. Kolodyazhnyi and M.P. Venzelovskii, U.S.S.R. Pat. 411,077 (1974); Chem. Abstr., 81 (1974) 93052.
152. J.A.W. George, Br. Pat. 1,504,137 (1978); Chem. Abstr., 89 (1978) 112381.
153. M.R. Michael, Br. Pat. 2,013,701 (1979); Chem. Abstr., 92 (1980) 199751.
154. Y. Banshō and K. Kondō, Kogyo Kagaku Zasshi, 57 (1954) 751.
155. K. Naiki, Yuki Gosei Kagaku, 12 (1954) 12.
156. Y. Banshō, Kogyo Kagaku Zasshi, 57 (1954) 80.
157. C.G. Zubrod, S. Schepartz, J. Leiter, K.M. Endicott, L.M. Carrese and C.G. Baker, Cancer Chemother. Rep., 50 (1966) 349.
158. R.I. Geran, N.H. Greenberg, M.M. Macdonald, A.M. Schumacker and B.J. Abbott, Cancer Chemother. Rep., Part 3, 3 (1972) 1.
159. Drug Development Branch, Division of Cancer Treatment, NCI, Instruction Booklet 14: Screening Data Summary Interpretation and Outline of Current Screen (National Cancer Institute, 1980).
160. L. Rothenberg and R.A. Terselic, Cancer Chemother. Rep., 54 (1970) 303.
161. A. Goldin and J.M. Venditti, in: Recent Results in Cancer Research, eds. S.K. Carter and Y. Sakurai (Springer-Verlag, Berlin) Vol. 70 (1980) pp. 5-20.
162. H.S. El Khadem, D.L. Swartz and R.C. Cermak, J. Med. Chem., 20 (1977) 957.
163. E.F. Fuchs, D. Horton, W. Weckerle and E. Winter-Mihaly, J. Med. Chem., 22 (1979) 406.
164. D. Horton and W.R. Turner, Carbohyd. Res., 77 (1979) C8.
165. H.S. El Khadem and D.L. Swartz, J. Med. Chem., 24 (1981) 112.
166. R.K.Y. Zee-Cheng and C.C. Cheng, J. Pharm. Sci., 71 (1982) 708.
167. G.L. Tong, H.Y. Wu, T.M. Smith and D.W. Henry, J. Med. Chem., 22 (1979) 912.
168. V.I. Avramis, V.B. Crossie, Jr., S. Feldman, E. McKelvey and T.L. Loo, Proc. Am. Assoc. Cancer Res., 22 (1981) 210 No. 830.
169. F.E. Durr and R.E. Wallace, in: Current Chemotherapy and Infectious Disease, eds. J.D.. Nelson and C. Grassi (Am. Soc. Microbiol., Washington, DC) Vol. II (1980) pp. 1595-1596.
170. T.H. Corbett, B.J. Roberts, M.W. Trader, W.R. Laster, Jr., D.P. Griswold, Jr. and F.M. Schabel, Jr., Cancer Treat. Rep., 66 (1982) 1187.
171. E.M. Uyeki, A. Nishio, P.J. Wittek and C.C. Cheng, J. Pharm. Sci., 70 (1981) 1011.
172. B.M. Kimler, C.C. Cheng, R.E. Barnes and M.L. Barnes, Invest. New Drugs, in press.
173. B.M. Kimler and C.C. Cheng, Cancer Res., 42 (1982) 3631.
174. B.M. Kimler, Cancer Res., 40 (1980) 42.
175. F. Traganos, D.P. Evenson, L. Staiano-Coico, Z. Darzynkiewicz and M.R. Melamed, Cancer Res., 40 (1980) 671.
176. B.F. Kimler, C.C. Cheng, R.E. Barnes and M.L. Barnes, Cell Tissue Kinet., 15 (1982) 104, No. 27.
177. A. Nishio, F. DeFeo, C.C. Cheng and E.M. Uyeki, Mutation Res., 101 (1982) 77.
178. D.P. Evenson, F. Traganos, Z. Darzynkiewicz, L. Staiono-Coico and M.R. Melamed, J. Natl. Cancer Inst., 64 (1980) 857.
179. R.E. Wallace, R.V. Citarella and F.E. Durr, Proc. Am. Assoc. Cancer Res., 20 (1979) 12 No. 49.
180. B. Drewinko, M. Patchen, L.Y. Yang and B. Barlogie, Cancer Res., 41 (1981) 2328.

181. M. Bailey, D. Raghavan and A. Shorthouse, Proc. Am. Assoc. Cancer Res., 21 (1980) 265 No. 1061.
182. A.W. Hamburger and S.E. Salmon, J. Clin. Invest. 60 (1970) 846.
183. A.W. Hamburger and S.E. Salmon, Science, 197 (1977) 461.
184. A.W. Hamburger, S.E. Salmon, M.B. Kim, J.M. Trent, B.J. Soehulen, D.S. Alberts and H.J. Schmidt, Cancer Res., 38 (1978) 3438.
185. D.D. von Hoff, J. Cowan, G. Harris and G. Reisdorf, Cancer Chemother. Pharmacol., 6 (1981) 265.
186. D.D. von Hoff, C.A. Coltman, Jr. and B. Forseth, Cancer Res., 41 (1981) 1853.
187. S.E. Salmon, F.L. Meyskens, Jr., D.S. Alberts, B. Soehnlen and L. Young, Cancer Treat. Rep., 65 (1981) 1, 532.
188. B.A. Newton, Adv. Pharmacol. Chemother. 8 (1970) 149 and references cited therein.
189. T.W. Plumbridge, V. Knight, K.L. Patel and J.R. Brown, J. Pharm. Pharmacol., 32 (1980) 78.
190. J. Kapuscinski, Z. Darzynkiewicz, F. Traganos and M.R. Melamed, Biochem. Pharmacol., 30 (1980) 231.
191. W.O. Foye, O. Vajragupta and S.K. Sengupta, J. Pharm. Sci., 71 (1982) 253.
192. T.R. Tritton and G. Yee, Science, 217 (1982) 248.
193. R.T. Su, Biochem. Biophys. Res. Commun., 103 (1981) 249.
194. D.W. Yesair and R.F. Taylor, Proc. Am. Assoc. Cancer Res., 20 (1979) 149 No. 602.
195. R.F. Taylor, L.A. Teague and D.W. Yesair, Cancer Res., 41 (1981) 4316.
196. R.E. Wallace, D. Lindh and F.E. Durr, Proc. Am. Assoc. Cancer Res., 23 (1982) 195 No. 767.
197. N.R. Bachur, S.L. Gordon and N.V. Gee, Cancer Res., 38 (1978) 1745.
198. J. Goodman and P. Hochstein, Biochem. Biophys. Res. Commun., 77 (1977) 797.
199. S.S. Pan, L. Pedersen and N.R. Bachur, Mol. Pharmacol., 19 (1981) 184.
200. R.F. Taylor and L.A. Gaudio, J. Chromatogr., 187 (1980) 212.
201. S.R. Cox, Dissertation Abs. Int., 41 (1981) 3746B.
202. D.L. Reynolds, L.A. Sternson and A.J. Repta, J. Chromatogr., 222 (1981) 225.
203. F. Ostroy, J.G. Spenney and R.A. Gams, Proc. Am. Cancer Res., 21 (1980) 197 No. 789.
204. K. Lu, G.L. Raulston and T.L. Loo, Proc. Am. Assoc. Cancer Res., 21 (1980) 261 No. 1045.
205. Y.M. Peng, D. Ormberg, T.P. Davis and D.S. Alberts, Proc. Am. Assoc. Cancer Res., 23 (1982) 202 No. 795.
206. T.L. Loo, K. Lu and N. Savaraj, Third NCI-EORTC Symposium on New Drugs in Cancer Therapy, Brussels, Belgium (1981) 34.
207. D.L. Reynolds, K.K. Ulrich, T.F. Patton, A.J. Repta, L.A. Sternson, M.C. Myron and S.A. Taylor, Int. J. Pharmaceut. 9 (1981) 67.
208. N. Savaraj, K. Lu, M. Valdivieso, T. Umsawasdi, M. Burgess, J. A. Benvenuto, R.S. Benjamin and T.L. Loo, Proc. Am. Assoc. Cancer Res., 22 (1981) 180 No. 713.
209. N. Savaraj, K. Lu, M. Valdivieso, M. Burgess, T. Umsawasdi, R.S. Benjamin and T.L. Loo, Clin. Pharm. Ther., 31 (1982) 312.
210. J. Neidhart, A. Staubus, D. Young and L. Malspeis, Proc. Am. Soc. Clin. Oncol., 1 (1982) 363 No. C-121.

211. G. Zbinden and A.K. Beilstein, Toxicol. Lett., 11 (1982) 289.
212. B.F. Kimler and M.P. Hacker, Cancer Clin. Trials, 4 (1981) 173.
213. E.K. Reddy, B.F. Kimler, S.D. Henderson, R.W. Glover and M.F. Scanlan, Int. J. Radiat. Oncol. Biol. Phys. 7 (Supp. 1) 97 No. 132.
214. B.F. Kimler, S.D. Henderson, C.M. Mansfield, D.J. Svoboda and C.C. Cheng, Cancer Res., 42 (1982) 2656.
215. J.A. Stewart, J.J. McCormack, J.P. Griffin and I.H. Krakoff, Proc. Am. Soc. Clin. Oncol., 22 (1981) 361 No. C-112.
216. B.M. Henderson, W.J. Dougherty, V.C. James, L.P. Tilley and J.F. Noble, Cancer Treat. Rep., 66 (1982) 1139.
217. B.M. Sparano, G. Gordon, C. Hall, M.J. Iatropoulos and J.F. Noble, Cancer Treat. Rep., 66 (1982) 1145.
218. D. von Hoff, E. Pollard, J. Kuhn, E. Murray, C.A. Coltman, Jr. and C.K. Osborne., Cancer Res., 40 (1980) 1516.
219. T. Vietti, W. Nix, T. Kim, E. Murray and V. Cappiello, Proc. Am. Assoc. Cancer Res., 22 (1981) 237 No. 938.
220. M.Y. Whitacre, D.A. van Echo, J. Aisner, M. Klein and P.H. Wiernik, Proc. Am. Soc. Clin. Oncol., 22 (1981) 369 No. C-147.
221. M.A. Goldsmith, T. Ohnuma, J. Roboz, I. Jaffey, E.M. Greenspan and J.F. Holland, Proc. Am. Soc. Clin. Oncol., 22 (1981) 389 No. C-224.
222. R.S. Ungerleider, L.F. Cohen, D.L. Glaubiger, D.D. von Hoff, J.S. Holcenberg, J.S. MacDonald and C.B. Pratt, Proc. Am. Soc. Clin. Oncol., 22 (1981) 392 No. C-235.
223. J.S. Holcenberg, B. Kamen, D. von Hoff, C. Pratt, D. Glaubiger and R. Ungerleider, Proc. Am. Soc. Clin. Oncol., 22 (1981) 410 No. C-304.
224. E.H. Estey, M.J. Keating, K.B. McCredie, G.P. Bodey and E.J. Freireich, Proc. Am. Soc. Clin. Oncol., 22 (1981) 490 No. C-615.
225. D.A. van Echo, M.Y. Whitacre, J. Aisner and P.H. Wiernik, Cancer Treat. Rep., 65 (1981) 831.
226. M. Valdivieso, A.Y. Bedikian, M.A. Burgess, N. Savaraj, W.B. Jeffers and G.P. Bodey, Cancer Treat. Rep., 65 (1981) 841.
227. R.L. Stephens, D.D. von Hoff, S.A. Taylor, W.A. Knight, III, R.B. Livingston, F.E. Bull, J. Cowan, R. Hilgers, C.K. Osborne, G.A. Gates and G. Clark, Third NCI-EORTC Symp. New Drugs in Cancer Therapy (Brussels, Belgium, 1981) p. 36.
228. T. Vietti, W. Nix, T. Kim, J. Holcenberg and B. Kamen in Ref. 227, p. 37.
229. R.A. Gams, A.A. Bartolucci and T.A. Terzakis, in Ref. 227, p. 38.
230. R. de Jager, P. Cappelaere, H. Earl, R. Rubens, R. Stuart-Harris, I. Smith, J. Smythe, J. Renard, M. van Galbbeke and G. Mathé, in Ref. 227, p. 39.
231. G.P. Bodey, M. Valdivieso, A.Y. Bedikian and H.Y. Yap, in Ref. 227, p. 40.
232. D. Raghavan, A. Coates, M. Friedlander, R.L. Woods, J. Levi, A. Hellestrand, A. Sullivan, R. Fox, D. Hedley and M. Tattersall, Proc. Am. Soc. Clin. Oncol., 1 (1982) 10 No. C-40.
233. W.R. Wynert, H.A. Harvey, A. Lipton, J. Schweitzer and D.S. White, Cancer Treat. Rep., 66 (1982) 1303.
234. D.A. van Echo, P.N. Shulman, A. Ferrari, D. Budman, S.D. Markus and P.H. Wiernik, Proc. Am. Soc. Clin. Oncol., 1 (1982) 132 No. C-513.
235. J.A. Stewart, J.J. McCormack and I.H. Krakoff, Cancer Treat. Rep., 66 (1982) 1327.

236. S.K. Callahan, D.D. von Hoff, G.M. Clark, T. Chen, J. Mira and R. Livingston, Proc. Am. Soc. Clin. Oncol., 1 (1982) 153 No. C594.
237. H.M. Earl, P.L. Amlot and R.D. Rubens, Br. J. Cancer, 45 (1982) 636.
238. M. Aapro, C. Mackel, D. Alberts and J. Woolfenden, Proc. Am. Soc. Clin. Oncol., 1 (1982) 14 No. C-55.
239. F.C. Schell, H.Y. Yap, G.R. Blumenschein and G.P. Bodey, Proc. Am. Soc. Clin. Oncol., 1 (1982) 21 No. C-83.
240. D. Mayernik, T. Stevenson and J.A. Neidhart, Proc. Am. Soc. Clin. Oncol., 1 (1982) 14 No. C-53.
241. C.B. Pratt, E. Etcubanas, E. Thompson, F.A. Hayes, J.E. Champion and D.B. Crom, Proc. Am. Assoc. Cancer Res., 23 (1982) 480.
242. E. Goormaghtich, P. Chatelain, J. Caspers and J.M. Ruysschaert, Biochem. Pharmacol., 29 (1980) 3003.
243. D.C. Lehotay, B.A. Levey, B.J. Rogerson and G.S. Levey, Cancer Treat. Rep., 66 (1982) 311.
244. D.V. Unverferth, S.P. Balcerzak and J.A. Neidhart, Proc. Am. Assoc. Cancer Res., 23 (1982) 125 No. 532.
245. M. Piccart, M. Rozencweig, R. Abele, E. Cumps, P. Dodion, D. Dupont, D. Kisner and Y. Kenis, Eur. J. Cancer Clin. Oncol., 17 (1981) 775.
246. H.Y. Yap, G.R. Blumenschein, F.C. Schell, A.U. Buzdar, M. Valdivieso and G.P. Bodey, Ann. Int. Med., 95 (1981) 694.
247. J.A. Neidhart and R.W. Roach, Proc. Am. Soc. Clin. Oncol., 1 (1982) 86 No. C-331.
248. W.A. Knight, III, D.D. von Hoff, B. Tranum and R. O'Bryan, Proc. Am. Soc. Clin. Oncol., 1 (1982) 87 No. C-335.
249. R. de Jager, P. Cappelaere, H. Earl, R. Rubens, R. Stuart-Harris, I. Smith, J. Carmichael, J. Smythe, R. Keiling, J. Renard, M. van Glabbeke and G. Mathé, Proc. Am. Soc. Clin. Oncol., 1 (1982) 89 No. C-345.
250. E.H. Estey, M.J. Keating, K.B. McCredie, G.P. Bodey and E.J. Freireich, Proc. Am. Assoc. Cancer Res., 23 (1982) 113 No. 442.
251. W.C. Rose, Cancer Treat. Rep., 65 (1981) 299.
252. K.C. Anderson, G. Cohen and M.B. Garnick, Cancer Treat. Rep., 66 (1982) 1929.
253. E.D. Kharasch and R.F. Novak, Biochem. Biophys. Res. Commun., 108 (1982) 1346.
254. E.D. Kharasch and R.F. Novak, Arch. Biochem. Biophys., in press.

ADDENDUM

Very recently, it was found that DHAQ and AQ are potent inhibitors of lipid peroxidation *in vitro*, whereas adriamycin stimulates the lipid peroxidation [253]. Furthermore, adriamycin enhances superoxide formation, whereas DHAQ, MHAQ and AQ are relatively inert in the presence of NADPH-cytochrome *P*-450 reductase and NADH dehydrogenase [254]. These results correlate well with the diminished cardiotoxicity of DHAQ and related compounds.

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3 Chemical Control of Virus Diseases of Plants

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INTRODUCTION

An antiviral agent has been described as a 'substance, other than a virus, a virus-containing vaccine or a specific antibody which can produce either a protective or therapeutic effect to the clear detectable advantage of the

infected host' [1]. Since higher plants lack a defined immune system and the presence of the rigid cell wall prevents the uptake of macromolecules, the definition can be simplified for the purposes of this review. Here, putative antiviral agents will be considered under three subdivisions, chemicals which (1) inhibit virus replication, (2) suppress virus symptoms and (3) induce host defence mechanisms.

It should be acknowledged at the outset that most of the chemicals tested in plants have resulted from earlier investigations by animal virologists. These chemicals may have originated from random screening which evolved into a programme of synthesis of derivatives and analogues of the active chemicals [2]. Plant virologists have then chosen, when available to them, the most active compound(s) produced in such synthetic programmes and have tested them in plant screening systems. This poses the problem that, in general, the chemicals screened by plant virologists are not designed for application to whole plants or use in plant screening systems and hence may fail to show activity or they may exhibit low activity because of failure to maintain inhibitory concentrations in the system due to poor uptake, metabolic conversion or some other as yet undefined reason.

In addition to the chemicals, plant virologists have historically adopted much of their rationale from their animal virologist colleagues. For example, in attempts to progress from totally random screening, viral chemotherapists have made certain basic assumptions about the events in virus replication in advance, in some cases, of knowledge of the replication strategies of the viruses which they were seeking to control (e.g., Ref. 2). Witkowski reportedly outlined the rationale for the synthesis of triazole-β-D-ribosides as follows: 'in the search for a broad-spectrum antiviral agent, an effort was made to concentrate on the synthesis of compounds which have the potential to affect enzymatic processes which are common to all known viruses such as viral-induced nucleic acid and protein synthesis. These processes are carried out by enzymes specifically coded for in the viral genome. Another common feature of all viruses is their lack of protein-synthesizing capacity. It is conceivable that initiation of virus specific protein synthesis and/or RNA synthesis may utilize unique viral enzymes which could be specifically inhibited' [3]. This statement of objectives should be borne in mind when considering our current knowledge of plant virus replication [4] and of the studies on the effects of ribavirin on plant viruses discussed below.

Temporally, the pioneer studies on a pseudo-rational approach to the design of antiviral chemicals (by animal virologists) paralleled the development of animal cell culture and the development of *in vitro* virus-host cell screening systems. These latter systems have been used extensively in the development

of antiviral benzimidazoles and ribavirin (a triazole-β-D-riboside), compounds which, along with some others to be mentioned later, have been subsequently investigated for antiviral activity in plant systems. Plant antiviral chemotherapy has been largely derived from studies on compounds synthesized for animal virologists, but progress has been made on uniquely plant virology.

For the purpose of this review, emphasis is placed on existing gaps in plant virus disease control where there is a commercial need for chemotherapeutants. An overview of the replication strategy of the main plant virus type is given and the relation between the plant virus and its host is outlined as a background to a review of targets for chemical action, screening methods used by plant virologists, and results obtained. Selective aspects of the literature on antiviral benzimidazoles and their derivatives, ribavirin and plant hormones will be reviewed, comparing results obtained in animals and plants where appropriate. To complete the perspective, there will be a discussion of symptom suppression in plants by benzimidazole derivatives and of the search for endogenous antiviral mechanisms in plants. In conclusion, the implications of plant propagation via tissue culture and the potential for chemotherapeutants in the latter will be discussed.

Aspects of antiviral chemotherapy in plants have been renewed by Matthews [5, 6], Matthews and Smith [7], Sinha [8], Gupta [9] and Hirai [10].

CURRENT STRATEGIES FOR PLANT VIRUS DISEASE CONTROL

A combination of chemical control and plant breeding for resistant varieties has been relatively successful in the control of fungal diseases of crop plants [11]. In the case of virus disease control, plant breeding for virus resistance, while still a commercial preference in that the introduction of stable resistant varieties avoids recurrent (treatment) cost, has so far made less contribution. This is possibly because of the practical problems involved [12], of the lesser economic importance in general of virus diseases of plants, and hence of their lower priority in policy making. Consequently, chemical control has assumed a great importance. In the absence of antiviral agents for field use, the main chemical control of plant virus diseases presently centres around vector control with the practical objective of delaying virus entry into the crop (e.g., Ref. 13).

This review is concerned with direct chemical control, i.e., disregarding vector control, of virus diseases of plants. A review on aphids as virus vectors is available [14]. As a preface to any discussion of possible developments in the chemical control of virus diseases of plants, it is considered appropriate to identify the current problems. In this context, it is usual to distinguish three types of crop – annual seeded crops, perennial crops and vegetatively propa-

gated crops (*Figure 3.1*). Many perennial crops are propagated vegetatively.

Seeded crops generally start virus-free, i.e., many viruses are not seed-transmitted or are transmitted at low frequency in seed, from one generation to the next [15]. In general, early entry of the virus is associated with greater crop loss. Traditionally, the objective has been to delay entry into the crop by controlling the virus vectors combined with attempts to reduce or eliminate virus reservoirs in the environment. This strategy works reasonably successfully for persistent viruses, i.e., viruses which are acquired relatively slowly and have a latent period in the vector before being transmitted [14]. In this case, for example, aphicides can act on the vector before it becomes viruliferous. Aphicides are less effective against the rapidly acquired and immediately transmissible non-persistent viruses. However, recently developed applications of mineral oils to infected plants, which appear to act by preventing virus acquisition, offer some prospect of improved control of the latter [16].

While aphids constitute an important group of virus vectors, many other vectors are known, ranging from fungi to possibly most insects and animals which enter, feed on or damage plants [4]. Indeed, even contact between plants may lead to virus transmission as, for example, in the field transmission of potato virus X [17].

In all cases of vector control in annual crops, the process can be made more cost-effective by the introduction of disease risk forecasting systems, as has

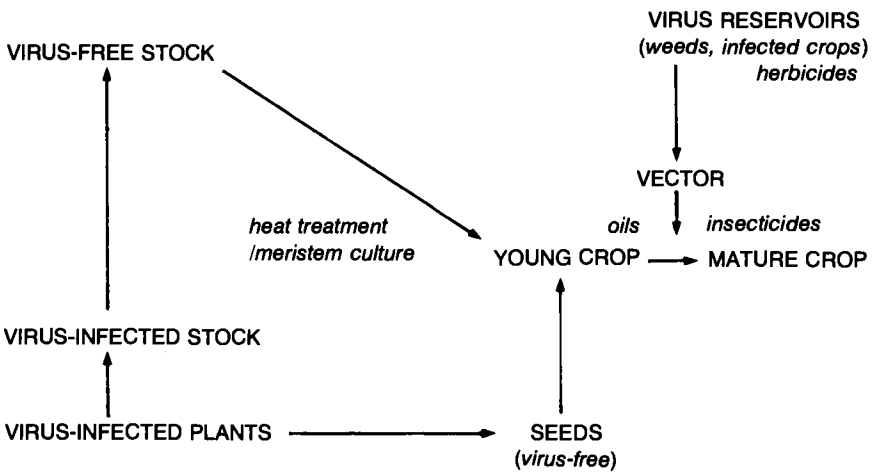


Figure 3.1. Strategies for the control of virus diseases of plants based on the use of virus-free planting material and vector control.

been suggested in the case of sugar beet viruses in the U.K. [18]. In the longer term, the search for stable resistant varieties is the most cost-effective solution.

The protection of perennial crops, and here one can also include vegetatively propagated annual crops, is more problematic. Given that aphids can transmit virus effectively at low population densities [14], the task of protecting perennial crops is seen as a formidable one. The normal and costly strategy is based on replanting at appropriate intervals.

In the potato crop, (annual, vegetatively propagated), either virus-free nuclear stocks are grown in vector-free (low vector incidence) areas, or vectors are carefully monitored and the crops are disease-indexed prior to certification [19, 20]. Growers then plant certified seed and may retain smaller ware tubers as seed in successive years until virus accumulation results in decline in yield and indicates the need for re-purchase of certified seed.

With annual vegetatively propagated crops, stock replacement is a commercial option. However, with longer-term crops, e.g., fruit trees or strawberry, the need for premature stock replacement can result in severe financial loss to the grower. Such long-term plants are in general not readily amenable to plant breeding techniques, thus compounding the need for alternative control measures.

Many high-value ornamental plants are also vegetatively propagated and with these there is also the problem of degeneracy of stock due to virus presence and accumulation. Whereas, in the case of potato, certified stock is maintained under the auspices of national governments, the structure of the horticulture industry and the diversity of material has mitigated against this approach for ornamental plants. In recent years, nuclear stock associations have been formed for many ornamental crops with a view to producing virus-free stock for the growers [21]. The long-term storage of such disease-free stock is less satisfactory at present.

Virus elimination from vegetatively propagated plants has been achieved in two ways which may be combined with varying degrees of success (*Figure 3.2*). These are (a) heat therapy of the infected plant to yield 'virus-free' buds or shoots which are then propagated, virus-tested and clonal propagated, and/or (b) by aseptic culture (tissue culture) of bud tips [21, 22]. In general, the smaller the bud-tip the greater the likelihood that the virus will be absent, although both heat-tolerant strains, e.g., potato leaf roll virus [23], and very invasive viruses such as potato virus S are known [24].

From this brief overview of the current position of virus disease control in plants, it can be appreciated that there is some potential for antiviral chemotherapy. Emphasis, it is suggested, should be placed on chemicals for the elimination of viruses in vegetatively propagated crops and for the protec-

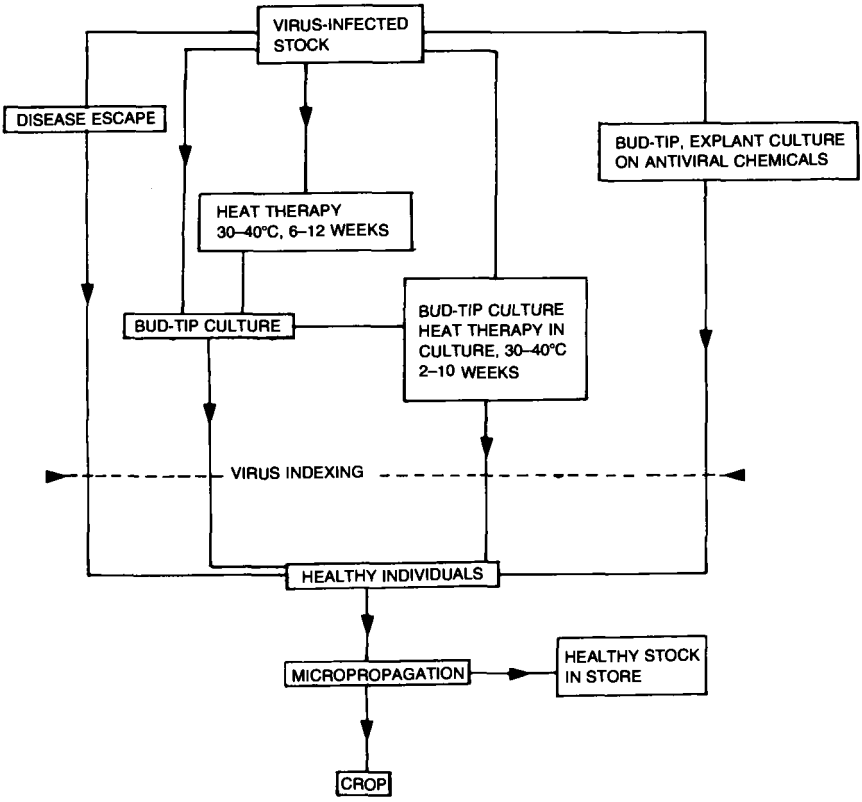


Figure 3.2. Strategies for the production of virus-free plants. The use of antiviral chemicals is a recent procedure.

tion and chemotherapy of long-term crops. In the fight against viruses of annual crops, chemotherapy could also have a role to play, depending on cost benefit.

POTENTIAL TARGETS FOR ANTIVIRAL CHEMICALS

Since the rationale used and the chemicals investigated have been derived largely from studies on animal systems, a comparative discussion is presented of the main types of plant virus and of virus-plant interactions in the context of chemical control.

Mammalian viruses show a range of replication strategies [25]. The main

RNA virus replication strategies are: ssRNA (+RNA) viruses, where the viral RNA genome acts as a messenger, and ssRNA (−RNA) genome viruses, where a complementary strand is synthesized before replication. These latter viruses may carry an RNA-dependent RNA-transcriptase in the nucleocapsid [25]. Viruses with single- and double-stranded DNA genomes can also occur [25]. In some, there are subtypes in which the genome is distributed among several similar or dissimilar particles [26]. The evolutionary relationship of such divided genome viruses to their undivided genome counterparts is at present uncertain. Many types of animal virus contain more than one type of capsid and some are enveloped [26].

Plant viruses show a similar range of diversity [4, 27]. In terms of economic importance and distribution in crop varieties, the ssRNA (+RNA) viruses are dominant (see *Table 3.1*). In general, these have divided or undivided genomes and a single protein species in the particle. This group constitutes many of the non-persistent viruses [27], that is, viruses which traditionally have been less amenable to the vector control strategy. The known events in the tobacco mosaic virus (TMV) replication strategies are represented in *Figure 3.3*.

Cell-surface virus receptor sites have been postulated as a target for animal antiviral chemicals [28], but no such sites appear to exist for plant viruses, the majority of which enter through sites of vector entry or damage to the plant. The possibility of an uncoating site in the plant cell has been discussed but the evidence for a specific site is equivocal [4]. If such a specific site existed, it could be a potential site for selective antiviral action.

The predominance of +RNA viruses and the absence of any cellular enzyme system for the replication of mRNAs led plant virologists, as with their counterparts in animal virology, to postulate that a central function of the viral genome was to code for an RNA-dependent RNA-replicase. This putative virus-coded, virus-specific enzyme was hypothesized as being a primary target for antiviral action. Thus, RNA synthesis inhibitors were sought which might selectively inhibit this virus-specific enzyme.

With the discovery of RNA-dependent RNA-replicase activity in non-infected 'healthy' tobacco plants [29], this central step in virus replication has had to be re-evaluated. Some controversy exists, and cucumber mosaic virus appears to code for its own replicase [30], whereas in the case of TMV, stimulation of a host enzyme has been suggested [31], although a virus-coded protein may act as a modifier of this enzyme.

The discovery of subgenomic messengers in TMV-infected cells and in other plant virus infections (see Ref. 4) and the findings mentioned above have led to a reappraisal of the number and role of virus-coded proteins in virus repli-

Table 3.1. AN ARRANGEMENT OF THE FAMILIES AND GROUPS OF PLANT VIRUSES [4]

Characterization	*Family or group*	*Number of members*	*Number of probable or possible members*
dsDNA non-enveloped	Caulimovirus	3	4
ssDNA non-enveloped	Geminivirus	4	5
dsRNA non-enveloped	Reoviridae	7	2
ssRNA enveloped	Rhabdoviridae	9	40
	Tomato spotted wilt virus group	1	0
ssRNA non-enveloped			
Monopartite genomes			
Isometric virus particles	Tymovirus	16	0
	Luteovirus	10	18
	Tombusvirus	5	3
	Southern bean mosaic virus	1	4
	Tobacco necrosis virus	1	1
	Maize chlorotic dwarf virus	1	1
Rod-shaped virus particles	Closterovirus	9	4
	Carlavirus	15	6
	Potyvirus	35	38
	Potexvirus	12	12
	Tobamovirus	10	6
Bipartite genomes			
Isometric virus particles	Nepovirus	17	1
	Pea enation mosaic virus	1	0
	Comovirus	11	1
Rod-shaped virus particles	Tobravirus	2	0
Tripartite genomes			
Isometric particles	Cucumovirus	3	1
	Bromovirus	3	0
	Ilarvirus	14	3
Isometric and bacilliform particles	Alfalfa mosaic virus	1	0
Rod-shaped virus particles	Hordeivirus	3	0
		193	150

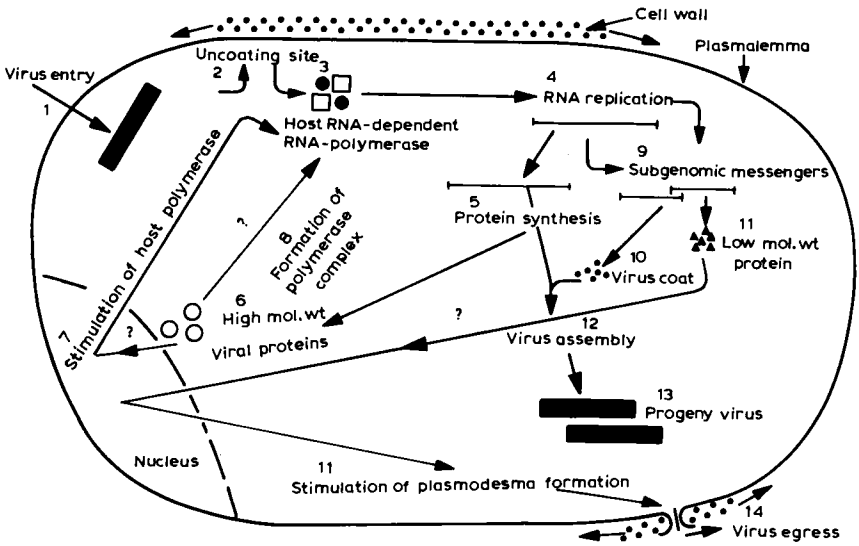


Figure 3.3. Hypothetical scheme for the replication of tobacco mosaic virus.

cation. In addition to the coat protein and large-molecular-weight protein(s) previously considered to function as replicases, proteins derived from other subgenomic messengers have as yet uncharacterized roles in the replication strategies. For example, recently a 30,000 molecular weight protein, a product of one of the TMV subgenomic messengers, has been postulated to play a role in the increase of plasmodesmatal (cell-to-cell) connections in virus-infected plants [32].

While 'new' virus-coded proteins may, as their roles are clarified, become potential sites of antiviral chemical action, these recent advances in basic knowledge of virus replication in plants, particularly the ambiguity regarding the origin of RNA-dependent RNA polymerases, have undermined much of the rationale of previous studies (see above).

The possibility of preventing virus particle assembly has been previously mooted [28]. It seems unlikely that the ionic environment in the plant could be selectively manipulated to affect viral nucleic acid-coat protein interactions to prevent particle assembly without serious disruption of cell metabolism. Particularly important in this regard could be the role of stress-induced ethylene and other hormonal changes [33]. Also, since plant viral RNA *per se* can replicate systemically, virus particle assembly is not a prerequisite for systemic spread and symptom production [34].

Following establishment, plant viruses may move from the primary site of replication and spread systemically through the plant, although not all cells may be infected [4]. The virus particles or nucleic acid with or without associated proteins move slowly from cell to cell and more rapidly in the phloem with the solute supply to the growing points or metabolite sinks. As the maturing apical cells become competent, virus may enter and continue the disease progression.

Some viruses [4] are restricted to the phloem. This may be because the stable form is too large to migrate from the phloem via the plasmodesmata. Alternatively, one might speculate, in the light of the recent finding that TMV codes for a protein associated with the stimulation of plasmodesmata, that they lack the latter protein.

Finally, in some infections, while the virus initially may spread from cell to cell, it subsequently becomes localized. By analogy with refractive virus states known in animal cell culture, plant virologists have sought an interferon-like mechanism to explain this phenomenon (see later).

In summary, the replication of relatively few plant viruses is known in any depth. Of those studied, the coat protein(s) and other products of the genome have been identified, but none of the latter proteins has as yet been definitely characterized, with the possible exception of putative viral replicases or proteins which may modify constitutive RNA-dependent RNA polymerase. No specific virus-uncoating sites have been positively identified, nor is enough known about virus movement to suggest that there are unique stages amenable to selective inhibition. Thus at present, the main attempts at 'rational' control are based on attempts to synthesize selective inhibitors of viral RNA metabolism. Many of the compounds are base or nucleoside analogues.

SCREENING SYSTEMS

Screening of chemicals for antiviral activity in intact plants poses two problems. There is the difficulty, firstly, of getting the test compound into the plant, for most of the chemicals have been optimized for activity in animal studies, and secondly, of maintaining inhibitory concentrations in the growing plant when most of the chemicals are reversible inhibitors. Uptake and distribution of chemicals in plants has been a fundamental problem in the development of systemic fungicides [35] as have associated problems of metabolism and redistribution of applied chemicals. While it may be possible to achieve even and continuous apoplastic distribution by the use of slow-release chemicals taken up by the root system, uniform symplastic movement is problematic [36]. This should be borne in mind when evaluating the practical significance of reports of transient viristasis following chemical treatment.

To avoid some of these problems, chemicals have been applied to seedlings in pots as soil drenches and/or spray treatments. In these latter systems, one needs to distinguish also between viricidal and viristatic effects. A particular problem is that virus titres may be naturally low in young tissue, and repeated and sensitive screening may be required to determine whether the effect is on virus suppression or elimination [37]. In addition, the problem of extrapolation from seedling to mature plant outlined above is relevant. In attempts to overcome the problems of uptake and maintenance of defined concentration of chemicals, plant virologists have often screened for antiviral activity in leaf disks floating on the test chemical [38]. A basic difficulty is that the specialized leaf cells may be synthesizing little constitutive protein and thus selectivity may be spurious. However, this system has merits as a prescreen; it is quick, reproducible and cheap.

A recently described tissue culture screening system [39] has apparent merit (*Figure 3.4*). In the absence of antiviral chemicals, virtually 100% of the adventitious shoots produced in explant cultures of tobacco and potato are virus-

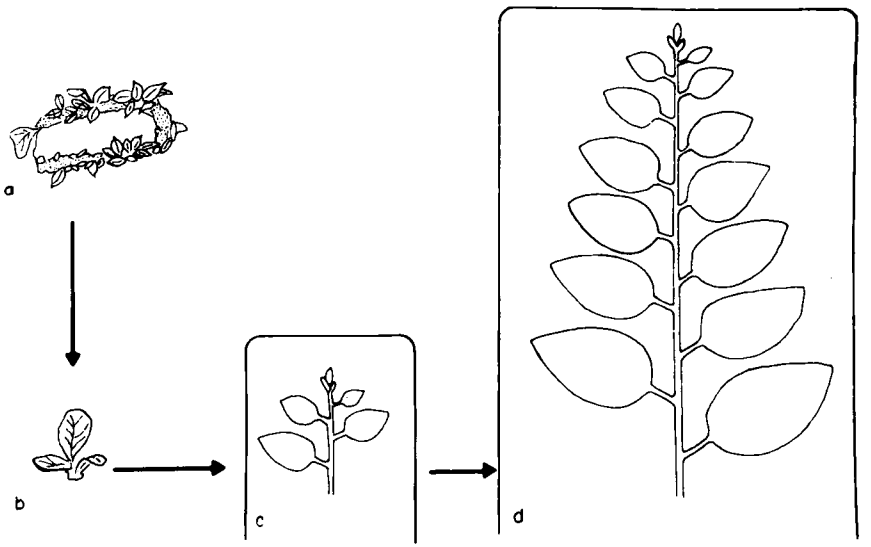


Figure 3.4. Tobacco petiole explant culture as a screening system for antiviral chemicals: (a) induction of adventitious shoot formation; (b) separation of adventitious shoots and transfer to insect-proof cages for rooting; (c) virus testing of progeny plants at the four-leaf stage (following removal from the tissue cultures, the plants are maintained in insect-proof cages); (d) if the virus test at the four-leaf stage is negative, the plants are grown on to the 15-leaf stage and retested.

infected. With ribavirin in the culture medium, this percentage can be reduced to zero [39]. In this latter system, possible side-effects of the test chemical on plant growth and development may also be detected.

Until relatively recently, plant virologists lacked a single cell system for the study of synchronous virus infection and replication. With advances in the

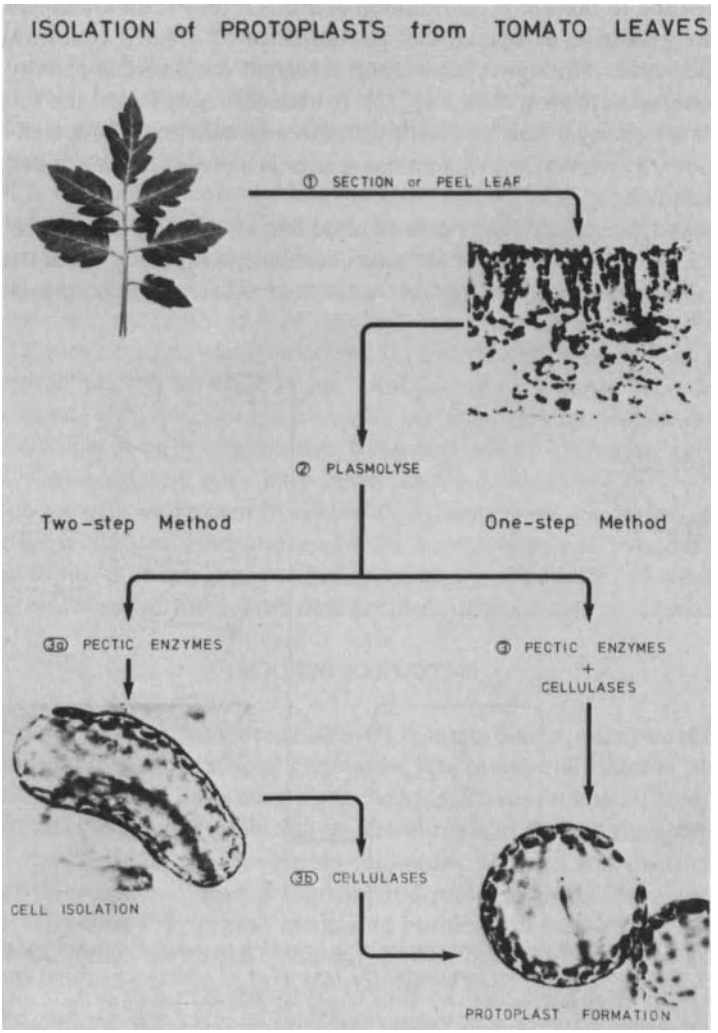


Figure 3.5. Procedures for the isolation of plant protoplasts using fungal enzymes for cell wall digestion.

methodology of plant protoplast (naked plant cell) isolation and culture (*Figure 3.5*), this was resolved [40].

Infection of plant protoplasts with viruses presented a problem. As mentioned previously, plant protoplast membranes lack virus receptor sites and there is charge repulsion between the negatively charged plasmalemma and most plant virus particles [40]. Initiation of infection has been achieved in three ways – (1) by clumping virus particles with polycations [41], the clumped particles entering the cell by pinocytosis or via wound damage [42]; (2) by precipitating the virus on to the plasmalemma and then inducing transient

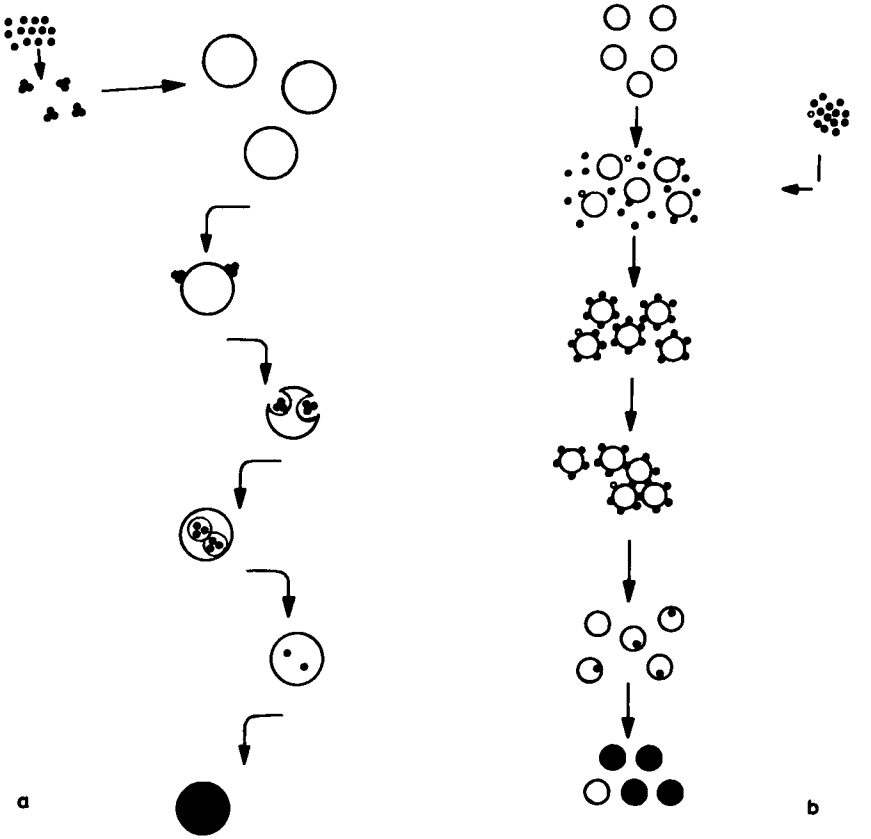


Figure 3.6. Inoculation of protoplasts with plant viruses. (a) Polycation procedure: involves clumping of virus particles with polycation followed by uptake of the virus clumps by pinocytosis or via wounds. Some of the particles taken up initiate infection. (b) Fusion procedure: virus particles are precipitated on the plasmalemma, then transient localized membrane destabilization between clumped protoplasts results in the initiation of infection.

localized membrane destabilization [43]; and (3) by incorporation of virus particles or RNA into targeted liposomes [44]. *Figure 3.6* illustrates procedures (1) and (2).

In parallel with the development of protoplast systems for the study of *in vitro* plant virus replication, the sensitivity and scope of the methodology for the study of parameters of virus replication has improved. In early studies on plant antiviral chemicals, effects on infectious virus production were determined by local lesion assay [4]. This posed problems of sensitivity and practical problems associated with the maintenance of large numbers of assay plants. It also restricted investigations to viruses which had local lesion hosts. Currently, sensitive methods including protein A-linked immunoelectron microscopy [37], and enzyme-linked immunosorbent assay [45] are used in addition to isotope studies to investigate the details of effects on viral and host proteins and nucleic acids in infected cells [46].

In spite of the methodological advances mentioned above, some caution must be exercised in evaluating the results of studies on putative plant antiviral chemicals. Where whole plants have been used as the test system, a distinction must be made between chemicals which show protective action, i.e., show activity when applied before virus entry, and chemicals which are therapeutant. Protectant chemicals, in view of the foregoing discussions of the problems of uptake and maintenance of inhibitory action in the growing plant, have less practical potential than therapeutants.

A consideration, when evaluating reports of antiviral activity in leaf disks or isolated protoplasts, is that selectivity may be spurious where the host metabolism is limited to a steady state. The main potential of protoplasts here, in the author's view, is as a mode of action study system with the tissue culture system referred to above as the primary screening system. The latter has potential also as a practical application for antiviral chemicals in plants (see *Figure 3.2*) in view of the commercial expansion of micropropagation [47].

CHEMICAL INHIBITION OF VIRUS REPLICATION IN PLANTS

NATURAL INHIBITORS

Since the report by Allard [48] of a virus inhibitor in *Phytolacca* sap, a range of inhibitors has been reported from other members of the Centrospermae [49], tobacco [50], cucumber [51], pepper [52] and sugarbeet [53]. Few of these compounds have as yet been fully characterized.

Ragetli [49] suggested that the inhibitor from *Dianthus caryophyllus*

blocked a virus 'receptor site', but there is no evidence for specific surface virus receptor sites in plants. However ectodesmata, projections from the plasmalemma into the cuticle, have been described and their role as virus entry sites discussed [54].

Stahmann and Gothoskar [55] have discussed the inhibitory action of natural polyanions and have postulated that these can act as reversible and irreversible inhibitors of viruses by clumping particles. Yet other compounds may affect the stomata, e.g., the capsicum inhibitor [56], and influence susceptibility through changes in the water relations of the plant [57]. Some of the proteinaceous inhibitors have been compared with interferon in animals [58].

The small molecules which have been shown to inactivate plant viruses, e.g., phenols and oxalic acid, are frequently either sap constituents or are induced on wounding of the sap. The former are generally cytotoxic and appear to have low selectivity. The latter are produced in plants which have mechanisms for overcoming their toxic side-effects and appear to be potentially too toxic for field application to crops.

In attempting to summarize these studies, one point is paramount, that is, most natural inhibitors act on the virus particles or cell surface and few appear to influence vector acquisition or transmission of virus [59]. In transmitting virus, the vector may bypass the surface site of action. The potential of these studies is that they may lead to the identification of host defence mechanisms capable of chemical induction.

SYNTHETIC COMPOUNDS

No attempt will be made here to review all the miscellaneous chemicals which have been screened for activity in plants (for reviews see Refs. 5–10). Instead, emphasis will be placed on benzimidazoles and ribavirin. These compounds have been chosen because they are amongst the best known antiviral chemicals to come out of synthetic programmes and because they serve to link common problems in the study of plant and animal antiviral agents.

Benzimidazole and derivatives

Ooi [60] in his review of antiviral benzimidazoles, distinguished five classes: (a) unsubstituted benzimidazole; (b) 2-(α-hydroxybenzyl)benzimidazole and its derivatives; (c) benzimidazole nucleosides; (d) substituted benzimidazoles and (e) bisbenzimidazoles. This classification is followed here. The structures are shown in *Figure 3.7.*

Benzimidazole. Benzimidazole (1), which can be considered to be a structural

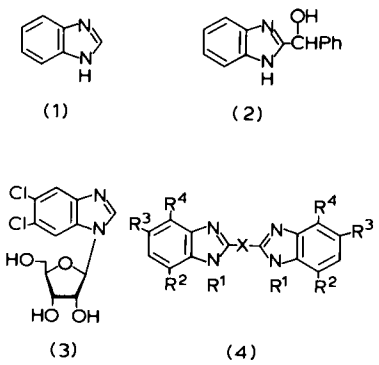


Figure 3.7. Structures of representative benzimidazoles: (1) benzimidazole; (2) a 2-substituted benzimidazole, 2-α-hydroxybenzylbenzimidazole; (3) a benzimidazole riboside, 5,6-dichloro-1-(β-D-ribofuranosyl)benzimidazole; (4) bisbenzimidazole (R^1 – R^4 = H, X = bond).

analogue of purine [61], was shown by Thompson [62] to inhibit vaccinia virus (New York strain) in tissue culture. Gifford, Robertson and Syverton [63] failed to demonstrate selective inhibition of polio virus type in HeLa cells. Subsequently, Tekerlekov, Mitev, Andonor, Dundarov, Bakalov and Boyanova [64] and Hall and Kier [65] reported selective inhibition by benzimidazole of foot-and-mouth disease and flu virus type B, respectively.

Antiviral activity of benzimidazole in plants has been reported against tobacco mosaic virus [66–68] and bromegrass mosaic virus [67]. However, Aldwinckle and Selman [69] failed to detect antiviral activity against tomato spotted wilt virus. In both cases, the treatment was given before inoculation as a spray to the intact plant [67] or via the petiole of the detached leaf [69]. Failure to show inhibition of tomato spotted wilt virus may reflect the unusual nature of this virus (see *Table 3.1*). Alternatively, this compound may be acting indirectly via effects on host metabolism but the physiological state of the host may be critical. In plants, benzimidazole is known to be converted into a ribonucleotide and to be incorporated into benzimidazole adenine dinucleotide [70]. Benzimidazoles exhibit cytokinin (hormone) activity in delaying the senescence of detached leaves [71]. (These latter points will be referred to later in discussing the possible mode of action of plant antiviral chemicals.)

2-(α-Hydroxybenzyl)benzimidazole and derivatives. Hollingshead and Smith discovered antiviral activity in 2-(α-hydroxybenzyl)benzimidazole (HBB, 2) in a random screening programme [72]. A practical criterion of antiviral activity used by animal virologists is that the substance can produce 70% inhibition of virus-induced cytopathic effect (CPE) without toxicity to the host at the virus

inhibitory concentrations (VIC). HBB exceeded this criterion [73]. However, almost immediately the potential of HBB was questioned when its spectrum was shown to be restricted [74] and resistant mutants appeared [75–77]. The spectrum of activity of HBB against animal viruses is shown in *Table 3.2*. In 1971, HBB was reported to be inactive against TMV in plants [78]. Recently, Cassells and Cocker have shown significant inhibition of TMV replication in tomato leaf disks by HBB [79]. In view of the relationship between benzimidazoles and plant hormones on the one hand and the plant antiviral activity

Table 3.2. THE HBB-SUSCEPTIBILITY OF ANIMAL VIRUSES [60]

++, complete inhibition; +, active; ±, slight or just detectable inhibition; –, inactive.

Group	*Sub-group*	*Type*	*Susceptibility*
(I) RNA-viruses			
(a) Picornavirus	Poliovirus	1, 2 & 3	+
	Coxsackievirus A	9	+
	Coxsackievirus A	7, 11, 13, 16,	+
		18, 21	–
	Coxsackievirus B	1–6	
	ECHO virus	1–9, 11–21, 24–27	+
	ECHO virus	22, 23 & 28	–
	Rhinovirus*	(some strains)	+
	Encephalomyocarditis	mouse	+
(b) Reovirus		1, 2 & 3	–
(c) Arbovirus	B & C		–
(d) Myxovirus	Influenza virus A		±
	Influenza virus B		–
(e) Paramyxovirus	Parainfluenza	3	±
	Parainfluenza	2 (& 3)	–
	Mumps		–
	Measles virus	(two strains)	+
(f) Arenavirus	Lymphocytic choriomeningitis virus (LCM)	(four strains)	+
(II) DNA-viruses			
(a) Picodnavirus	H1		++
	RV		++
	X14		++
(b) Adenovirus		2–5	–
(c) Herpesvirus	Herpes simplex		–
(d) Poxvirus	Vaccinia		–

* Inconsistent susceptibilities were reported for various strains.

of benzimidazoles on the other [79], it is appropriate here to discuss the studies of the structure-activity relationship and mode of action of HBB and its derivatives in animal antiviral chemotherapy as a preface to discussion of the plant work.

Of the optical isomers of HBB, D-HBB has been shown to be more active than the L-isomer [80]. While the alpha-carbon appears spatially necessary, the role of the α-OH has been the subject of debate. O'Sullivan and Sadler have implicated it in intramolecular bonding, at the target site [81], whereas an American team has rejected this hypothesis in view of the activity of 2-(α-methoxybenzyl)- and 2(α-acetoxybenzyl)benzimidazoles [82]. Substitution of the heterocyclic ring resulted in loss of activity [82]. The view has been expressed that the overall configuration of HBB is of critical importance in relation to its antiviral activity in animal systems [83].

O'Sullivan and Wallis have ruled out the possibility that HBB is metabolized to nucleoside or nucleotide derivatives in animal cells by showing that N^1-substitution can increase antiviral activity as follows HBB < 1-*n*-pentyl < 1-benzyl, 1-*n*-butyl < 1-*n*-propyl < 1-crotyl < 1-allyl < 1-phenyl [84]. This N^1-substitution effect suggests that the lipid-to-water coefficient might be related to antiviral activity.

Substitution at the 5-position with a halogen in the order of increasing electron-withdrawing potential, i.e., Br < Cl < F, increases the activity of both HBB and N^1-*n*-propyl-HBB [85].

While systematic studies of the antiviral activity of HBB and its derivatives in plants have been limited, Cassells and Cocker, in studies on inhibition of TMV in leaf disks, have shown that the nature of the substituent at the 2-position and antiviral activity, parallels that found in the animal studies [79]. They have also shown that apparent hormone and antiviral activity in 2-substituted benzimidazoles are separable (see Symptom suppression, below).

Results of a more extensive systematic examination in plants of the effects of substituents on the benzyl and benzimidazole rings of HBB are given in *Table 3.3*. No distinctive group behaviour is seen between electron-withdrawing groups, Cl, Br and NO_2, and electron-donating groups, MeO, Me, on the heterocyclic ring. In no case was antiviral activity significantly increased over that shown by HBB. By comparison with animal studies, 5-bromo substitution decreased antiviral activity. The most striking result was the significant stimulation of virus synthesis by the 5(6)-methoxy derivative.

Substitution of the benzene ring resulted in reduction in antiviral activity in most cases, e.g., *m*- and *p*-chloro, *p*-methyl and *m*-methoxy, and *p*-nitro derivatives. Substitution of fluorine at the *ortho*-position resulted in slightly increased antiviral activity. The *p*-nitro derivative stimulated virus production (*Table 3.3*).

Table 3.3. INHIBITORY ANTIVIRAL ACTIVITY OF HBB DERIVATIVES AGAINST TMV IN TOBACCO OF LEAF DISKS

+ indicates stimulation of virus replication (Cassells, Long and O'Driscoll, unpublished work).

HBB derivatives	*Inhibitory activity relative to HBB (100%)*
2-(α-Hydroxybenzyl)-5,6-dichlorobenzimidazole	11
2-(α-Hydroxybenzyl)-5(6)-bromobenzimidazole	71
2-(α-Hydroxybenzyl)-5(6)-nitrobenzimidazole	66
2-(α-Hydroxybenzyl)-4,7-dimethoxybenzimidazole	40
2-(α-Hydroxybenzyl)-4(7)-methylbenzimidazole	100
2-(α-Hydroxybenzyl)-5(6)-methoxybenzimidazole	+343
2-(α-Hydroxy-*m*-methoxybenzyl)benzimidazole	71
2-(α-Hydroxy-*p*-methylbenzyl)benzimidazole	77
2-(α-Hydroxy-*o*-methylbenzyl)benzimidazole	100
2-(α-Hydroxy-*p*-nitrobenzyl)benzimidazole	+71
2-(α-Hydroxy-*o*-fluorobenzyl)benzimidazole	106
2-(α-Hydroxy-*p*-chlorobenzyl)benzimidazole	3
2-(α-Hydroxy-*m*-chlorobenzyl)benzimidazole	17

The development of a selective inhibitor of viral RNA-dependent RNA polymerase has been a primary objective of the development of HBB and derivatives as antiviral compounds. Evidence that HBB can inhibit RNA-dependent RNA polymerase in animal cells *in vitro* has been reported by Dmitrieva and Agol [86].

While the studies reported so far on HBB and derivatives in plant systems have been limited and even contradictory (see above), two aspects are worthy of comment. Firstly, if HBB is acting as an inhibitor of RNA-dependent RNA polymerase, the evidence suggests that in the case of TMV, it is acting on a host-coded enzyme stimulated by the virus [29]. It is questionable whether selectivity can be achieved if this enzyme has a metabolic function in the host. Secondly, some HBB derivatives stimulate plant virus replication (*Table 3.3*), which may indicate a host cell target. Stimulation has also been reported in animal virus studies [87]. HBB-resistant strains were detected early in studies of effects on animal virus replication [77]. It might be anticipated that any agent which acted directly as an inhibitor of a virus-coded enzyme would put a selection pressure on the virus genome and result in the appearance of mutant strains.

Benzimidazole nucleosides. The relationship between the benzimidazole ring and purines also stimulated a synthetic programme by animal virologists, based on substituted benzimidazole ribosides [88]. The antiviral activity of halogen-

substituted benzimidazole ribosides increased, regardless of the halogen, as the number of substituents increased in the fused benzene ring. Replacement of the ribose with other sugar residues did not increase activity over that of the corresponding unsubstituted free benzimidazole (*Table 3.4*).

DRB (5,6-dichloro-1-β-D-ribofuranosylbenzimidazole, 3) has been screened for antiviral activity in plants but has been shown to be a non-selective inhibitor of RNA synthesis (R.S.S. Fraser, personal communication).

Substituted benzimidazoles. Many reports have appeared of antiviral studies on substituted benzimidazoles other than those already discussed. However, with the exception of certain 2-substituted benzimidazoles to be discussed under Symptom suppression, these studies have not led to any notable development in plant antiviral chemotherapy and will not be discussed here.

Bisbenzimidazoles. A number of bisbenzimidazoles (4) have been reported to

Table 3.4. INHIBITORY ACTIVITY OF BENZIMIDAZOLE NUCLEOSIDES AGAINST INFLUENZA B VIRUS [60]

Benzimidazole nucleosides	*Inhibitory activity relative to benzimidazole*
1-β-D-Ribofuranosyl	unavailable
5(6)-Chloro-1-β-D-ribofuranosyl	13
5,6-Dichloro-1-β-D-ribofuranosyl (DRB)	92
5,6-Dichloro-1-α-D-ribopyranosyl	15
5,6-Dichloro-1-β-D-ribopyranosyl	15
5,6-Dichloro-1-α-D-arabinopyranosyl	3.1
5,6-Dichloro-1-β-D-arabinopyranosyl	3.1
5,6-Dichloro-1-β-D-galactopyranosyl	$>1.8<3.5$
5,6-Dichloro-1-β-D-glucopyranosyl	~3.2
5,6-Dichloro-2-methyl-1-β-D-ribofuranosyl	9.7
5,6-Dichloro-2-methyl-1-β-D-glucopyranosyl	2.7
4,5,6(5,6,7)-Trichloro-1-α-D-ribofuranosyl	165
4,5,6(5,6,7)-Trichloro-1-β-D-ribofuranosyl (TRB)	760
4,5,6,7-Tetrachloro-1-α-D-ribofuranosyl	950
5,6-Dibromo-1-β-D-ribofuranosyl	230
4,5,6(5,6,7)-Tribromo-1-β-D-ribofuranosyl	1000
5(6)-Bromo-4,6(5,7)-dichloro-1-α-D-ribofuranosyl	250
5(6)-Bromo-4,6(5,7)-dichloro-1-β-D-ribofuranosyl	1950
5(6)-Iodo-4,6(5,7)-dichloro-1-α-D-ribofuranosyl	73
5,6-Dimethyl-1-α-D-ribofuranosyl	inactive
5,6-Dimethyl-1-D-lyxopyranosyl	inactive
5,6-Dimethyl-1-D-arabinopyranosyl	inactive
2,5(2,6)-Dimethyl-1-β-D-ribofuranosyl	inactive

have antiviral activity in animal systems (*Table 3.5*). To the author's knowledge, none of these compounds has been studied in plant systems.

Ribavirin

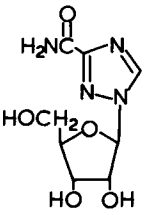
Following the discovery that showdomycin and pyrazomycin – nucleosides with five-membered heterocyclic rings – had antiviral activity, a programme of synthesis of imidazole and triazole ribonucleosides was undertaken [3]. The

Table 3.5. ANTIVIRAL BISBENZIMIDAZOLES (4) [60]

PV1, Poliovirus Type 1 (Mahoney strain). LCM, Lymphocytic choriomeningitis virus (UBC, WCP & CA1371 strains). Rhinovirus, Rhinovirus serotypes 1A and 42.

R^1	R^2	R^3	R^4	X	*Virus*
H	H	amidino	H	CH_2	Respiratory syncytial virus
H	H	H	H	$(CH_2)_2$	PV1
H	H	Cl	H	$(CH_2)_2$	PV1
H	H	NO_2	H	$(CH_2)_2$	PV1
H	H	OMe	H	$(CH_2)_2$	Rhinovirus & LCM
H	H	Me	H	CH_2CHOH	PV1 & Adenovirus Type 1
H	H	OMe	H	CH_2CHOH	PV1, Rhinovirus & LCM
H	H	H	H	$(CHOH)_2$	PV1
H	H	Me	H	$(CHOH)_2$	PV1
H	H	Cl	H	$(CHOH)_2$	PV1 & Rhinovirus
H	H	Me	H	$(CHOH)_2$	PV1, Rhinovirus, LCM & Coxsackie A9 & B3
H	H	OEt	H	$(CHOH)_2$	Rhinovirus, Coxsackie A9 & B3
Me	H	H	H	$(CHOH)_2$	PV1
H	OMe	H	OCH_3	$(CHOH)_2$	Rhinovirus
H	H	H	H	$(CH_2)_3$	PV1
H	H	NO_2	H	$(CH_2)_3$	PV1
H	H	H	H	$(CHOH)_3$	PV1
H	H	H	H	$(CH_2)_4$	PV1
H	H	Cl	H	$(CH_2)_4$	PV1
H	H	NO_2	H	$(CH_2)_4$	PV1
H	H	OMe	H	$(CH_2)_4$	PV1
H	H	H	H	$(CHOH)_4$	PV1
H	H	OMe	H	$(CHOH)_4$	PV1
H	H	Me	H	$(CH_2)_5$	PV1
H	H	Me	H	$(CH_2)_6$	PV1
H	H	NO_2	H	$(CH_2)_6$	PV1
H	H	OMe	H	$(CH_2)_6$	PV1
H	H	OMe	H	$(CHOAc)_2$	Rhinovirus

rationale has been referred to (see Introduction). From this programme, ribavirin, (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide, virazole, 5) has been shown to have wide spectrum antiviral activity against both DNA and RNA animal viruses, but is generally not active against those viruses with +RNA genomes (see Plant studies, later) [89]. That ribavirin shows low activity against some DNA viruses in cell culture has led Smith to remark that, 'its antiviral activity must be ascribed to a relatively viral-specific event or possibly to a combination of host cell and viral events' [90].



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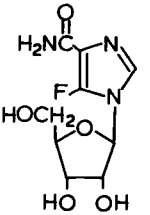

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Extensive structure activity studies have been carried out on ribavirin derivatives and analogues. Metabolic studies have shown that ribavirin is phosphorylated to mono-, di- and triphosphates which are in energy charge equilibrum with the adenosine nucleotides [90]. Studies on the sugar moiety have shown that the 1,2,4-triazole-3-carboxamide derivative is active, possibly through conversion to a β-D-riboside *in vivo*, but that other sugar derivatives show little or no activity [3]. The metabolism of ribavirin is shown in *Figure 3.8*.

Substitution of the heterocyclic ring has in most cases resulted in loss of activity, an exception being 5-fluoro-1-β-D-ribofuranosylimidazole-4-carboxamide (6) [91] which shows a broad antiviral spectrum, but is not as active as ribavirin.

As with HBB, ribavirin appears to have very stringent structural requirements for uptake and activity. This may be because either the molecule needs to be 'processed' by cellular enzymes before it reaches its target(s) or it acts both on host enzymes of nucleotide metabolism and enzymes of nucleic acid metabolism. This is discussed further below. Ribavirin does not significantly accumulate in RNA or DNA [3]. A monograph has recently been produced on ribavirin [92].

Considerable controversy surrounds the possible mode of action of ribavirin. It has been shown to act as a reversible inhibitor of animal cell RNA metabolism [93] and as an inhibitor of inosine monophosphate (IMP) dehy-

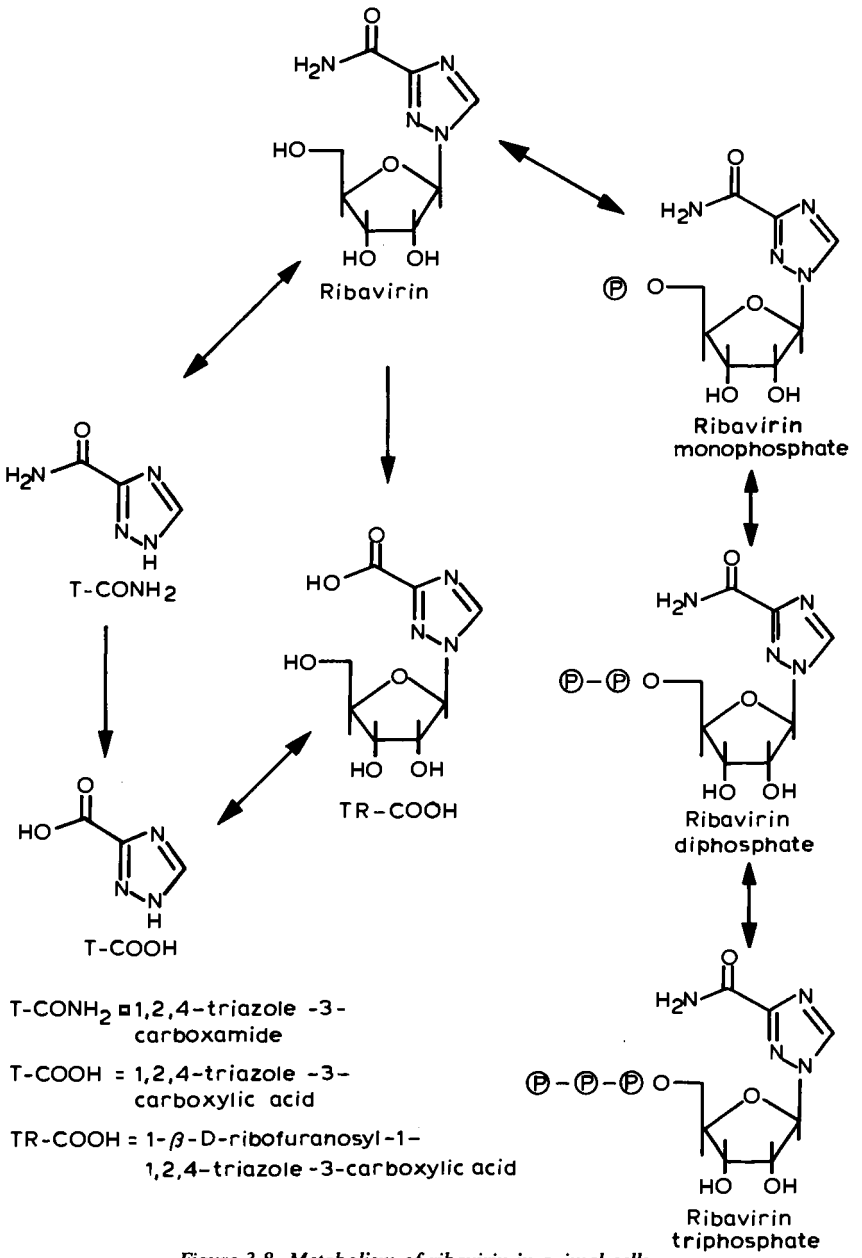


Figure 3.8. Metabolism of ribavirin in animal cells.

drogenase [94]. The consequence of the latter is to reduce the pool of guanosine and other nucleotides [95]. Xanthosine, and in some cases guanosine, reverses the antiviral effects of ribavirin [96]. However, as Smith has pointed out, it would be difficult to achieve selectivity if the sole effect were inhibition of IMPDH [90]. Also, ribavirin does not inhibit polio virus RNA, which is relatively unusual in that it lacks a 5′ 'cap', that is, a 5′-terminal 7-methylguanosine normally required for translation *in vitro* of viral RNA and a common feature of all eukaryotic mRNAs. The structural relationship between ribavirin and guanosine raises the possibility that ribavirin may inhibit 5′ capping enzyme(s). There is evidence of impaired capping of viral RNA in the presence of ribavirin leading to reduced translation efficiency [97]. In this connection it is important to emphasize that many animal viruses inhibit host RNA synthesis and thus ribavirin under these circumstances could show spurious selectivity.

Finally, Stollar and Malinoski have raised the interesting possibility that perturbation by ribavirin of intracellular nucleotides *per se* may result in virus inhibition [98].

Ribavirin has been shown to reduce the virus content of whole plants [99–105] and leaf disks [106] and to increase the frequency of virus-free plants produced from protoplasts [107], explants and bud-tips of virus infected plants [108, 109]. Ribavirin was applied to the plant via the roots [99] or as a foliar spray [101–105] prior to virus inoculation. It has been reported that the chemical must be applied within 8 h of inoculation [102].

Selective inhibition of viral RNA in tobacco plants infected with TMV has been demonstrated [100]. Cassells, Long and Austin have detected inhibition of TMV replication in TMV-inoculated tobacco protoplasts cultured in the presence of ribavirin [106]. However, Cassells and Long [68] have failed to reverse the growth-inhibitory effects of ribavirin by culturing tobacco tissues in the presence of guanosine or xanthosine.

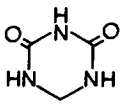
Ribavirin has been shown either to inhibit or to stimulate TMV replication in tomato leaf disks, depending on the concentration [106]. This type of response has been shown also with some 2-substituted benzimidazoles and cytokinins (plant hormones) [110, 111]. The possible interaction between antiviral chemicals such as ribavirin and 2-substituted benzimidazoles and plant hormone metabolism will be discussed later.

In the absence of a defined target, the mode of action of ribavirin poses an interesting problem, the answer to which may have basic implications for the design and screening of antiviral agents. Smith [90] has identified IMPDH and the enzyme(s) of 5′ cap synthesis as possible targets for ribavirin in animals. These enzymes involved may both be constitutive in tobacco. Thus, antiviral

selectivity may depend on the greater vulnerability of the virus, compared with the host, to inhibition of these enzymes, and antiviral activity may be expressed only during specific physiological states of the host cell. Stollar and Malinoski have proposed that ribavirin acts primarily through its effects on nucleotide pools. They have hypothesized, 'the antiviral effects of ribavirin suggest that in *A. ablopictus* cells each of the four ribonucleotide triphosphates must be present at a certain minimal concentration in order for virus replication to occur; when the concentration of one or more of these nucleotides falls below a threshold level, viral replication is inhibited' [98].

Miscellaneous compounds

In addition to ribavirin, benzimidazole and its derivatives, a wide range of nucleic acid base derivatives and analogues have been screened in plants [5–10]. Other compounds tested include guanidine and its derivatives [113]. A number of these compounds, including 2-thiouracil and 8-azaguanidine, have been shown to be incorporated into viral nucleic acid and to modify its biological activity [114, 115]. The phytotoxicity of these compounds appears to preclude their practical use [5–10]. More recently, the antiviral activity of dihydro-1,3,5-triazine-2,4-dione (5-azadihydrouracil, 2,4-dioxohexahydro-1,3,5-triazine, DHT, 7) has been described [116, 117]. Schuster and Byhan have reported that DHT selectively inhibits viral RNA synthesis in TMV-infected tobacco leaves [118]. Schuster has also advocated the combination of DHT with ribavirin [119].



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Amongst the substituted nucleic acid bases possessing selective antiviral activity are compounds, such as 5-bromo-2′-deoxyuridine, which are mutagenic [120]. Incorporation of such agents into viruses without nucleic acid repair mechanisms could critically impair genome function. However, selectivity has in many cases been reported in circumstances where no host DNA synthesis is taking place. The effects on the host remain to be clarified. This approach has been reviewed by Mitchell [28].

The histories of HBB and guanidine have progressed in parallel. Both appear to inhibit selectively picornavirus viral RNA synthesis, albeit of a

limited number of viruses or virus strains. As in HBB studies, guanidine-resistant mutants have been reported. Relatively few guanidine derivatives have been screened, compared with HBB derivatives. Of these, most are less active than the parent compound, with the exception of methylglyoxal bis(guanylhydrazone) [83]. The only systematic screening reported in plants was carried out by Schuster, who screened 27 guanidine derivatives [119]. Of these, guanidine nitrate, *N*-cyanoguanidine and guanylurea sulphate inhibited PVX and TMV replication in secondary-infected leaves of *N. tabacum* 'samsun'.

Caliguiri and Tamm have published a comparative review of guanidine and HBB studies in animal virology [83].

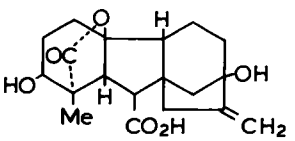
PLANT HORMONES AND SUPPRESSION OF VIRUS SYMPTOMS

PLANT HORMONES AND VIRUS REPLICATION

Many plant virologists have expressed the view that virus-induced alteration in endogenous hormone metabolism may underlie the abnormalities of growth and reduction in vigour of virus-infected plants [121, 122].

Both natural [123] and synthetic auxins [123–125] have been shown to inhibit virus production in infected plants. 2,4-D (2,4-dichlorophenoxyacetic acid) has been reported to mask potato leaf roll virus symptom(s) [126].

Cytokinins, both natural and synthetic derivatives, have been shown to affect virus replication in isolated leaf disks and whole plants. Both inhibition and stimulation of virus replication by cytokinins have been reported [110, 127–131]. Milo and Srivastava [132] have excluded the possibility that (in leaf disks at least) the effect of cytokinins is primarily on chlorophyll retention, i.e., in delaying senescence and thus maintaining the biosynthetic capability of the test system. Their studies led them to the conclusion that the effect of cytokinins on viruses appears to be virus-specific and may depend on the particular host-virus system, the cytokinin and its concentration, and other factors. (There will be further discussion of cytokinins later.)



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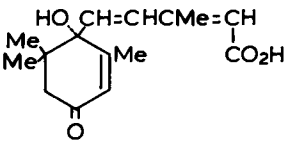

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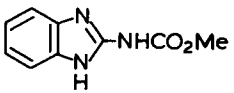

(10)

Gibberellic acid (GA, 8) has been shown to reverse the effects of virus inhibition of growth in several virus-host combinations [133–136]. Bailiss [137] has shown that the gibberellin content of CMV-infected cucumber plants is lower than that of healthy plants. GA, like cytokinins and auxins, can stimulate virus production [138]. Abscisic acid (ABA, 9), which enhances leaf senescence (see cytokinins above) has been shown to increase virus susceptibility [139].

Schuster [140] has investigated the interaction between ribavirin and plant hormones in antiviral chemotherapy in whole plants. In a study of PVX infection of tobacco plants, he found that indoleacetic acid, kinetin, abscisic acid and ethylene had only negligible effects on the concentration of virus in inoculated leaves, but tended to reduce the virus content in secondary-infected leaves. Gibberellin (GA_3) had no effect. In combination with virus-inhibitory but toxic concentrations of ribavirin, ABA reduced the toxicity and enhanced virus inhibition. Also, the antiviral activity of ribavirin tended to be increased by the hormones tested.

CHEMICAL SUPPRESSION OF VIRUS SYMPTOMS

Many of the symptoms of viruses in plants, e.g., stunting, premature senescence of tissues and organs, and abnormalities of shape, resemble hormonal upsets in the infected plants; hence the rationale of the antiviral hormone studies outlined above. Tomlinson, Faithfull and Ward advanced this approach when they used methyl benzimidazol-2-yl-carbamate (carbendazim, MBC, 10) to suppress the symptoms of TMV in tobacco and beet western yellows virus in lettuce [141]. MBC is a 'pivotal' compound in the context of this review, since it possesses cytokinin activity but is also a 2-substituted benzimidazole. Before proceeding to discuss the implication of the latter, it should be mentioned that the 'beneficial' effects of MBC treatment are double-edged, for it may also enhance virus replication in some hosts, e.g., TMV in tomato and CMV in cucumber [110]. Fraser and Whenham [112] have reported that MBC shows no inhibition of TMV RNA synthesis and hypothesized that 'possibly through the known cytokinin activity of the compound, MBC may act indirectly by maintaining the host in a state unsuitable for viral multiplication' (see also effects of hormones and ribavirin, discussed above).

Studies at Cork on plant antiviral activity in cytokinins, HBB and other 2-substituted benzimidazoles and ribavirin, showed that these compounds exhibit concentration-dependent stimulation or inhibition of TMV replication in tomato and tobacco leaf disks (*Figure 3.9*) [68, 110]. This prompted assay of the 2-substituted benzimidazoles for their effects on chlorophyll reten-

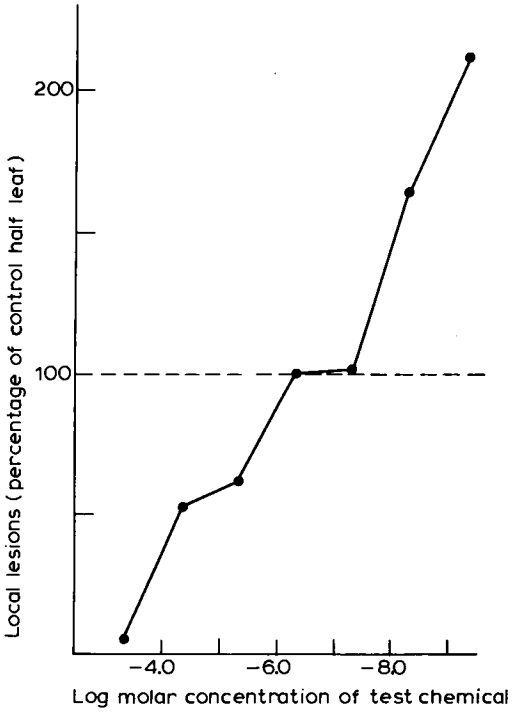

Figure 3.9. Effect of ribavirin on tobacco mosaic virus production in tobacco leaf disks. HBB shows a similar pattern of response.

tion in the leaf disks and for cytokinin activity in the Amaranthus β-cyanin bioassay and soyabean callus bioassay. None of the compounds tested – 2-(α-hydroxybenzyl)-, 2-benzyl-, 2-phenyl-, 2-methyl- or 2-hydroxymethylbenzimidazole – showed cytokinin activity, but there were effects on chlorophyll concentration. The relationship between antiviral activity and the nature of the 2-substituent was as reported in animal studies [108].

Data cited in this review for the activity of ribavirin, MBC and some 2-substituted benzimidazoles, which may either suppress or stimulate virus replication depending on concentrations, support the view that the action of these compounds may be physiological. Interestingly, cytokinins have been reported to alter nucleotide pools and energy charge in plants [142, 143] and there is evidence that energy charge is altered in virus-infected tissues [144]. MBC may be acting to maintain a high energy charge in infected cells, thus allowing normal host synthesis to occur alongside virus replication. Under these condi-

tions it is hypothesized that virus-induced metabolite pool depletion would not occur and consequently there would be no symptoms produced.

In addition to altering nucleotide pools, or energy charge of such pools, test compounds may be affecting host RNA metabolism. Stimulation of host RNA metabolism at appropriate cytokinin concentrations [145] might explain the increase in the relative competitiveness of host RNA for ribosomes. Cytokinins have also been reported to stimulate RNase [128] which may function in an antiviral role by increasing viral RNA turnover, although it has been questioned whether this has a selective antiviral effect [146].

A critical balance between virus synthesis and breakdown may be exploitable. Delay in completing the replication cycle may make the virus susceptible to endogenous processes leading to viral RNA degradation (or failure to achieve particle maturation, although it is known that viral RNA *per se* can cause systemic spread) [34]. Nevertheless, the concept of critical rates of viral RNA production for disease development may be important.

Two aspects of the possible indirect antiviral activity of compounds such as ribavirin exist. The first is whether compounds which function, in this case, as inhibitors of nucleotide metabolism can be used through the different developmental phases of the host plant without affecting growth and cropping behaviour. The second is whether their spectrum of antiviral activity varies with the physiological state of the host tissue or with the virus strain. These latter considerations, combined with the problem of maintaining appropriate concentrations of such reversible inhibitors in developing plants, constitute a formidable challenge to the investigator seeking antiviral chemicals for field application.

CHEMICAL INDUCTION OF HOST DEFENCE MECHANISMS

Yarwood [147] demonstrated that inoculation of the leaves of hypersensitive tobaccos resulted in zones around the primary virus lesions which were resistant to superinfection. Prior to this, Gilpatrick and Weintraub [148] showed that upper uninoculated leaves developed a resistance to a second inoculation. This has been called induced or acquired resistance [149, 150]. Recent reviews have been published on virus interaction in plants by Hamilton [151] and on induced resistance by Loebenstein [152]. For completeness, this topic is discussed here in relation to the possibility of chemical induction of host defence mechanisms.

In a series of papers dating from 1968, van Loon demonstrated the presence of up to four 'new' proteins in leaves showing acquired (induced) resistance,

depending on the host cultivar (e.g., Ref. 153). Gianinazzi [154] suggested that the induction of these proteins was analogous to the interferon system in animals and subsequently, with Kassanis [155], he investigated the effect of synthetic polyanion interferon inducers [156] on acquired resistance and production of these 'new' proteins in plants. Polyacrylic acid (PA) was shown to induce both the 'new' or b-proteins, as they came to be known, and resistance to hypersensitive TMV or tobacco necrosis virus in tobacco, but only partial inhibition of systemic PVX was reported [157]. Kassanis and White, following investigation of the effect of PA on PVX and PVY and other viruses in tobacco, speculated that, 'the system that localized TMV in Xanthi tobacco (hypersensitive) is activated by virus infection or by treatment with PA causing the production of additional proteins that are probably responsible for increase in resistance to virus infection'. That PA-induced resistance was related to the N (hypersensitive) gene in tobacco was challenged by Cassells and Flynn [158], who demonstrated PA-induced resistance to a range of viruses in non-N-gene containing tobaccos and a non-solanaceous host. Barker [159] first suggested that the b-proteins were present in healthy plants. Shortly after, Cassells, Barnet and Barlass suggested that PA was acting as a wilt-inducing toxin and that it reduced the susceptibility of plants to mechanically-inoculated viruses [160]. They further demonstrated that protoplasts isolated from tobacco leaves showing induced resistance were susceptible to TMV and capable of supporting the same level of replication as those from control leaves [160].

Fraser, who has shown that b-proteins accumulate in healthy plants, particularly at flowering, and are induced by MBC, has failed to demonstrate a correlation between these proteins and induced resistance [161, 162]. Studies by Cassells and Atkins (unpublished) suggest that the b-proteins may be stress-related.

Results to date of investigations of acquired resistance have failed to identify any specific defence system. The evidence seems to suggest that induced resistance may be a consequence of the altered physiological state of the plant by primary inoculation or PA treatment. However, separate research on TMV infection of hypersensitive *Nicotiana* (tobacco) has led Sela to suggest that interferon-like materials may be involved in resistance [50]. In addition, the suggestion that cAMP may be involved in resistance to virus in plants has been made [50].

POSSIBILITIES FOR THE FUTURE

Of the currently characterized viral pathogens of crop plants, the majority appears to have a +RNA replication strategy (*Table 3.1*). Most enter via vector-caused wounds, thus bypassing any surface 'receptor' site. When in the cell, they are uncoated, but no specific site(s) have been unequivocally identified [4], the +RNA may then code for a number of proteins (subgenomic messengers) [4]. In general, the particle contains only one species of protein [27]. A second protein may function as a RNA-dependent RNA polymerase or may form a complex with a host enzyme with similar activity, e.g., alter the specificity of the host enzyme. It is interesting to speculate that the virus strain-host polymerase induction may be on a protein-for-protein basis [163]. In the case of TMV, a third protein has recently been implicated in increased plasmodesmatal formation [32]. One might hypothesize that this has the effect of increasing (or controlling) virus egress from infected cells. Movement *per se* is a passive phenomenon influenced primarily by the endogenous mechanism controlling photosynthate movement [164]. The physiology of the plant may be affected and this results in altered metabolism and solute movement [165]. Virus egress from infected cells to mature competent cells and production of virus competent cells through cell division, constitute the steady-state progression of the infection. Viruses in some cases exhibit turnover or cyclical changes in concentration in the infected plant [4], but whether this is a passive or active mechanism is unknown.

Compared with the diversity of viruses affecting animals and the complexity of the animal body, the range of the problem is much less for those seeking antiviral agents for plant use. However, this does not necessarily mean the problem is less intractable.

The primary hypothetical target for antiviral chemicals has been the putative virus-coded enzyme(s) involved in viral nucleic acid replication (see Introduction). Where this is a virus-coded enzyme, the possibility of producing selective inhibitors is still a primary objective. However, such agents may act as a selection pressure for mutant viruses, thus limiting their field potential. (This may be an explanation of the appearance of HBB mutants referred to above [77].) If this is a constitutive enzyme or if the virus contributes a subunit to a virus-host polymerase complex, it may be difficult to inhibit this constitutive enzyme selectively without affecting the host during its development.

Fundamental to a programme of rational synthesis of antiviral chemicals remains the task of identifying and characterizing potential target sites (see *Figure 3.3*). Once this has been realized, the synthesis of trypsin inhibitors provides a model for the design of irreversible inhibitors of viral function [28].

A second parallel strategy should involve elucidation of possible antiviral defence mechanisms in plants and the mechanism of cross-protection ('immunization') of plants [166]. Cross-protection involves inoculation of plants with mild strains of viruses which prevent the development of virus symptoms upon subsequent inoculation with severe or symptom-producing strains [167]. Hamilton [151] has suggested five possibilities offered by the latter: sequestration of challenge virus RNA by inducer virus capsid protein; sequestration of challenge virus RNA by inducer virus RNA replicase; inducer virus coded-inhibitors; metabolic stress; and (natural) inhibitors. These mechanisms all offer the possibility of developing chemical inducers or of the appropriate genes being utilized in genetic engineering of plants [168].

Theoretically, the screening system problems have been solved in recent years with the development of protoplast culture for the study of plant virus replication, although protoplast isolation *per se* still presents practical problems [169]. While this single-cell system may have advantages over the whole plant in terms of chemical uptake and distribution, it is doubtful whether it is a great advance over the leaf disk system. Both animal and plant cells, and leaf disks, may have essentially steady physiological states with little (quantitatively and qualitatively) active host protein synthesis. In this system, it is questionable whether selectivity against viruses can be determined. They are probably best regarded as pre-screening systems with the proviso that a compound may show desirable indirect antiviral activity in the whole plant masked in such systems or, conversely, toxic effects may be hidden.

Tissue culture, or more precisely, bud or bud-tip and explant culture [47], has revolutionized the production of traditionally vegetatively propagated crops and has increased the pressure for virus-free stock for mass clonal propagation.

The possible advantages of tissue culture for the screening of antiviral chemicals have been discussed above. Explant culture and bud tip culture involves direct culture of the exposed plant cells on the medium containing antiviral chemical; thus, exposure to the inhibitory concentration can be maintained, effectively solving two of the main problems confronting plant virologists testing reversible inhibitors designed as a result of *in vitro* animal screening. This system has the advantage over other *in vitro* screening procedures in that the effects on developmental processes can be assessed.

In conclusion, an attempt has been made to define the priorities for action in virus disease control in relation to the development of commercial antiviral chemicals. One of the most intractable problems in virus disease control is that of preventing virus accumulation in long-term crops. There is some evidence that once virus-free stock has been obtained, it may remain virus-free in the

field where the virus-vector relationship has been lost. Here, an understanding of the mechanism of cross-protection may lead to advances, for instance, in the production of cross-protecting strains where there is little risk of mutation or pseudo-recombination to produce virulent strains [167].

It is likely that the existing strategies for the elimination of virus from vegetatively propagated stock will be expanded by the inclusion of antiviral chemicals in the tissue culture medium combined with *in vitro* storage of virus-free stock (see *Figure 3.2*). In the short term, the existing methods of vector and reservoir control will constitute the mainstays of control of virus diseases of annual crops. The impetus for refinement of genetic engineering techniques and protoplast fusion for plant improvement may result in advances in the production of resistant varieties in the longer term. In the medium term, somaclonal variation may yield commercially useful resistant clones [170]. The balance in the priorities for action could change if advances in basic plant virology lead to the elucidation of targets for selective chemical inhibition or chemically inducible host defence mechanisms.

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REFERENCES

1. E.C. Herrmann, Jr., Annu. Rep. Med. Chem., 122 (1966).
2. I. Tamm, Antibiot. Chemother., 12 (1962) 437.
3. S. Harris and R.K. Robbins, in: Ribavirin: A Broad Spectrum Antiviral Agent, eds. R.A. Smith and W. Kirkpatrick (Academic Press, New York, 1980) pp. 1–21.
4. R.E.F. Matthews, Plant Virology, 2nd Edn. (Academic Press, New York, 1981).
5. R.E.F. Matthews, Nature (London), 167 (1951) 892.
6. R.E.F. Matthews, J. Gen. Virol., 8 (1953) 277.
7. R.E.F. Matthews and J.D. Smith, Adv. Virus Res., 3 (1955) 51.
8. B.M. Gupta, in: Aphids as Virus Vectors, eds. K.F. Harris and K. Maramorosch (Academic Press, New York, 1977) pp. 455–471.
9. T. Hirai, in: Plant Disease, an Advanced Treatise, eds. J.C. Horsfall and E.B. Cawling (Academic Press, New York, 1977) Vol. 1, pp. 285–306.

10. R.C. Sinha, in: Metabolic Inhibitors, eds. E. Hochester, J. Kates and J. Questel (Academic Press, New York, 1972) Vol. 3, pp. 277–304.
11. S.A.J. Tarr, The Principles of Plant Pathology (Macmillan, London, 1972).
12. K. Bjorling, Acta Agric. Scand. Suppl., 16 (1966) 119.
13. R.T. Plumb, in: Strategies for the Control of Cereal Diseases, eds. J.F. Jenkyn and R.T. Plumb (Blackwell Scientific Publications, Oxford, 1981) pp. 135–145.
14. K.F. Harris and K. Maramorosch, eds., Aphids as Virus Vectors (Academic Press, New York, 1977).
15. L. Bos, ed., in: Plant Health and Quarantine in International Transfer of Genetic Resources (CRC Press, Cleveland, 1980) pp. 39–68.
16. J.J. Vanderveken, in Ref. 14, pp. 435–454.
17. A.B.R. Beemster and A. Rozendaal, in: Viruses of Potatoes and Seed-potato Production, ed. J.A. de Bokx (PUDOC, Wageningen, 1972) pp. 115–142.
18. G.E. Russell, Plant Pathol., 14 (1965) 95.
19. D.E. van der Zaag, in Ref. 17, pp. 188–205.
20. J. Hiddema, in Ref. 17, pp. 206–214.
21. M. Hollings, Annu. Rev. Phytopathol., 3 (1965) 367.
22. G. Nyland and A.C. Goheen, Annu. Rev. Phytopathol., 7 (1969) 331.
23. F.C. Mellor and R. Stace-Smith, Phytopathology, 62 (1971) 246.
24. R. Stace-Smith and F.C. Mellor, Phytopathology, 58 (1968) 199.
25. H.L. Bachrack, Adv. Virus Res., 20 (1978) 163.
26. E.M. Jaspars, Adv. Virus Res., 19 (1974) 37.
27. A.J. Gibbs and B. Harrison, Plant Virology (Edward Arnold, London, 1976).
28. W.M. Mitchell, in: Selective Inhibitors of Viral Functions, ed. W.A. Carter (CRC Press, Cleveland, 1973) pp. 51–79.
29. C.T. Duda, M. Zailtin and A. Siegel, Biochim. Biophys. Acta, 319 (1973) 62.
30. R.H. Symons, D.S. Gill and K.H.J. Gordon, 5th Int. Congr. Virol. Abstr. (Strasbourg, 1981), p. 250.
31. C.P. Romaine and M. Zaitlin, Virology, 86 (1978) 241.
32. T.A. Shalla, L.J. Petersen and M. Zaitlin, J. Gen. Virol., 60 (1982) 355.
33. W.P. Jacobs, Plant Hormones and Plant Development (Cambridge University Press, Cambridge, 1979).
34. H. Jockusch, Virology, 35 (1968) 94.
35. J. Dekker, in Ref. 9, pp. 307–326.
36. S.H. Crowdy, in: Systemic Fungicides, ed. R.W. Marsh (Longman, London, 1972) pp. 92–115.
37. A.C. Cassells and R.D. Long, Potato Res., 25 (1982) 165.
38. B. Commoner and F. Mercer, Nature (London), 168 (1951) 113.
39. A.C. Cassells and R.D. Long, in: Tissue Culture for Plant Pathologists, eds. J.P. Helgeson and D.S. Ingram (Blackwell, Oxford, 1981) pp. 131–135.
40. I. Takebe, Annu. Rev. Phytopathol., 13 (1975) 105.
41. I. Takebe and Y. Otsuki, Proc. Natl. Acad. Sci. U.S., 64 (1969) 843.
42. J. Burgess, F. Motoyoshi and E.N. Fleming, Planta, 112 (1973) 323.
43. A.C. Cassells and F.M. Cocker, Z. Naturforsch., 35c (1980) 1057.
44. F. Rollo and R. Hull, J. Gen. Virol., 60 (1982) 359.
45. M.F. Clarke and A.N. Adams, J. Gen. Virol., 34 (1977) 475.
46. I. Takebe, in: Comprehensive Virology, eds. H. Fraenkel-Conrat and R.R. Wagner (Plenum Press, New York, 1977) Vol. 11, pp. 237–277.

47. J. Reinert and Y.P.S. Bajaj, eds., Plant Cell, Tissue and Organ Culture (Springer-Verlag, Berlin, 1977).
48. H.A. Allard, Phytopathology, 8 (1918) 51.
49. H.W.J. Ragetli, Curr. Adv. Plant Sci., 19 (1975) 321.
50. I. Sela, Perspect. Virol. XI (Liss, New York 1981) pp. 129–139.
51. R.I.B. Francki, Virology, 24 (1964) 193.
52. C.D. McKeen, Can. J. Bot., 34 (1956) 891.
53. T.J. Grant, Phytopathology, 24 (1934) 311.
54. P.E. Thomas and R.W. Fulton, Virology, 34 (1968) 459.
55. M.A. Stahmann and S.S. Gothoskar, J. Biol. Chem., 189 (1958) 362.
56. H. Fischer and F. Nienhans, Phytopathol. Z., 78 (1973) 25.
57. T.W. Tinsley, Ann. Appl. Biol., 40 (1953) 750.
58. G. Loebenstein and A.F. Ross, Virology, 20 (1963) 507.
59. S.P. Dolittle and M.N. Walker, J. Agric. Res., 31 (1925) 1.
60. G. Ooi, Ph.D. Thesis, University of Salford, 1981.
61. D.W. Woolley, J. Biol. Chem., 152 (1944) 225.
62. R.L. Thompson, J. Immunol., 55 (1947) 345.
63. G.E. Gifford, H.E. Robertson and J.T. Syverton, Proc. Soc. Exp. Biol. Med., 86 (1954) 515.
64. P. Tekerlekov, G. Mitev, P. Andonor, S. Dundarov, B. Bakalov and F. Boyanova. Vet. Med. Nanki, 13 (1976) 60.
65. L.H. Hall and L.B. Kier, J. Pharm. Sci., 67 (1978) 1743.
66. R. Schneider, Phytopathology, 44 (1954) 243.
67. R.J. Chiu and W.H. Sill, Jr., Phytopathology, 52 (1962) 432.
68. A.C. Cassells and R.D. Long, unpublished data.
69. H.S. Aldwinckle and W. Selman, Ann. Appl. Biol., 60 (1967) 49.
70. M. Kapoor and E.R. Waygood, Can. J. Biochem., 43 (1965) 165.
71. C. Person, D.J. Samborski and F.R. Frosyth, Nature (London), 180 (1957) 1294.
72. A.C. Hollingshead and A.K. Smith. J. Pharmacol. Exp. Ther., 123 (1958) 54.
73. I. Tamm, R. Bablanian, M.M. Nemes, C.H. Shunk, F.M. Robinson and K. Folkers, J. Exp. Med., 113 (1961) 625.
74. H.J. Eggers and I. Tamm, Virology, 13 (1961) 545.
75. H.J. Eggers, Cold Spring Harbor Symp. Quant. Biol., 27 (1962) 309.
76. H.J. Eggers and I. Tamm, J. Exp. Med., 113 (1961) 657.
78. H. Lapierre, C. Kusaik, J. Albouy, G. Macquaire, A.C. Ferault and T. Staron, Ann. Phytopathol., 3 (1971) 3.
79. A.C. Cassells and F.M. Cocker, Z. Naturforsch., 37c (1982).
80. S.B. Kadin, H.J. Eggers and I. Tamm, Nature (London), 201 (1964) 378.
81. D.G. O'Sullivan and P.W. Sadler, Nature (London), 192 (1961) 341.
82. I. Tamm, H.J. Eggers, Bablanian, A.F. Wagner and K. Folkers, Nature (London), 223 (1969) 785.
83. A. Caliguiri and I. Tamm, in Ref. 28, pp. 257–293.
84. D.G. O'Sullivan and A.K. Wallis, Nature (London), 198 (1963) 1270.
85. D.G. O'Sullivan, C.M. Ludlow and A.K. Wallis, Experentia, 27 (1971) 1025.
86. T.M. Dmitrieva and V.I. Agol, Archiv. Ges. Virusforsch., 45 (1974) 17.
87. I. Tamm, J. Exp. Med., 138 (1973) 858.
88. I. Tamm, K. Folkers, C.H. Shunk and F.L. Horsfall, Jr., J. Exp. Med., 99 (1954) 227.

89. R.W. Sidwell, J.H. Huffman, G.P. Khare, L.B. Allen, J.T. Witkowski and R.K. Robbins, Science, 177 (1972) 705.
90. R.A. Smith in Ref. 3, pp. 99–118.
91. E. de Clercq, M. Luczak, J.C. Reepmeyer, K.L. Kirk and L.A. Cohen, Life Sci., 17 (1976) 187.
92. R.A. Smith and W. Kirkpatrick, eds., Ribavirin: A Broad Spectrum Antiviral Agent (Academic Press, New York, 1980).
93. A. Larsson, K. Stenberg and B. Oberg, Antimicrob. Agents Chemother., 13 (1978) 154.
94. D.G. Streeter, J.T. Wilkowski, G.P. Kanare, R.W. Sidwell, R.J. Baner, R.K. Robbins and L.N. Simon, Proc. Natl. Acad. Sci. U.S.A., 70 (1973) 1174.
95. J.K. Lowe, L. Brox and J.F. Henderson, Cancer Res., 37 (1977) 736.
96. F. Malinoski and V. Stollar, Virology, 110 (1981) 281.
97. B.B. Goswami, E. Borek, O.K. Sharma, J. Fujitaka and R.A. Smith, Biochem. Biophys. Res. Commun., 89 (1979) 830.
98. V. Stollar and F. Malinoski, Virology, 115 (1981) 57.
99. B. Lerch, Phytopathol. Z., 89 (1977) 44.
100. O. Byhan, S. Kluge, B. Pustowoit, W. Pustowoit and G. Schuster, Biochem. Physiol. Pflanz., 173 (1978) 521.
101. G. de Fazio, J. Caner and M. Vicente, Arch. Virol., 58 (1978) 153.
102. A.J. Hansen, Plant Dis. Rep., 63 (1979) 17.
103. S. Kluge and K. Marcinka, Acta Virol., 23 (1979) 148.
104. G. de Fazio, M. Kudamatsu and M. Vicente, Fitopathol. Bras., 5 (1980) 343.
105. G. de Fazio, J. Caner and M. Vicente, Arch. Virol., 63 (1980) 305.
106. A.C. Cassells, R.D. Long and S. Austin, in: Plant Tissue Culture in Relation to Biotechnology, eds. A.C. Cassells and J.A. Kavanagh (Royal Irish Academy, Dublin, 1983) p. 42.
107. J.F. Shepard, Virology, 78 (1977) 261.
108. A.C. Cassells and R.D. Long, Z. Naturforsch., 35c (1980) 350.
109. I. Simpkins, D.G.A. Walkey and H.A. Neely, Ann. Appl. Biol., 99 (1981) 107.
110. K.W. Bailiss, F.M. Cocker and A.C. Cassells, Ann. Appl. Biol., 87 (1977) 383.
111. A.C. Cassells and F.M. Cocker, Z. Naturforsch., 37c (1982) 390.
112. J.P. Varma, Virology, 36 (1968) 305.
113. G. Schuster, Phytopathol. Z., in press.
114. R.I.B. Francki, Virology, 17 (1962) 9.
115. H.H. Zimmerman, Biochim. Biophys. Acta, 157 (1960) 378.
116. G. Schuster, W. Horingklee, H. Winter, G. Esser, U. Steinke, W. Kochmann, W. Kramer and W. Steinke, Acta Virol. (Engl. Edn.), 23 (1979) 412.
117. G. Schuster and C. Hanzsch, Phytopathol. Z., 10 (1981) 226.
118. G. Schuster and O. Byhan, Biochem. Physiol. Pflanz., 176 (1981) 286.
119. G. Schuster, Phytopathol. Z., in press.
120. W.D. Rupp and W.H. Prusoff, Nature (London), 202 (1964) 1288.
121. R.N. Goodman, Z. Kiraly and M. Zaitlin, The Biochemistry and Physiology of Infectious Plant Disease (Van Nostrand, New Jersey, 1967).
122. L. Sequeira, Annu. Rev. Phytopathol., 1 (1963) 5.
123. C.W. Nichols, Phytopathology, 42 (1952) 579.
124. P.F. Limasset and M. Sechet, Comptes Rendus, 227 (1948) 643.
125. R.J. Kutsky and T.E. Rawlins, J. Bacteriol., 60 (1950) 763.
126. C.W. Nichols, Phytopathology, 42 (1952) 579.

127. Z. Kiraly and J. Szirmai, Virology 23 (1964) 286.
128. I.W. Selman, Ann. Appl. Biol., 53 (1964) 67.
129. M.J. Daft, Ann. Appl. Biol., 52 (1963) 393.
130. M.J. Daft, Ann. Appl. Biol., 55 (1965) 51.
131. H.S. Aldwinckle, Virology, 66 (1975) 341.
132. G.E. Milo, Jr. and B.I.S. Srivastava, Virology, 38 (1969) 26.
133. K. Maramorosch, Science, 126, (1957) 651.
134. M. Chessin, in: Proceedings of the 3rd Conference on Potato Virus Diseases (PUDOC, Wageningen, 1957) pp. 80–84.
135. S.R. Chant, W. Kimmins, W. Stephens, T.C. Wrigley, Phyton, 20 (1963) 105.
136. I.W. Selman and P.V. Arulprayasam, Ann. Bot., 34 (1970) 1107.
137. K.W. Bailiss, Physiol. Plant Pathol., 4 (1974) 73.
138. A. Kyrlov, V.A. Smirnova and G.A. Takakanova. Rev. Appl. Mycol., 40 (1960) 628.
139. E. Balazs, R. Gaborjanyi and Z. Kiraly, Physiol. Plant Pathol., 3 (1973) 341.
140. G. Schuster, Phytopathol. Z., 94 (1979) 72.
141. J.A. Tomlinson, E.M. Faithfull and C.M. Ward, Ann. Appl. Biol., 84 (1976) 31.
142. D.E. Atkinson, Biochemistry, 7 (1968) 4030.
143. E.G. Brown and A.C. Cassells, Phytochemistry, 10 (1971) 1251.
144. A.C. Cassells and D.C. Burke, J. Gen. Virol., 18 (1973) 135.
145. W.A. Jensen, E.G. Pollock, P. Healey and M. Ashton, Exp. Cell Res., 33 (1964) 523.
146. V. Santilli, C.M. Neopolcroeff and N.C. Gaglordi, Nature (London), 193 (1962) 656.
147. C.E. Yarwood, Phytopathology, 50 (1960) 741.
148. J.D. Gilpatrick and M. Weintraub, Science, 115 (1952) 701.
149. A.F. Ross, Virology, 14 (1961) 329.
150. A.F. Ross, Virology, 14 (1961) 340.
151. R.I. Hamilton, in: Plant Disease, an Advanced Treatise, eds. J.G. Horsfall and E.B. Cowling (Academic Press, New York, 1980) Vol. 5, pp. 279–303.
152. G. Loebenstein, Annu. Rev. Phytopathol., 10 (1972) 177.
153. L.C. van Loon, J. Gen. Virol., 30 (1976) 375.
154. S. Gianinazzi, These de Docteur-es-Sciences (University of Geneva, 1970).
155. S. Gianinazzi and B. Kassanis, J. Gen. Virol., 23 (1974).
156. E. de Clercq, F. Eckstein and T.C. Merigan, Ann. N.Y. Acad. Sci., 173 (1970) 444.
157. B. Kassanis and R.F. White, Ann. Appl. Biol., 79 (1975) 215.
158. A.C. Cassells and T. Flynn, Pestic. Sci., 9 (1978) 365.
159. H. Barker, J. Gen. Virol., 28 (1975) 155.
160. A.C. Cassells, A. Barnet and M. Barlass, Physiol. Plant Pathol., 13 (1978) 13.
161. R.S.S. Fraser, Physiol. Plant Pathol., 19 (1981) 69.
162. R.S.S. Fraser, J. Gen. Virol., 58 (1982) 305.
163. J.E. Vanderplank, Genetic and Molecular Basis of Plant Pathogenesis (Springer-Verlag, Berlin, 1978).
164. G. Samuel, Ann. Appl. Biol., 21 (1934) 90.
165. T. O'Diener, Annu. Rev. Phytopathol., 1 (1963) 197.
166. A.Th. Rast, Neth. J. Plant Pathol., 78 (1972) 110.
167. A.C. Cassells and C.C. Herrick, Virology, 78 (1977) 253.
168. E.C. Cocking, Phil. Trans. R. Soc., Lond., B292 (1981) 557.
169. A.C. Cassells and F.M. Cocker, Physiol. Plant., 56 (1982) 69.
170. P.J. Larkin and W.R. Snowcroft, Theor. Appl. Genet., 60 (1981) 197.

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4 The Benzodiazepines

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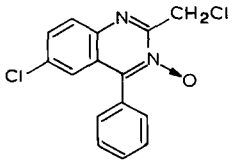
INTRODUCTION

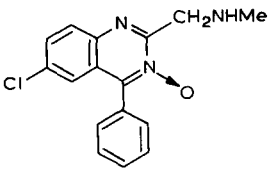
The 1,4-benzodiazepines entered clinical practice in 1960 with the introduction of chlordiazepoxide (Librium). Since that time, they have become the most frequently prescribed of all psychotropic drugs, being used as anxiolytics, hypnotics, anticonvulsants and muscle relaxants. The popularity of these compounds can be ascribed to their essential lack of disturbing peripheral side-effects and to their high therapeutic indices; both of these factors provide them with considerable advantages over the barbiturates which they have now largely replaced.

Like many drug discoveries, that of the 1,4-benzodiazepines owes much to serendipity. Leo Sternbach, leading a synthetic programme at Hoffmann-La Roche in 1957, submitted for clinical evaluation what was then thought to be a quinazoline *N*-oxide (2). The compound had been prepared some 2 years earlier by the reaction of (1) with methylamine. Sternbach recounts that he held little hope for a positive outcome [1, 2]. It had soon become clear, however, that the compound possessed a wide spectrum of pharmacological activity [3]. In comparison with chlorpromazine, it appeared to have greater anticonvulsant activity but similar efficacy as a muscle relaxant, while it was readily distinguished from phenobarbital in exhibiting no direct hypnotic activity below toxic doses. Such encouragement led to further studies which revealed that the initial structural assignment was incorrect; the compound possessed a 7-membered heterocycle and was in fact 7-chloro-2-methylamino-5-phenyl-3H-1,4-benzodiazepine 4-oxide (3.1) [4]. Further substitutions in rings A and C led to a successful patent application which was placed in 1958 [5], and subsequent toxicological and clinical investigations of the parent compound, chlordiazepoxide, allowed its introduction into the market in 1960 – a considerable achievement in less than 3 years from the first pharmacological studies.

It was clear from the initial clinical studies that chlordiazepoxide had considerable potential, and efforts were made to produce an elixir or syrup which would be suitable for paediatric and geriatric use. The marketed hydrochloride salt was extremely bitter to taste and the finely powdered free base proved to be unstable due to the substituent at position 2. This however, was readily removed by acid hydrolysis to give (4) [6], which showed similar biological potency to that of the parent compound. Further removal of the *N*-oxide to form (5.1) appeared to result in a significant increase in biological activity and thus the two main chemical characteristics of the parent compound, namely, the methylamino and N4-oxide groups, were merely unnecessary appendages to the biologically active structure. It was with this knowledge that an intense investigation of a series of analogues of compounds (4) and (5) was initiated, and, as a preliminary to such a study, alternative synthetic routes were sought.



(1)



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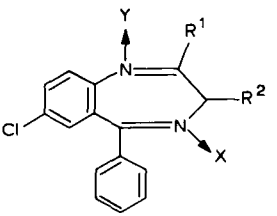

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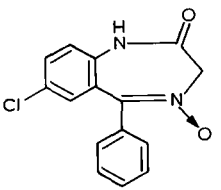

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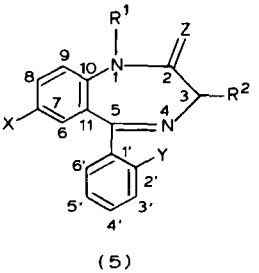


(5)

	R^1	R^2	X	Y
(3.1)	NHMe	H	O	–
(3.2)	NH_2	1-imidazolyl	–	–
(3.3)	NMe_2	OH	–	–
(3.4)	NEt_2	H	O	–
(3.5)	NHMe	NMe_2	–	–
(3.6)	NH_2	H	O	O
(3.7)	NHMe	OH	–	–

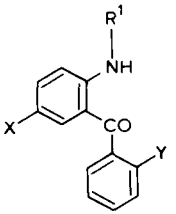
	R^1	R^2	X	Y	Z
(5.1)	H	H	Cl	H	O
(5.2)	H	OH	Cl	H	O
(5.3)	H	OH	Cl	Cl	O
(5.4)	Me	OH	Cl	H	O
(5.5)	Me	OH	Cl	Cl	O
(5.6)	Me	H	Cl	H	O
(5.7)	H	COOK	Cl	H	O
(5.8)	CH_2.(c-C_3H_5)	H	Cl	H	O
(5.9)	$(CH_2)_2NEt_2$	H	Cl	F	O
(5.10)	H	H	NO_2	H	O
(5.11)	H	H	NO_2	Cl	O
(5.12)	Me	H	Cl	H	H_2
(5.13)	Me	H	Cl	H	S
(5.14)	Me	H	NO_2	F	O
(5.15)	$CH_2C{\equiv}CH$	H	Cl	H	O
(5.16)	Me	H	NO_2	H	O
(5.17)	Me	$OCONMe_2$	Cl	H	O
(5.18)	Me	H	Cl	F	O
(5.19)	H	H	Cl	Cl	O
(5.20)	CH_2CF_3	H	Cl	H	O
(5.21)	CH_2POMe_2	H	Cl	H	O
(5.22)	CH_2CF_3	H	Cl	F	S
(5.23)	CH_2.(c-C_3H_5)	H	Cl	F	O
(5.24)	H	H	Br	Cl	O
(5.25)	H	H	NHOH	H	O
(5.26)	H	$O(CH_2)_2OH$	Cl	Cl	O
(5.27)	Me	$OCOCCl_3$	Cl	H	O
(5.28)	CH_2.(c-C_3H_5)	OH	Cl	H	O
(5.29)	Me	NMe_2	Cl	H	O
(5.30)	H	NHOH	Cl	H	O
(5.31)	H	Me	Cl	H	O
(5.32)	H	CH_2Ph	Cl	H	O
(5.33)	H	Me	CH(Me)-COOH	H	O
(5.34)	OH	OH	Cl	H	O
(5.35)	H	H	Cl	H	$CHNO_2$
(5.36)	$CHMe_2$	H	Cl	H	O
(5.37)	CH_2Ph	H	Cl	H	O
(5.38)	Et	H	Cl	H	O
(5.39)	Et	H	Cl	4'-Cl	O
(5.40)	CH_2OMe	H	NO_2	H	O
(5.41)	Me	Me	Cl	H	O
(5.42)	H	H	H	H	O
(5.43)	H	H	CF_3	H	O
(5.44)	Me	H	CN	H	O
(5.45)	H	$OCO(CH_2)_2$-COO^-Na^+	Cl	H	O
(5.46)	Me	H	Br	H	O
(5.47)	Me	H	Cl	4'-F	O
(5.48)	Me	H	H	H	O
(5.49)	Me	H	Cl	Cl	O
(5.50)	H	NHCOCO-NHMe	Cl	H	O
(5.51)	$CH_2CONHMe$	H	Cl	H	O

Various aspects of both the chemistry and pharmacology of the benzodiazepines have been reviewed recently [1, 7–10]. This review will be restricted mainly to 1,4- and 1,5-benzodiazepines, which lack cyclic structures on the *b*- or *c*-faces, as these have markedly different pharmacological profiles.

SYNTHETIC PATHWAYS

COMPOUNDS IN CLINICAL USE

Compounds of type (4) were readily obtained from 2-chloromethylquinazoline *N*-oxides by treatment with alkali [6]. Although these did not prove of great value, they undergo a rearrangement on treatment with acid chlorides or acid anhydrides to yield 3-acetoxy derivatives which on hydrolysis yield the highly active 3-hydroxy compounds [11]. Of these, oxazepam (5.2) [11], lorazepam (5.3) [12, 13], temazepam (5.4) [11, 13] and lormetazepam (5.5) [14] are marketed as anxiolytics, anticonvulsants or hypnotics.



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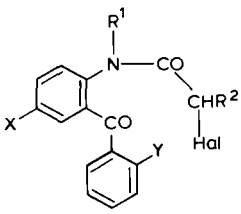

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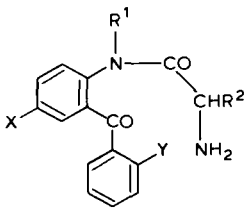
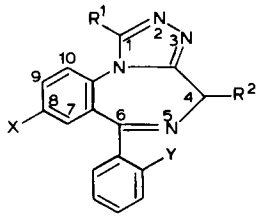


(8)

More general methods of preparing compounds of type (5, Z = O) were also developed [1, 15]. These are mostly based on 2-aminobenzophenones as starting material. Of the two most extensively used methods, one involves treatment of the appropriately substituted benzophenone (6) with a haloacetyl halide and then reaction with ammonia (6) → (7) → (8). Compound (8) readily cyclizes to form the 5-phenyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one (5, Z = O). The second method, treatment of (6) with an α-amino acid ester salt in pyridine to give (5) directly, is particularly useful for introducing various substituents at C3 [16]. A number of other, related, synthetic routes have been described in the review literature [1, 15].

Benzodiazepines of type (5, Z = O) have proved clinically the most useful. Diazepam (5.6) [6, 16], clorazepate potassium (5.7) [17] and prazepam (5.8) [18, 19] are marketed in the U.K. as anxiolytics, flurazepam (5.9) [20] and

nitrazepam (5.10) [21] as hypnotics, and clonazepam (5.11) [21] as an anticonvulsant. Replacement of the 2-carbonyl group by a methylene group lowers the activity, but one such compound, medazepam (5.12) is marketed in the U.K. as an anxiolytic. It can be obtained by reduction of (5.13), the 2-thione analogue of diazepam [22] and also by a number of other methods [1, 15].



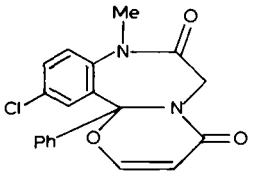
(9)

	R^1	R^2	X	Y
(9.1)	Me	H	Cl	Cl
(9.2)	H	H	Cl	H
(9.3)	Me	H	Cl	H
(9.4)	Ph-3,4,5-$(OMe)_3$	H	Cl	H
(9.5)	CH_2OH	H	H	Cl
(9.6)	CH_2NMe_2	H	Cl	H
(9.7)	CH_2NHMe	H	Cl	H
(9.8)	CH_2NH_2	H	Cl	H
(9.9)	Me	Me	Cl	H
(9.10)	Me	H	N=N(O)Me	Cl
(9.11)	$CH_2N(O)Me_2$	H	Cl	H
(9.12)	$CH_2N(Me)$-(c-C_3H_5)	H	Cl	H
(9.13)	CH_2ONMe_2	H	Cl	H
(9.14)	CH_2NMe_2	H	SMe	H
(9.15)	CH_2NMe_2	H	H	H
(9.16)	$CH_2CH_2NMe_2$	H	Cl	H
(9.17)	Me	OH	Cl	Cl

A further three benzodiazepines are presently available commercially in the U.K. for clinical use [23]. Triazolam (9.1), and ketazolam (10) contain an additional hetero-ring fused across the N1-C2 bond (*a*-face) and N4-C5 bond (*d*-face), respectively, of the benzodiazepine system; clobazam (11) is a 1,5-benzodiazepinedione. Triazolam was prepared by the reaction of the thione (5.13, R^1 = H, Y = Cl) with $AcNHNH_2$ [24], and ketazolam from diazepam (5.6) and diketene, or (5.6) and acetyl chloride and triethylamine [25]. Clobazam was obtained by cyclization of the *N*-phenyl-*N*-(2-amino-5-chlorophenyl)malonic ethyl ester amide (12) followed by *N*-methylation [26–28].

In all, fifteen benzodiazepines are available (June, 1982) for clinical use in the U.K. as anxiolytics, sedative-hypnotics, anticonvulsants and muscle relaxants. Listed [23] are chlordiazepoxide (Librium) (3.1), oxazepam (Serenid) (5.2),

lorazepam (Ativan) (5.3), temazepam (Normison) (5.4), lormetazepam (Noctamid) (5.5), diazepam (Valium) (5.6), clorazepate potassium (Tranxene) (5.7), prazepam (Centrax) (5.8), flurazepam (Dalmane) (5.9), nitrazepam (Mogadon) (5.10), clonazepam (Rivotril) (5.11), medazepam (Nobrium) (5.12), triazolam (Halcion) (9.1), ketazolam (Anxon) (10) and clobazam (Frisium) (11).



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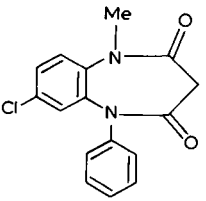

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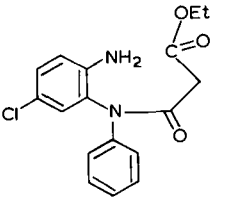
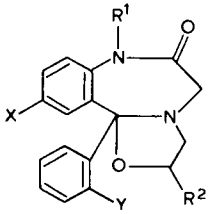


(12)

The United States Pharmacopeia (1981) [29] lists the following benzodiazepines (with U.S. trade name in parenthesis where this differs from the U.K. name): chlordiazepoxide, oxazepam (Serax), lorazepam, diazepam, clorazepate, prazepam (Verstran) and flurazepam. These seven drugs, however, also cover the pharmacological spectrum.

Some ten other benzodiazepines are marketed in other countries [1]. Five of these, desmethyldiazepam (5.1) [6], flunitrazepam (5.14) [21], pinazepam (5.15) [30], nimetazepam (5.16) [21] and camazepam (5.17) [31] are 5-phenyl-1,4-benzodiazepin-2-ones of type (5). Estazolam (9.2) [32], a hypnotic, is a close analogue of triazolam (9.1). Oxazolam (13.1) and cloxazolam (13.2) contain an oxazolo-ring attached to the *d*-face of the benzodiazepine system. They can be prepared from the benzophenones (7, $R^1 = R^2 = H$, X = Cl, Y = H or Cl) by treatment with NH_2CH_2CHROH, R = Me or H, and cyclization of the product [33], and also by other methods [1, 15].

Bromazepam (14) and tetrazepam (15) are the only two benzodiazepines on the market which do not have a phenyl group at the 5-position. Bromazepam, an anxiolytic, was prepared by standard methods from 2-(2-amino-5-bromobenzoyl)pyridine [34] and tetrazepam, a muscle relaxant, was prepared from the 5-cyclohexyl analogue of desmethyldiazepam (5.1) by chlorination followed by elimination of hydrogen chloride and N-methylation [35].



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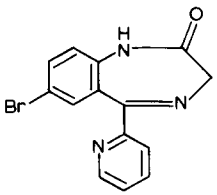

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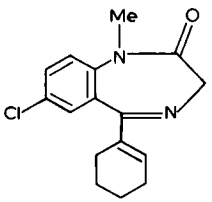


(15)

	R^1	R^2	X	Y
(13.1)	H	Me	Cl	H
(13.2)	H	H	Cl	Cl
(13.3)	CH_2CH_2OH	H	Cl	H
(13.4)	CH_2CH_2OH	H	Cl	F
(13.5)	H	H	Br	F
(13.6)	H	Me	Br	H

EXPERIMENTAL COMPOUNDS

All the benzodiazepines currently marketed in the U.K. and the U.S.A. have been known for more than 10 years, although their introduction dates range up to more recent times. Despite this apparent lack of progress, intensive research has continued with the general objective of producing superior drugs, in particular, a narrower spectrum of biological activity, acting more specifically as anxiolytics, muscle relaxants, hypnotics or sedatives [1]. Drugs which would act both as anxiolytics and antidepressants have also been sought.

Synthetic methods used in the preparation of new drug molecules will not be described. These are mostly based on the general methods outlined previously, but whenever possible, a reference to the synthesis is given.

New compounds mostly fall into the five categories (a)–(e):

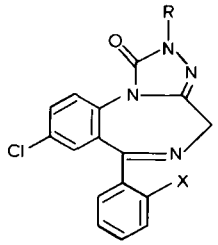
(a) those containing an additional ring fused to the seven-membered ring of the 'classical' benzodiazepine system (5);

(b) 'heterodiazepines'* where the fused benzo-ring is replaced by a heterocyclic ring, with or without an additional ring fused to the seven-membered ring;

* 'Heterodiazepines' are included in this review, since in overall shape and chemical properties they resemble the benzodiazepines sufficiently well that their pharmacological spectrum is generally similar.

(c) 'classical benzodiazepines' of types (3) and (5) incorporating different substituents, especially at the 3-position;
(d) benzodiazepines not containing a phenyl substituent at the 5-position;
(e) benzodiazepines with nitrogen atoms at positions other than 1 and 4.

In view of the favourable properties of triazolam (9.1), estazolam (9.2) and alprazolam (9.3) [24], considerable interest in recent years has been in the synthesis and pharmacological testing of compounds containing an additional ring fused to the *a*-face of the benzodiazepine system. Analogues of oxazolam (13.1) and cloxazolam (13.2) involving *d*-face fusion have also been studied. In particular, however, *a*-face fusion of a triazolo-ring has produced promising compounds of type (9). Thus the 6-aryl-4H-s-triazolo[4,3-*a*][1,4]benzodiazepines (9.4), (9.5) [36], (9.6)–(9.8) [37], (9.9) [38], (9.10) [39], the 2,4-dihydro-1H-s-triazolo[4,3-*a*][1,4]benzodiazepin-1-ones (16.1)–(16.4) [40], the oxazirine (17) [38] and the 6-piperidino derivative (18) [41] all have central nervous system (CNS) activity in test animals.



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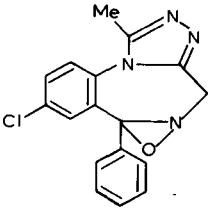

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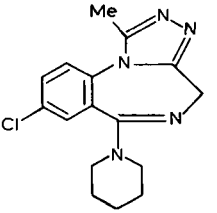


(18)

(16.1) X = Cl, R = Me

(16.2) X = H, R = $(CH_2)_2\overline{NCH_2CH_2NMeCH_2CH_2}$

(16.3) X = Cl, R = $(CH_2)_2\overline{NCH_2CH_2NMeCH_2CH_2}$

(16.4) X = Cl, R = $(CH_2)_3\overline{NCH_2CH_2NMeCH_2CH_2}$

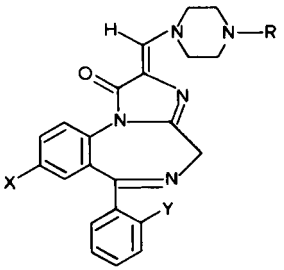
Compounds (9.6)–(9.8) were found to act both as anxiolytics and as antidepressants [37]. Incorporating an *N*-oxide group into the side-chain at C1 (to give 9.11), replacing one of the methyl groups of (9.6) by a larger group, for example, cyclopropyl (as in 9.12) or incorporating an oxygen atom between the nitrogen and carbon atoms (as in 9.13) diminished the antidepressant potency but did not greatly affect the anxiolytic properties. On the other hand, compounds (9.14)–(9.16) had diminished anxiolytic activity but largely retained antidepressant activity. Thus each type of activity could be varied independently by appropriate selection of substituents [37].

Substitution at C4 generally reduces anxiolytic activity [38]; the C4-OH derivative (9.17) has markedly lower anxiolytic activity [38, 42], unlike the corresponding 5-phenyl-1,4-benzodiazepin-2-ones of type (5) where an OH group at the 3-position (corresponding to the 4-position of the triazolobenzodiazepines) results in compounds of high activity [14].

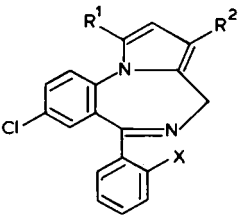
Thus (9.17), a potential metabolite of triazolam (9.1), possesses relatively low activity in contrast to oxazepam (5.2), the main metabolite of diazepam [42] (see p. 179). The C4-Me derivative (9.9) is, however, highly active [38].

Compound (16.1) is a potent anxiolytic, whereas (16.2)–(16.4) have both anxiolytic and antidepressant activities [40], but these latter compounds are not as effective as the 1-aminomethyl-substituted (9.6)–(9.8). In general, electronegative substituents at C1 have a detrimental effect on anxiolytic activity, whereas electron-releasing substituents have a favourable effect [43].

Replacing the 6-(2-chlorophenyl)-ring of triazolam (9.1) by nitrogen substituents resulted in compounds with little anxiolytic activity apart from the 6-piperidino derivative (18) [41]. 1,2- and 1,3-Dimethyl-4H-s-triazolo[4,3-*a*][1,4]benzodiazepinium salts [44] and free-base N5-oxides [45, 46] have also been reported.

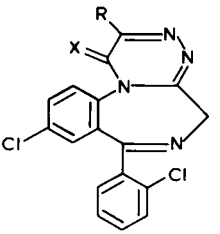


(19)



(20)

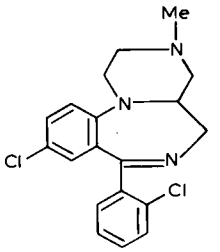
(20.1) $R^1 = R^2 = H$, X = H
(20.2) $R^1 = R^2 = H$, X = Cl
(20.3) $R^1 = R^2 = H$, X = F
(20.4) $R^1 = R^2 = Me$, X = Cl
(20.5) $R^1 = R^2 = Me$, X = F



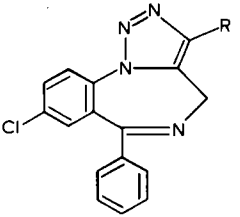
(21)

(21.1) R = Me, X = O
(21.2) R = CH_2NMe_2, X = H_2
(21.3) R = CH_2Cl, X = H_2

Central nervous system (CNS) activity has been found also in compounds with other types of ring fused to the 1,4-benzodiazepine *a*-face. In the class of imidazo[1,2-*a*][1,4]benzodiazepines [47–49], compounds (19, R = Me, Et, X = Cl, NO_2, Y = Cl, F) have anxiolytic and hypnotic activity in mice [50] and pyrrolo[1,2-*a*][1,4]benzodiazepines of type (20) [51, 52] are also CNS-depressant, the most active member of the series (20.4) having a potency compar-



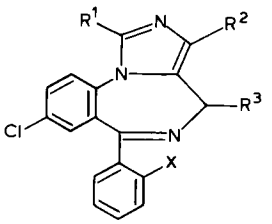
(22)



(23)

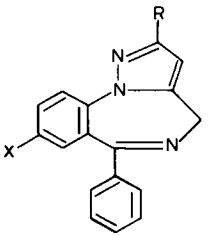
(23.1) R = H
(23.2) R = Me

able with that of diazepam [52]. The as-triazino[4,3-*a*][1,4]benzodiazepines of type (21), for example, (21.1)–(21.3), also show anxiolytic activity [53]. However, the pyrazino[1,2-*a*][1,4]benzodiazepine (22) was the only member of a series of compounds of this type, synthesized in an attempt to obtain drugs which would be both anxiolytic and antidepressant, to show any activity at all [54].



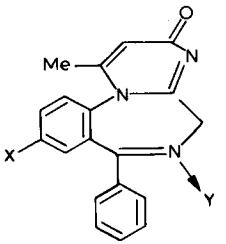
(24)

(24.1) R^1 = Me, R^2 = R^3 = H, X = F



(25)

(25.1) R = CH_2OH, X = Cl
(25.2) R = COOMe, X = Cl
(25.3) R = CH_2NH_2, X = NO_2



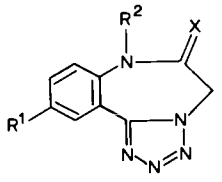
(26)

(26.1) X = Cl, Y = O
(26.2) X = Cl, Y = –
(26.3) X = NO_2 Y = O

Other ring systems to have been studied include the v-triazolo[1,5-*a*][1,4]benzodiazepine (23) [55], the imidazo[1,5-*a*][1,4]benzodiazepine of type (24) [49, 56-61], the pyrazolo[1,5-*a*][1,4]benzodiazepine of type (25) [56, 62] and the pyrimido[1,2-*a*][1,4]benzodiazepine of type (26) [63]. The imidazo[1,5-*a*]benzodiazepine (24.1) [57] has recently been used as an intravenous anaesthetic [64].

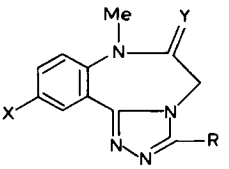
The oxazolam analogues (13.3) and (13.4) [65, 66] and (13.5) [67], containing an additional ring fused to the *d*-face, have been synthesized and found to

have useful CNS activity. The alcohol (13.4) has sedative and anticonvulsant properties comparable with those of diazepam; (13.5) has hypnotic and anticonvulsant properties.



(27)

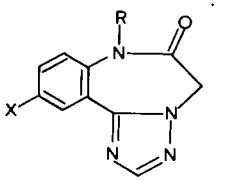
(27.1) $R^1 = CF_3$, $R^2 = H$, $X = O$
(27.2) $R^1 = H$, $R^2 = Me$, $X = H_2$



(28)

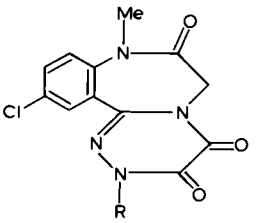
(28.1) R = Me, X = H, $Y = H_2$
(28.2) R = H, X = Cl, Y = O
(28.3) R = Me, X = Cl, Y = O

A number of compounds containing other ring systems fused to the *d*-face have also been reported. These include tetrazolo[1,5-*d*][1,4]benzodiazepines of type (27) [68, 69], s-triazolo[4,3-*d*][1,4]benzodiazepines of type (28) [69–71], triazolo[1,5-*d*][1,4]benzodiazepines of type (29) [71] and triazino[4,3-*d*][1,4]-benzodiazepines of type (30) [70]. It may be noted that none of the compounds (27)–(30) contains a phenyl ring in the 5-position of the 1,4-benzodiazepine system. The *a*-face-fused derivatives of type (31) [72] also lack a phenyl ring in this position. Of these compounds, only those of type (29) have been reported to show CNS activity [71].



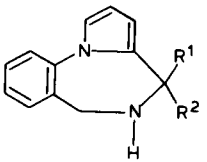
(29)

(29.1) R = H, X = H
(29.2) R = Me, X = Cl



(30)

(30.1) R = H
(30.2) $R = (CH_2)_2NMe$

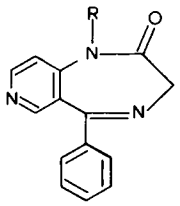


(31)

(31.1) $R^1 = Ph$, $R^2 = H$
(31.2) $R^1 = Me$, $R^2 = COOEt$

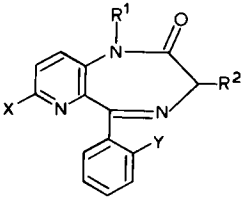
The first 'heterodiazepines' to be reported were the 7-azabenzodiazepines (32.1), (32.2) [73], but these showed only weak CNS activity. However, the isomeric 6-aza derivatives of type (33) [74] are more active, and (33.1) has

useful anxiolytic and hypnotic properties [75]. Heterodiazepines based on five-membered hetero-rings have, however, aroused wider interest.



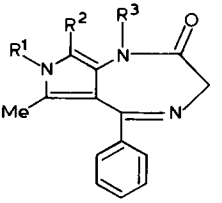
(32)

(32.1) R = H
(32.2) R = Me



(33)

(33.1) R^1 = H, R^2 = OH, X = Y = Cl



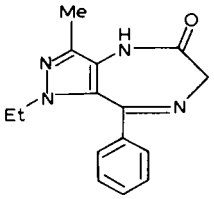
(34)

(34.1) R^1 = Me, R^2 = R^3 = H

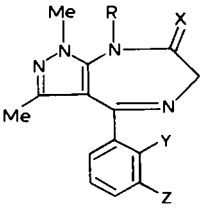
The pyrrolodiazepines of type (34) [76, 77] have good anticonvulsant activity in mice but show little or no CNS-depressant effect. The pyrazolo derivatives of types (35) [78] and (36) [79, 80] have been described; (35) and (36.1) have CNS-depressant properties. The former is being tested as an anxiolytic and (36.1) has found use as an animal anaesthetic. When, however, the carbonyl function is absent, compounds of type (36) are inactive as anxiolytics or anticonvulsants but possess antidepressant properties [80], (36.2) being studied clinically as an antidepressant. The isoxazolodiazepines (37.1) and (37.2) have also been reported [81].

The thienodiazepine system has been extensively studied. The compound (38.1) [82] has been tested clinically as an anxiolytic and hypnotic. A large number of derivatives, for example, (38.2) and (38.3), have been synthesized [83], as have certain 5-(2-pyridyl) analogues [84] and the isomeric thienodiazepines of types (39) [85, 86] and (40) [87]. Thiazolodiazepines of type (41) have also been reported [86].

Recently, there has been considerable interest in hetero[1,4]diazepines with an additional ring fused to the seven-membered ring, structurally analogous to the *a*-face-fused 1,4-benzodiazepines. The triazolothienodiazepine system of type (42) has yielded a number of promising new compounds with strong CNS activity: (42.1) [88] has been tested clinically and found to be effective as a hypnotic; (42.2) [88, 89] has good anxiolytic activity in test animals. Triazolothiazolo- (43) [86], imidazothieno- (44) [90], imidazopyrazolo- (45) [90] and pyrrolothieno[1,4]diazepines (46) [91] have also been reported.

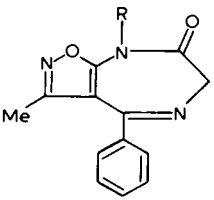


(35)



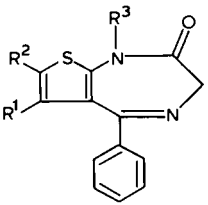
(36)

(36.1) R = Me, X = O, Y = F, Z = H
(36.2) R = H, X = H_2, Y = H, Z = Cl



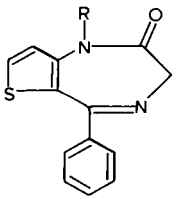
(37)

(37.1) R = H
(37.2) R = Me

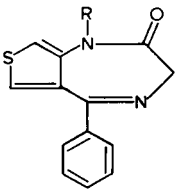


(38)

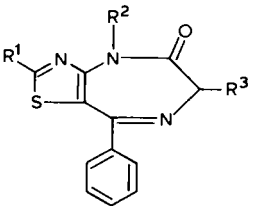
(38.1) R^1 = H, R^2 = Et, R^3 = Me
(38.2) R^1 = Me, R^2 = Me, R^3 = H
(38.3) R^1 = NO_2, R^2 = Me, R^3 = Me



(39)

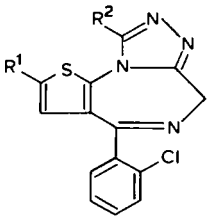


(40)



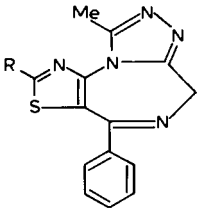
(41)

(41.1) R^1 = NMe_2, R^2 = Me, R^3 = H
(41.2) R^1 = piperidino, R^2 = R^3 = H
(41.3) R^1 = NMe_2, R^2 = H, R^3 = Me



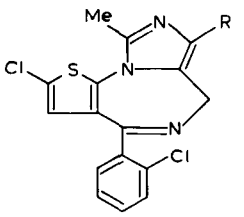
(42)

(42.1) R^1 = Br, R^2 = Me
(42.2) R^1 = Et, R^2 = Me
(42.3) R^1 = Br, R^2 = c-C_3H_5



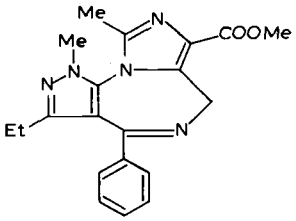
(43)

(43.1) R = morpholino

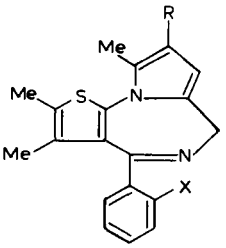


(44)

(44.1) R = COOMe
(44.2) R = $CONH_2$



(45)



(46)

(46.1) R = H, X = H
(46.2) R = Me, X = Cl

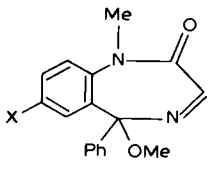
'Classical' 1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-ones have continued to arouse interest. Compounds (5.18) and (5.19), known since 1962 [16] have been recently tested clinically as anxiolytics [92, 93]. Variants (5.20) [94] and (5.21) [95] have useful anxiolytic properties and (5.21) is also a hypnotic [96, 97]. The 1-(dimethyloxophosphinylmethyl) group confers good water-solubility on this compound. The thione (5.22) [94] has useful hypnotic properties. The 5-(2-fluorophenyl) analogue (5.23) [98] of prazepam shows potent anticonvulsant, muscle relaxant and sedative activity [99] and the 7-bromo compound (5.24) has anticonvulsant and sedative activity [100] (both compounds tested in mice); 7-hydroxyaminobenzodiazepines, for example, (5.25), have been found to be less active than the 7-nitro analogues [101].

A number of new C3-substituted derivatives have been reported recently. Compounds (5.26) [102], (5.27) [103] and certain analogues exhibit potent anticonvulsant, muscle-relaxant and sedative activity in mice [103]. The C3-hydroxy analogue of prazepam (5.28) has been reported [104], as have 3-amino derivatives, for example, (5.29) [105] and (5.30) [106], 3-alkyl derivatives, for example, (5.31), (5.32) [107] and (5.33) [108]. Compound (5.34), the 1-hydroxy analogue of oxazepam, has also been prepared [109].

C3-substitution results in chiral molecules. Both the (*R*)- and (*S*)-enantiomers of (5.31) and (5.32) have been prepared, and it was found that the (*S*)-form is considerably more potent in anticonvulsant, muscle-relaxant and sedative activity but similar in hypnotic activity [107]. 3-Hydroxy compounds, however, racemize readily, so that the use of enantiomers of oxazepam (5.2), for example, as possibly more active species in clinical applications does not seem feasible [110].

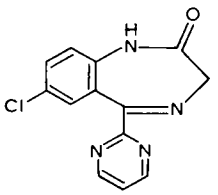
The carbonyl function has also been modified. For example, ring expansion with nitromethane produces the N4-oxide of the 2-nitromethylene derivative (5.35) from compound (1) [111].

The 3H-1,4-benzodiazepine system (3) of chlordiazepoxide has received some attention. Representative examples of new compounds include (3.2) [112], (3.3)–(3.5) [113] and (3.6) [109].



(47)

(47.1) X = Cl
(47.2) X = NO_2

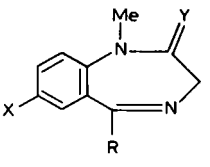


(48)

Although generally 1,5-dihydro-2H-1,4-benzodiazepine-2-ones are much less active than the 1,3-dihydro isomers of type (5), compounds (47.1) and (47.2) have somewhat greater anticonvulsant activity than diazepam in mice [106].

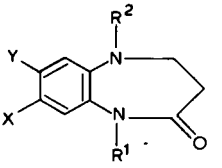
A characteristic feature of nearly all CNS-depressant benzodiazepines is the phenyl substituent at the 5-position. Nevertheless, some promising compounds have been obtained which do not contain a 5-phenyl ring. Thus the 5-(2-pyrimidinyl)-1,4-benzodiazepine (48) has good anticonvulsant activity in mice [114]. 5-Substituted 4-pyrimidinyl-, 2-thiazolyl-, 5-isothiazolyl-, 2-imidazolyl- and 5-pyrazolyl-1,4-benzodiazepines have also been reported [114, 115], as have 5-amino- [116], 5-alkyl- [117] and 5-alkoxy- [118] 1,4-benzodiazepines, for example, (49.1)–(49.6). The ethoxy compound (49.5) has CNS activity in mice similar to that of diazepam [118]. It is noteworthy that the 8-chloro analogue of (49.5) retains activity, unlike the situation in the 5-phenyl-1,4-benzodiazepines [1, 2].

1,5-Benzodiazepines have attracted considerable interest. Clobazam (11) has been on the market since 1975 as an anxiolytic and the 7-trifluoromethyl analogue reported at the same time [26, 28] also has CNS-depressant activity. A large number of simple 1,5-benzodiazepines involving various modifications of the clobazam-type framework have been reported. Only a relatively small number, however, have useful CNS activity. Compound (50.1) [119, 120] is active as a sedative, muscle relaxant and anticonvulsant, but appears to have undesirable after-effects [121]. The amine (51.1) [122] shows moderate anticonvulsant and CNS-depressant activity [123], as does (52.1) [124]. However, the amino analogues (52.2) and (52.3) are convulsants and CNS stimu-



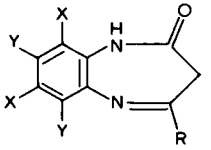
(49)

	R	X	Y
(49.1)	NH_2	H	H_2
(49.2)	piperidino	H	H_2
(49.3)	H	Cl	O
(49.4)	CH_2Ph	Cl	O
(49.5)	OEt	Cl	O
(49.6)	OPh	Cl	O



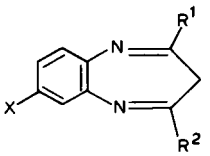
(50)

	R^1	R^2	X	Y
(50.1)	Ph	H	Cl	H
(50.2)	Ph	Pr	CF_3	H
(50.3)	H	Ph	H	Cl
(50.4)	H	Me	H	H
(50.5)	H	H	H	H



(51)

(51.1) R = NEt_2, X = H, Y = H
(51.2) R = Me, X = Cl, Y = H
(51.3) R = Me, X = H, Y = Cl

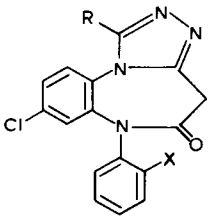


(52)

(52.1) R^1 = SMe, R^2 = NEt_2, X = H
(52.2) R^1 = NMe_2, R^2 = NEt_2, X = H
(52.3) R^1 = piperidino, R^2 = NEt_2, X = H
(52.4) R^1 = Ph, R^2 = SMe, X = NO_2

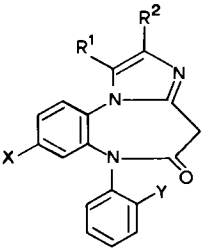
lants [124]. Analogues (51.2), (51.3) [125] and (52.4) [126] have also been reported.

As with the 1,4-benzodiazepines, 1,5-benzodiazepines containing an additional ring fused to the *a*-face show promising pharmacological properties. The s-triazolo[4,3-*a*][1,5]benzodiazepines (53.1)–(53.3) [127, 128] have anticonvulsant and moderate CNS-depressant properties in animals; the first two compounds, in particular, appear to be useful anticonvulsants [129]. A series of imidazo[1,2-*a*][1,5]benzodiazepines of type (54) has been synthesized [130, 131]; (54.2) and (54.3) are the most active of these but are several-times less active than diazepam in mice [131]. The thiazolo[3,2-*a*][1,5]benzodiazepine (55) has also been reported [130]. As examples of hetero-1,5-diazepines, compounds of type (56) have been prepared [132] for use as tranquillizers.



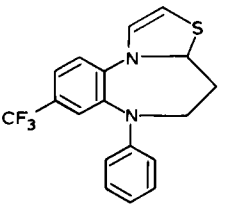
(53)

(53.1) R = H, X = Cl
(53.2) R = H, X = CF_3
(53.3) R = Me, X = H

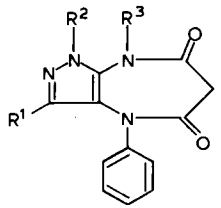


(54)

	R^1	R^2	X	Y
(54.1)	Me	H	CF_3	H
(54.2)	H	Et	Cl	H
(54.3)	H	Et	Cl	F

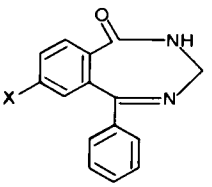


(55)



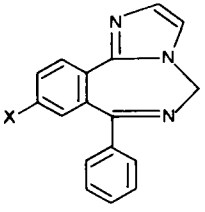
(56)

(56.1) $R^1 = R^2 = R^3 = Me$
(56.2) $R^1 = R^2 = Me$, $R^3 = H$



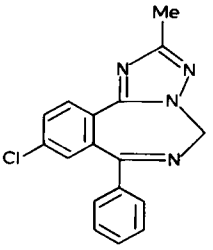
(57)

(57.1) X = H
(57.2) X = Cl

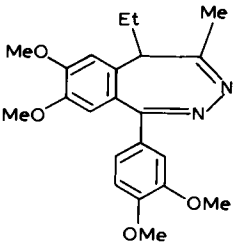


(58)

(58.1) X = H
(58.2) X = Cl



(59)



(60)

The 2,4-benzodiazepines (57.1), (57.2) [113], the imidazo[2,1-*a*][2,4]benzodiazepines (58.1) and (58.2) [134], and the triazolo[5,1-*a*] derivative (59) [135] have been described; (57.2) shows anticonvulsant activity comparable with that of diazepam in mice [133]. Attempts to synthesize the triazolo[4,3-*a*] derivative, analogous to the ring system of triazolam (9.1), were not successful [135].

A 2,3-benzodiazepine (60) [136], tofisopam, has been tested clinically as an anxiolytic [137]. However, it does not appear to behave like a typical 1,4-benzodiazepine CNS depressant [138] and does not bind to the benzodiazepine receptor [139].

A very large number of publications both in the patent literature and as scientific papers have appeared and continue to appear describing new methods of preparing known benzodiazepines. This aspect will, however, not be considered further in this review.

METHODS OF BIOLOGICAL TESTING

GENERAL INTRODUCTION

The benzodiazepines, with few exceptions, are very poorly soluble in water and as a result, systemic administration is frequently carried out with the aid of organic solvents or emulsifiers. It is therefore essential that the appropriate vehicle is used in control experiments during biological testing of the compounds, and such precautions have not been universally followed. A second difficulty which frequently complicates the interpretation of experimental data is the well-documented interaction of this class of drugs with general anaesthetics such as the barbiturates [140–142], halothane [143, 144], equithesin [145], steroid anaesthetics [146] and nitrous oxide [147, 148]. This has made interpretation of the effects of benzodiazepines on the cardiovascular and respiratory systems particularly difficult to quantify.

The benzodiazepines produce their major therapeutic effects as anticonvulsants, anxiolytics, muscle relaxants and sedatives and at the doses used clinically to achieve such responses there is little evidence of any direct actions on peripheral systems [149].

ANTICONVULSANT ACTIVITY

The quantification of the anticonvulsant activity of the benzodiazepines can be carried out with facility and two standard procedures have been widely used.

In the first, convulsions are induced in an animal population, the dose of the convulsant being selected to produce an easily recognizable end-point, e.g., clonic and/or tonic convulsions or death, in a large proportion of the group. Dose-response curves are then constructed for the compound of interest in protecting against the convulsions. The second procedure uses the constant infusion of a known concentration of a convulsant until a predetermined end-point is reached. The ability to antagonize the actions of the convulsant is then determined by prior injection of the drug under investigation. Both methods have their advantages and disadvantages though the latter paradigm provides more detailed information if the appropriate convulsant is selected.

The most popular choice of convulsant has been pentylenetetrazole (PTZ), and initial studies [150] have been extended to many members of the series of 5-phenyl-1,4-benzodiazepines [151–153] and the triazolobenzodiazepines [154, 155]. Though the mechanism by which PTZ induces convulsions is poorly understood, both bicuculline [156–161] and picrotoxin [159, 162–164] have been used as alternative convulsant agents, which produce their effects by antagonism of GABA (γ-aminobutyric acid)-mediated transmission, though by different mechanisms. The benzodiazepines appear to be less effective against convulsions produced by these two agents than against those initiated by PTZ. Alternatively, convulsions can be induced by blockade of GABA biosynthesis and the benzodiazepines are also effective against such convulsants as isoniazid [162, 165], thiosemicarbazide [157, 166] and 3-mercaptopropionic acid [149]. Many other convulsant agents have been used and the benzodiazepines appear to be remarkably effective in antagonizing their actions [149].

Electroshock-induced convulsions have also been used to measure anticonvulsant potency [167]. A comparative study with PTZ-induced convulsions indicated that the ED_{50} for a number of benzodiazepines against electroshock-induced convulsions was approximately one order of magnitude higher than those against PTZ (Ref. 168, see also Ref. 169).

While the benzodiazepines exhibit profound anticonvulsant activity in such acute conditions, attempts have been made to develop more relevant models of seizure disorders in man where the condition is invariably of a chronic nature. The most promising of these approaches is that known as the 'kindling effect'. In this procedure, repeated stimulation of discrete brain areas with subconvulsive electrical discharges induces a sensitization to the stimulation after some time, with the eventual production of paroxysmal activity. A number of benzodiazepines have been tested against kindling and have been found extremely effective [170–173].

While the benzodiazepines are clearly potent anticonvulsant agents, their

use in the control of convulsions in man is limited (see following section). There remain considerable difficulties in the prediction of the therapeutic potential of such agents from animal models to the multiple forms of human convulsive disorders [174].

MUSCLE RELAXANT AND SEDATIVE ACTIVITY

Discussions have continued over many years concerning the differentiation of muscle relaxation, as defined by loss of muscle tone and/or co-ordination, from sedation, normally identified with a decrease in the state of arousal or vigilance. The matter remains unresolved and, as is frequently the case in situations where there is a marked lack of agreement, multiple procedures have been developed in attempts to define drug actions at different points along this behavioural axis.

A subjective measure of muscle relaxation can be obtained by an experienced individual by gently holding an animal and judging the muscle tone in its body simply by the 'feel' of the animal. Such a procedure has been modified for use in the cat, in which the animal is held gently by the neck and the degree to which the hindlegs hang downwards taken as a measure of muscle relaxation; data on several benzodiazepines have been published using this procedure [175].

Objective determination of muscle relaxation-sedative state of the animal has been assessed by a number of more-or-less complex procedures and, while it is clear that a measurement is being made, little further definition can be given concerning precisely what is being measured. This is not to denigrate the procedures used, as compounds under investigation can be compared with an accepted standard drug treatment and therefore useful comparative data can be obtained.

Animals placed on inclined screen tend to fall off and the time which this takes can be used in quantification [176]. In an allied procedure, mice may be placed with their fore-paws in contact with a horizontal wire and the time taken for them to climb onto the wire with their hindlegs is assessed [177]. A rather more complex task involves the animal maintaining its balance on a slowly rotating rod; there are of course innumerable variables which can be superimposed on this procedure, but, within a given laboratory, useful comparisons can be made [178, 152]. The chimney test, in which mice must climb a glass tube in a certain time, yields essentially identical ED_{50} values, for a series of benzodiazepines, as the rotorod test [179, 152].

While the above tests certainly suffer some contribution from muscle relaxant effects of the drugs used, their sedative effects are normally assessed by

modification of spontaneous motor activity. This is an extremely complex behaviour and is made up of a number of individual components (see Ref. 180). The motor activity can be assessed simply by observation or by a number of mechanized procedures in which quantification does not require an experienced operator [150]. Exploratory activity has also been used to gain information on the sedative effects of the benzodiazepines in a technique whereby mice are placed on a board containing a number of holes, and the number of times the animal dips its head into a hole used to assess activity [181]. The benzodiazepines increase the number of head dips made [182]. Such procedures described above are not easily quantified and minor variations of conditions produce considerable variations in the values obtained for an individual compound. It is, however, fairly easy for a skilled individual to obtain a relatively accurate assessment of a drug profile with procedures of this type. The very considerable difficulty arises, however, when attempts are made to correlate such behavioural measures with precise neurochemical or chemical indices for the compounds as required for the investigation of structure-activity relationships.

ANXIOLYTIC ACTIVITY

The behavioural testing of potential anxiolytic agents suffers from the inevitable complexities attendant upon the modelling of an abnormal human psychological condition in laboratory animals. It is not possible to review such complex discussions here and the interested reader must consult more specialized texts [183].

The first procedure, which contributed very considerably to the definition of the potent anxiolytic activity of the benzodiazepines, was devised by Geller and Seifter, and now bears their name [184]. Animals are trained to press a lever in order to obtain some reward, normally food; the response rate of the animal can then be reduced markedly by pairing the response with a punishing electric shock. The release of this punishment-suppressed behaviour by drugs is suggested to be an appropriate model of their anxiolytic activity [185]. This paradigm also allows the quantification of non-punished responses, an index of sedative activity, and it has been demonstrated that upon chronic administration of oxazepam, the sedative effects rapidly attenuate while punished responding appears to increase [186].

The above procedure, while giving reliable information, is time-consuming to perform in that animals must first be trained and then the behaviour maintained. An alternative procedure [187] suffers from neither of these disadvantages. Here thirsty rats are allowed to lick water from a dispenser and the

behaviour is periodically suppressed by the administration of an electric shock to the mouth contingent upon licking. A number of benzodiazepines together with meprobamate and pentobarbital markedly increased the number of shocks an animal was prepared to endure in order to obtain its reward.

One of the main arguments which have developed against such procedures rest on the use of electric shocks to suppress the behaviour. It has been suggested that drugs acting to increase drive levels or pain thresholds would be likely to produce the same increase in punished responding, and therefore such tests may not be descriptive only of anxiolytic activity. Alternative procedures have therefore been developed to circumvent such arguments.

One of these involves the observation of the social interaction which occurs between two male rats placed in a cage. As the novelty of the environment or the light intensity therein was increased so the social interaction between the partners decreased. Chlordiazepoxide given chronically, markedly attenuated this reduction in social interaction compared with that of undrugged animals, as the light intensity or unfamiliarity of the environment was increased [188]. It is proposed that such a model of anxiety has a number of advantages over those using behavioural suppression with electric shock, as obviously the painful suppression has been removed and a degree of uncertainty introduced by the novel environment.

Much more sophisticated behavioural analysis can be performed in which the animals suffer frustration of an expected reward in performing some task by the fact that their successful completion of the task is rewarded only on randomly assigned occasions. Under these conditions, the extinction of the learned behaviour is delayed compared with animals trained using a continuous reward. This effect is attenuated in animals treated with ethanol, the barbiturates or the benzodiazepines [189]. Such analyses have the ability to provide detailed information about the behavioural profile of drug action, though their very sophistication renders them unpopular for routine drug testing.

EFFECTS ON AGRESSIVE BEHAVIOUR

The potent anti-aggressive effects of the benzodiazepines, though not clearly understood, played an important part in defining the profile of action of these compounds in early studies. The behavioural patterns which can be described as aggression have been categorized by Moyer [190] and it has become clear that the benzodiazepines elicit reproducible effects on only certain types of aggressive behaviour.

Chordiazepoxide was first shown to produce taming effects in spontaneously aggressive rhesus monkeys [150] at doses below those required to produce

overt behavioural effects, and similar investigations on other members of the series indicated that this property was common [191, 192]. Aggressive behaviour in mice induced by mild electric footshock [193, 194] is also remarkably susceptible to the benzodiazepines (for reviews see Refs. 15, 175, 195), as is that produced in a previously isolated group of mice [196]. These drugs also appear to be effective in reducing certain measures of aggression produced by electrical stimulation of such areas as the hypothalamus, septum or periaqueductal grey matter of freely moving animals [197–199]. However, they are ineffective in reducing aggression caused by olfactory bulbectomy or lesions of the ventromedial hypothalamus [200].

CLINICAL STUDIES

METABOLISM AND PHARMACOKINETICS

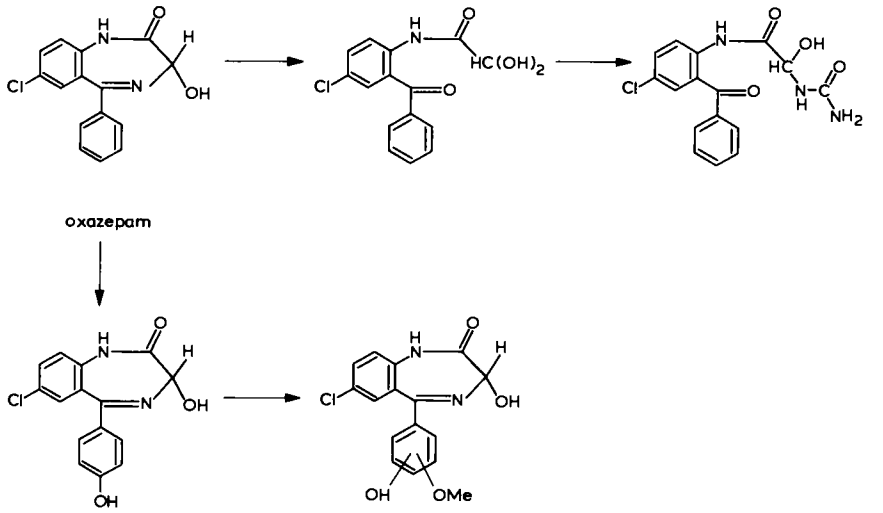
There have been extensive studies concerning the metabolic fate of each of the benzodiazepines currently used in clinical practice and it is not possible to review these adequately here. A comprehensive coverage of this subject, together with references to the original material, has recently been published [201].

The major metabolic products from diazepam result from N1-demethylation, 3-hydroxylation [202] and a combination of these two. The above-named metabolic products retain considerable biological activity. Equivalent hydroxylation of the triazolo derivatives (4-position) results in a marked decrease in biological activity [42, 203, 204]. The hydroxylated metabolites (and similarly for the 3-hydroxylated parent drugs, e.g., oxazepam) are excreted as conjugates and are devoid of activity. The major metabolic pathways of four representative members of the series are shown in *Schemes 4.1–4.4*.

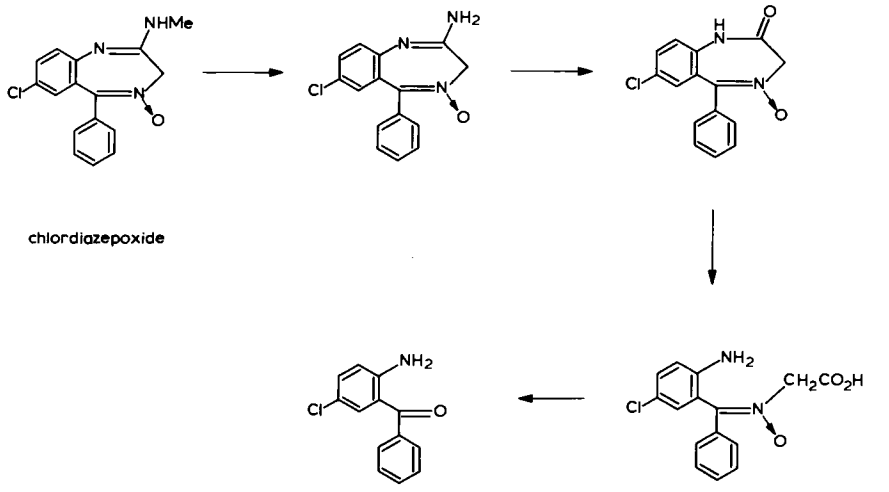
Heterocyclic ring opening to form metabolites of the benzophenone type has been documented and is of interest, as it has been suggested that the metabolites may act as prodrugs for the benzodiazepines. Many such structures exhibit marked *in vivo* biological activity [205–208]. They are thought to be cyclized to benzodiazepine derivatives *in vivo*, as the benzophenones show a low affinity for the benzodiazepine receptor *in vitro* [209].

The pharmacokinetics of the benzodiazepines have been amply documented and for detailed reviews on individual compounds the reader is referred to Schutz [210]. The clinical implications of the pharmacokinetics of the benzodiazepines have been briefly reviewed [211].

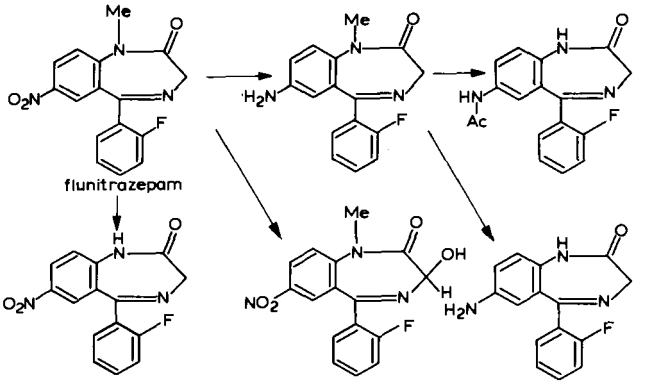
The benzodiazepines are normally rapidly absorbed after oral administra-



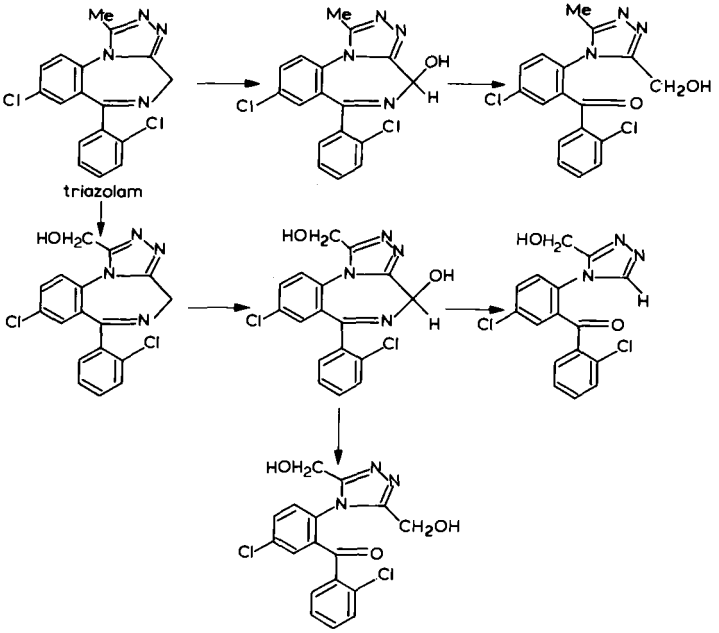
Scheme 4.1. Metabolism of oxazepam.



Scheme 4.2. Metabolism of chlordiazepoxide.



Scheme 4.3. Metabolism of flunitrazepam.



Scheme 4.4. Metabolism of triazolam.

tion, though their biological half-lives differ enormously. Those without hydroxyl substituents at the 3-position or additional rings on the *a*-face frequently exhibit long residence times in the body and yield biologically active metabolic products with similar characteristics. For example, diazepam has an elimination half-life of 21–46 h, while the comparative figure for its *N*-desmethyl metabolite is 50–99 h [212]. These properties result in marked accumulation of both parent and metabolite on repeated administration. Substitution of hydroxyl groups in the 3-position results in marked decreases in the elimination half-lives, that for oxazepam being about 8 h. Here, also, no active metabolites are formed and the accumulation in the body is less pronounced. Triazolam, however, with a heterocycle on the *a*-face, possesses a half-life of only 2.7 ± 0.5 h [213], with the result that it is the drug of choice as a night-time hypnotic.

CLINICAL USAGE

The most frequent use of the benzodiazepines is in the treatment of anxiety. This condition is a normal response to stressful situations and it is only when the condition becomes excessive or chronic, frequently termed 'pathological anxiety', that some type of clinical intervention is required. Currently, the most cost-effective treatment is the prescription of benzodiazepines.

The compounds also possess anticonvulsant activity, clonazepam being used in the alleviation of certain seizure disorders [214] while diazepam remains the drug of choice in status epilepticus. The anticonvulsant effects, however, show tolerance on chronic usage in both animals [215, 216] and man, with the result that therapeutic efficacy in this regard is lost within a period of months [214].

The sedative effects of the benzodiazepines also display tolerance; in this case, however, the drug response declines much more rapidly, within a few days [186, 215, 216]. Several members of the series, however, are frequently used, very effectively, as night-time hypnotics [217]. Though flurazepam, nitrazepam and flunitrazepam are frequently used in this way, their prolonged presence in the body can lead to impairment of performance on the following day, and triazolam appears to be the sensible choice for the treatment of simple insomnia [218].

Higher doses of the benzodiazepines are used to produce muscle relaxation in conditions such as cerebral palsy, certain degenerative neurological disorders, muscle pain and tetanus [219].

Together with the anxiolytic, muscle-relaxant and sedative effects, their additional property of anterograde amnesia has made the benzodiazepines increasingly popular as preanaesthetics. However, the additive depressive effects of these compounds with both the barbiturates [142] and halothane [144] require the careful manipulation of dosage.

The pharmacological profile of the benzodiazepines has assured them an important place in the clinical armoury, and their usage is widespread. The most readily available figures suggest that one in ten Canadians receive a prescription for a benzodiazepine each year, while in excess of 30% of all hospitalized patients are prescribed one of these drugs [220]; there is no reason to believe that such figures are not representative of other developed countries.

The acute toxicity of the benzodiazepines is extremely low. Enormous quantitites of the compounds have been taken in overdose and there remains no documented evidence of a fatality occurring due to the ingestion of a benzodiazepine alone [149]. The compounds do not appear to induce liver microsomal enzymes to any considerable extent (but see Ref. 221) and they are essentially free of disturbing peripheral side-effects [149]. Nevertheless, their remarkably wide usage has led to concern recently over their over-use [222]. Psychological dependence on the compounds has been reported, though physical withdrawal symptoms are very uncommon except following the cessation of long-term, high-dose treatment. The major population at risk, therefore, would appear to be those receiving benzodiazepines as a treatment for anxiety of a chronic nature. Recent evidence would suggest that these drugs actually impair the ability of laboratory animals to adjust to stressful situations and this may also apply to man [223, 224]. If such hypotheses gain further support, they would supply most compelling reasons to restrict the prescription of these compounds.

MECHANISM OF ACTION OF THE BENZODIAZEPINES

The first clues to the possible mechanism of action of the benzodiazepines were provided by Schmidt, Vogel and Zimmermann [225]. They observed that diazepam was able to potentiate presynaptic inhibition in the spinal cord, a process later shown to be mediated by the inhibitory neurotransmitter, GABA [226]. Since that time there have been numerous electrophysiological observations indicating that facilitation of GABA-mediated transmission by the benzodiazepines occurs in many areas of the mammalian CNS [227], and it is now generally accepted that these compounds produce their pharmacological effects by interaction with GABAergic transmission.

Biochemical evidence suggested that the mechanism by which this occurred was due to the action of the benzodiazepines on the post-synaptic GABA receptor, by increasing the efficacy of the membrane-mediated events which occurred subsequent to the interaction of GABA with its receptor [228].

Undoubtedly the most significant finding in attempts to elucidate the mechanism of action of these drugs was the discovery of specific, high-affinity

binding sites for the benzodiazepines in the mammalian central nervous system [229, 230]. The sites were identified by equilibrium-binding techniques which had previously been used in the biochemical identification of neurotransmitter receptors in brain [231]. It was shown that [^{3}H]diazepam bound to membranes, obtained from the CNS, in a saturable manner and that the diazepam so bound was displaceable specifically by benzodiazepines, and not by other psychoactive drugs or by any of the known neurotransmitter candidates, including GABA. That this binding site was the pharmacological receptor through which the benzodiazepines produced their effects was indicated by the fact that the ability of a number of benzodiazepines to displace [^{3}H]diazepam, in these experiments, was highly correlated with their efficacy in producing muscle relaxation in the cat *in vivo* and with other pharmacological tests used to predict activity in these compounds [229, 232, 233].

The benzodiazepine receptor appears to be located on neuronal cells [232–235] rather than on glia [236, 237] and exhibits a differential topographical distribution in the CNS of both animals [229, 230, 238, 239] and man [240–242]. Initially, the receptor population appeared to be homogeneous, only one type of receptor being identifiable in the various regions of the CNS [229, 230], though recently this has been questioned. The first suggestion that there may be multiple benzodiazepine receptors came from studies in which certain triazolopyridazines were found to give shallow displacement curves of the benzodiazepines from their membrane binding sites, an observation which was compatible with the existence of two binding sites [243]. Additional evidence was provided for this hypothesis when ethyl β-carboline-3-carboxylate (βCCE) was also shown to exhibit similar behaviour [244]. Such an hypothesis was very attractive, as the triazolopyridazines were reported to possess the anxiolytic profile of the benzodiazepines without exhibiting any sedative action [245], and it was therefore proposed that the different subtypes of benzodiazepine receptor may mediate separate aspects of their behavioural profile. Further detailed analysis has suggested that the interaction of these ligands with their receptor is complex and interpretation of experimental data has been difficult. However, the current evidence suggests that there is but one receptor, which may exist in two different conformations [246–249]. These conformations may themselves have different recognition properties or, alternatively, the benzodiazepine receptor may possess separate recognition site domains [250, 251].

While the demonstration of a specific receptor for the benzodiazepines was a significant advance, it of course did not explain why these compounds were able to facilitate GABA-mediated transmission; for that, some interaction with the receptor for GABA had to be demonstrated. Studies have shown that, while the ontogenetic development of the benzodiazepine receptor pre-

cedes that of the GABA receptor [252, 253], GABA itself is able to increase the affinity of the benzodiazepine receptor for these compounds [254–257]. Probably of equal significance, however, was the observation that certain anions, including chloride, were able to act synergistically with GABA in this regard [258–260]. The reason for this is that GABA is thought to produce its actions on membrane conductance by affecting chloride channels in the neuronal membrane and it has now been elegantly demonstrated that the benzodiazepines are able to increase the frequency with which these channels are opened by GABA [261]. Interaction of benzodiazepines with their receptor, therefore, in some way modulates the efficacy of the GABA receptor-effector system by an action at this chloride ionophore.

Subsequent to the identification of the opioid peptides as natural ligands for the opiate receptor [262], attempts have been made to identify substances which may serve a similar role at the benzodiazepine receptor. Though none of this work has yet come to a conclusion, in terms of the structural identification of these substances, several candidates have been proposed [263–266].

Specific antagonists for the benzodiazepines have now been prepared which appear to have no overt behavioural actions alone, but are able to block rapidly and completely the *in vivo* actions of the benzodiazepine by interaction at the receptor [267–269].

There is a considerable amount of information available concerning the interaction of the benzodiazepines with GABA-mediated transmission, though no direct actions have been demonstrated so far on any other neurotransmitter systems. It has been assumed, therefore, that the complete pharmacological spectrum of activity of the benzodiazepines is dependent solely upon this GABA axis. A question which remains unanswered, however, is whether or not there is a consistency in this pharmacological profile; does a potent sedative profile necessarily imply an equally potent anxiolytic? Such a question is extremely difficult to answer, due largely to our inability to quantify accurately the behavioural measures involved, and there have been few attempts to answer the question directly. It is nonetheless an extremely important question, for not only would such differences imply that different neurochemical mechanisms underlie the separate pharmacodynamic actions, but subsequent attempts to investigate the structure-activity relationships involved would supply the medicinal chemist with invaluable information.

STRUCTURAL CHARACTERISTICS

INTRODUCTION

Of the fifteen benzodiazepines currently marketed in the U.K. and the U.S.A. for clinical use, all have a phenyl substituent at the 5-position, all except one, clobazam (11), are 1,4-benzodiazepines and all except chlordiazepoxide (3.1), medazepam (5.12) and triazolam (9.1) have a ketonic oxygen at the 2-position. Apart from these four and ketazolam (10), the remaining ten compounds are all 1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-ones of type (5), with different substituents at the 1-, 3- and 7-positions and at the 2′-position of the 5-phenyl ring.

By the mid-1960's, a sufficient number of benzodiazepines of this type had been prepared and tested for biological activity in laboratory animals for it to become possible to delineate the effect on activity of various substituents at these and other positions in the basic framework. Electron-withdrawing groups at the 7-position (e.g., halogen, NO_2, CF_3) are of paramount importance for high activity. Electron-releasing groups (e.g., Me, OMe) at position 7, or any substituent at positions 6, 8 or 9 lead to a marked decrease in activity. Methylation at N1 increases the activity, but a larger group generally decreases activity. At the 3-position, a hydroxyl group tends to increase activity, but other substituents have generally not proved beneficial. In the 5-phenyl ring,an *ortho*-fluoro or chloro substituent leads to increased activity, but a *para*-halo substituent causes almost complete loss of activity. Other modifications such as reduction of the N4-C5 double bond or replacement of the ketonic oxygen also lower the activity considerably [14, 270]. At about the same time, structural studies by NMR spectroscopy and X-ray crystallography began to appear in the literature (for the earliest work see Refs. 271 and 272). By the end of 1981, the crystal structures of 11 of the clinically used benzodiazepines had been determined, as had those of many analogues covering a wide range of biological potency, and many of these compounds have also been examined by NMR spectroscopic techniques. The object of these studies has generally been to find relationships between structural features and activity in order to provide a rationale for the empirical structure-activity relationships already established [14, 270]. Recently, a number of studies correlating activity with electronic parameters (such as atomic charge or dipole moment), calculated by molecular orbital methods, have been reported (see, for example, Ref. 273).

This section is concerned with a survey of the structural results and with the attempted structure-activity correlations.

NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

NMR spectroscopy is a valuable method of studying stereochemistry in solution. Compared with solid-state structure as determined by X-ray crystallography, the results pertain to the molecule in an environment closer to the actual conditions under which it exhibits its biological activity when interacting with its receptor. However, the spectroscopic data are generally harder to interpret and the results less definitive. Different interpretations of the experimental data are often possible, especially as in solution the molecule may not exist in one unique conformation.

The main interest, particularly in the earlier investigations, has been in the conformation of the seven-membered heterocyclic ring. In the 1,3-dihydro-2H-1,4-benzodiazepin-2-ones it is formally analogous to cyclohepta-1,3-diene, but containing another trigonally hybridized carbon atom because of the presence of the carbonyl function. There is also likely to be electron delocalization across the N1-C2 bond, giving it partial double-bond character, and making the ring geometrically analogous to cycloheptatriene. Recently, the orientation of the 5-phenyl ring has also come under investigation.

In what appears to be the first detailed NMR investigation, Linscheid and Lehn [271] studied compounds (5.1), (5.6), (5.36)–(5.38) and the 5-(4-chlorophenyl) analogue (5.3q) of (5.38) over a range of temperature both in deuteropyridine and in hexachlorobutadiene solution. At 30°C, the C3 methylene signal of (5.1) consists of a singlet at about δ4.5. At −50°C, this line has split into an AB signal, indicating that the geminal methylene hydrogens are non-equivalent (diastereotopic). The methylene groups of compounds (5.6), (5.36)–(5.39) at 30°C show AB-type spectra, which at higher temperatures (90–120°C) coalesce into a single peak (A_2 type). These results are interpreted in terms of a conformational equilibrium involving inversion of the seven-membered ring between two non-planar, enantiomeric, but energetically equivalent forms, which on the NMR time-scale is rapid for (5.1) and slow for (5.6) and (5.36)–(5.39) at 30°C. The geometries of the extreme forms in the equilibria were considered to be intermediate between that of cycloheptatriene, which is boat-shaped [274], and a cycloheptadiene pseudo-boat [274].

The free energy of activation for the inversion both at the coalescence temperature and at 30°C was calculated for these compounds. At 30°C the value for (5.1) is 12.4 kcal/mol compared with *ca.* 6 kcal/mol for cycloheptatriene itself [274]. Replacing hydrogen at N1 of (5.1) by a methyl group to give (5.6) increases the inversion barrier to 17.3 kcal/mol; larger N1 substituents appear to cause only a relatively small increase over the N1-methyl value (maximum 1.7 kcal/mol for compound (5.37)). This effect is attributed to the

interaction of the N1 substituent with the fused benzene ring (presumably a *peri* interaction with the C9 hydrogen atom) in the planar transition state. The 4′-chloro substituent in the 5-phenyl ring (compound (5.39)) does not appear to affect the ring inversion process.

Other studies [275–279] using similar methods have confirmed the effect of N1 substituents on the barrier to ring inversion, and it has been shown [279] that addition of oxygen to the imino nitrogen, N4, does not affect the inversion barrier significantly. Analysis of the spectra of compounds (5.10), (5.11), (5.14), (5.16) and (5.40) shows [277] that replacing the 7-chloro substituent of (5.1) and (5.6) by a nitro group (compounds (5.10) and (5.16)) has little effect, but that a 2′-chloro or 2′-fluoro substituent in the 5-phenyl ring reduces the barrier to ring inversion slightly (by 1.8 and 1.6 kcal/mol for Cl and F, respectively). Rate constants for the inversion are given [277]. At 37°C, the average lifetime of a conformer ranges from 0.6 s for compound (5.40) to 3.3×10^{-6} s for clonazepam (5.11).

A recent study [280] using the paramagnetic shift reagent $Eu(fod)_3$ and computer simulation of the lanthanide-induced chemical shifts has confirmed the earlier results [271, 276, 277] that demethyldiazepam (5.1) exhibits a conformational equilibrium involving rapid inversion of the seven-membered ring at room temperature. N1-Substituted derivatives such as (5.6), (5.9) and (5.15) are considered [280] to exist in solution in only one conformation which does not interconvert at room temperature (or interconverts only slowly – see, for example, Refs. 271 and 277). It was also concluded [280] that N1 has a flattened pyramidal geometry in the unsubstituted (5.1) but becomes trigonal in the substituted derivatives, so that the seven-membered ring approaches a cycloheptadiene-like pseudo-boat form in (5.1) and a cycloheptatriene-like boat in (5.6), (5.9) and (5.15). It is suggested that the increased barrier to ring inversion of N1-alkyl-substituted derivatives may be due not only to the steric interaction with the adjacent benzene ring, but also to the different geometry of N1, pyramidal in (5.1) and trigonal in the substituted compounds*. Medazepam (5.12) and its demethyl derivative both exhibit conformational equilibrium at room temperature, involving rapid inversion of two pseudo-boat conformers [281, 282]. It is suggested [282] that complexation with the shift reagent $Eu(fod)_3$ shifts the equilibrium towards the conformer with the N1

* These conclusions are contrary to the X-ray crystallographic results, which show that in 1,3-dihydro-2H-1,4-benzodiazepin-2-ones of type (5), N1 is essentially trigonal, irrespective of the nature of the substituent, and the seven-membered heterocyclic ring is essentially a cycloheptatriene-like boat; N1-Me-substituted compounds tend to deviate more from ideal cycloheptatriene geometry than N1-H compounds (see *Table 4.2*, p. 195).

substituent oriented pseudo-equatorial. This is the conformer found in the solid state both for medazepam [283] and medazepam hydrochloride [284]. N1 is intermediate between trigonal and pyramidal in the solid state (*Figure 4.1*, compound (5.12)).

The 1,5-benzodiazepines, clobazam (11), its 7-dechloro and 7-trifluoromethyl analogues and compounds of type (50.3)–(50.5) have also been studied by use of lanthanide shift reagents [285, 286]. The diones of type (11) are considered to exist in only one cycloheptadiene-like pseudo-boat conformation at room temperature. The preferred conformer has the 5-phenyl ring oriented pseudo-axial, with N5 having a flattened pyramidal geometry. The methyl-substituted N1 is trigonal [285]. The heterocyclic ring of (50.3), however, assumes a cycloheptene-like chair conformation where the nitrogen atoms have a flattened pyramidal geometry. At room temperature, there is a conformational equilibrium which is shifted (85%) towards the conformer with the N1 hydrogen atom and the N5 phenyl ring oriented pseudo-axial [286].

Recent measurements [287–290] of carbon-13 nuclear magnetic resonance parameters of diazepam (5.6), flurazepam (5.9), nitrazepam (5.10), clonazepam (5.11), chlordiazepoxide (3.1) and some related compounds have shown that an earlier assignment [291] of resonances must be revised. The ring-inversion characteristics have been confirmed [287, 290] by the ^{13}C studies. In the N1-Me-substituted (5.6) and (5.14) at room temperature, there is a slow (on the NMR time-scale) interconversion between the two boat forms of the seven-membered ring. In chlordiazepoxide (3.1) and the N1-H compounds (5.10) and (5.11) the interconversion is rapid at room temperature, but becomes slow at −60 to −70°C.

Compounds with triazole, triazolone and triazinedione rings fused to the *d*-face of the 1,4-benzodiazepine nucleus (e.g., (28.2), (28.3), (30.1), (30.2))

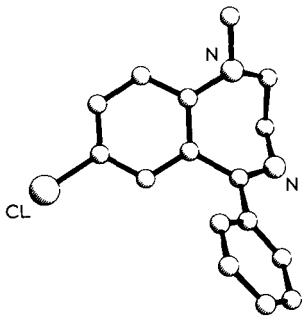


Figure 4.1. Compound (5.12).

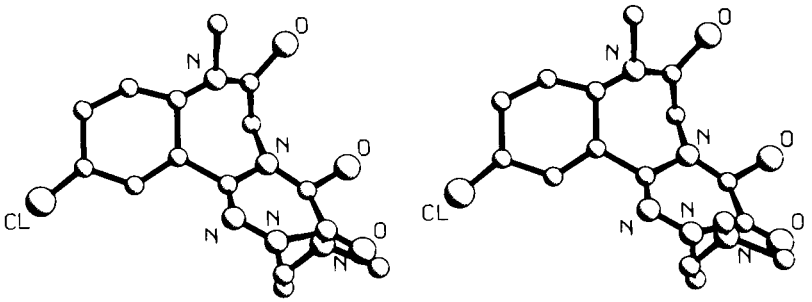


Figure 4.2. Stereoscopic view of compound (30.2).

have also been studied [70] and barriers to inversion of the seven-membered ring determined. This ring was shown by X-ray crystallography to adopt a pseudo-boat conformation in the hydrobromide of (30.2) [70] (*Figure 4.2* and p. 203). For nine compounds with fused triazole and triazolone rings, the free energy of activation at the coalescence temperature (−13.5 to 18°C) is in the range 12.5–14.2 kcal/mol. These values are lower than the ring-inversion barrier in diazepam (5.6) which is 17.3 kcal/mol [271]. This effect is ascribed to the absence of a double bond between N4 and C5 in these compounds. In the triazinediones (30.1) and (30.2) the activation energy for ring inversion is much greater at *ca.* 20 kcal/mol (coalescence temperatures 145 and 152°C), ascribed to increased electron delocalization in the bridgehead lactam, thus increasing the rigidity of the 7-membered ring, and to repulsive dipole-dipole interactions in the transition state of the adjacent carbonyl groups. Fusion of a triazole ring to the *a*-face appears to increase the barrier to ring inversion even more [70].

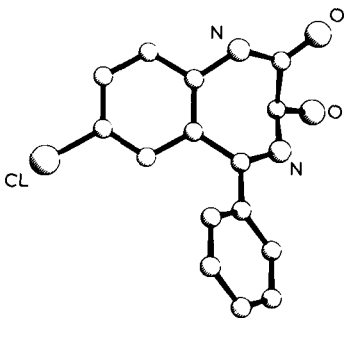


Figure 4.3. Compound (5.2).

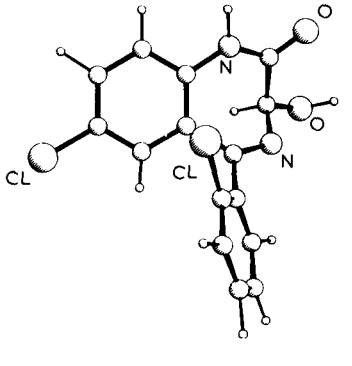


Figure 4.4. Compound (5.3).

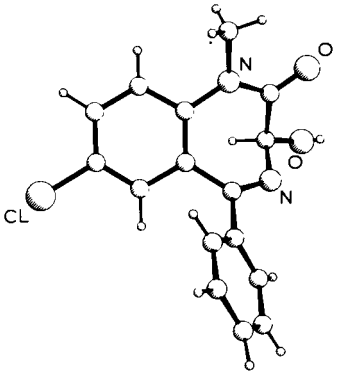


Figure 4.5. Compound (5.4).

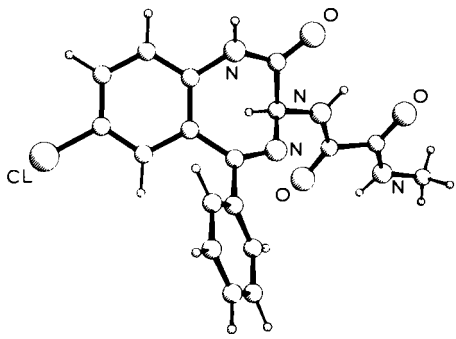


Figure 4.6 Compound (5.50).

In the benzodiazepines of type (5) so far discussed in this section, with the exception of medazepam (5.12), inversion of the seven-membered ring results in an equilibrium involving two enantiomeric conformers of equal energy. When, however, C3 is substituted unsymmetrically, the invertomers are diastereoisomeric and of unequal energy, one with R^2 quasi-equatorial and the other having R^2 quasi-axial. The 3-hydroxy and 3-methyl derivatives (5.2), (5.4), (5.31) and (5.41) are considered [292, 293] to exist in only one conformation at the temperature of the experiments (35–36.5°C), with the OH or Me groups in the quasi-equatorial position and H quasi-axial. X-Ray crystallographic data on C3-substituted benzodiazepines confirm this (*Figures 4.3–4.6*). The other has the substituent in the less favoured quasi-axial orientation.

In *Table 4.1* are listed kinetic and thermodynamic parameters pertaining to inversion of the seven-membered ring derived from the NMR studies. Although these measurements have been carried out using a variety of solvents, it is considered [277] that the results are not affected significantly by the choice of solvent. The free energies of activation, ΔG^*, are mostly accurate to about ± 0.3 kcal/mol, and in general the results for the same compound in different solvents do not differ significantly. This assumption, therefore, seems valid, with the possible exception of (5.38) where there is a difference of 0.7 kcal/mol between the values obtained in hexachlorobutadiene and deuteropyridine. Enthalpies and entropies of activation, ΔH^* and ΔS^*, are considerably less accurate than the ΔG^* values but are listed for completeness. ΔG^* and the kinetic parameters, the rate constant for the inversion, k, and the average lifetime of a conformer, T ($= 1/k$), have been recalculated, where necessary, so that all the data apply to the same temperature (37°C).

Table 4.1. KINETIC AND THERMODYNAMIC PARAMETERS FOR THE INVERSION OF THE SEVEN-MEMBERED RING

Solvents: a, deuteroacetone; b, carbon disulphide; c, deuteropyridine; d, deuterodimethylsulphoxide; e, deuterobenzene; f, hexachlorobutadiene.

Compound	*N1 substituent*	*Solvent*	T *(s)*	k *(s^{-1})*	ΔG^*_{310} *(kcal/mol)*	ΔH^*	ΔS^* *(e.u.)*	*Ref.*
(5.11)	H	a	3.3×10^{-6}	3.0×10^{5}	10.4	9.5	−2.9	277
(5.10)	H	a	4.6×10^{-5}	2.2×10^{4}	12.0	10.9	−3.4	276
		a	6.7×10^{-5}	1.5×10^{4}	12.2	9.1	−10.2	277
(5.1)	H	c	8.7×10^{-5}	1.1×10^{4}	12.4	11.0	−4.5	271
(3.1)	–	b	7.0×10^{-3}	1.4×10^{2}	15.1	11.0	−13.3	276
(5.14)	Me	d	2.0×10^{-2}	5.1×10^{1}	15.7	17.7	6.3	277
(5.16)	Me	d	2.4×10^{-1}	4.1	17.3	18.3	3.2	277
(5.6)	Me	c	2.5×10^{-1}	4.0	17.3	15.5	−5.8	271
		e	3.5×10^{-1}	2.9	17.5	15.9	−5.1	276
		f	4.1×10^{-1}	2.4	17.6	17.3	−1.0	271
(5.38)	Et	f	3.0×10^{-1}	3.4	17.4	13.7	−12.0	271
		c	9.2×10^{-1}	1.1	18.1	15.3	−9.0	271
(5.39)	Et	f	4.1×10^{-1}	2.4	17.6	12.6	−16.2	271
		c	6.7×10^{-1}	1.5	17.9	15.6	−7.3	271
(5.40)	CH_2OMe	d	6.3×10^{-1}	1.6	17.9	19.2	4.5	277
(5.36)	$CHMe_2$	f	9.2×10^{-1}	1.1	18.1	15.7	−7.7	271
		c	1.5	6.7×10^{-1}	18.4	15.5	−9.3	271
(5.37)	CH_2Ph	c	4.0	2.5×10^{-1}	19.0	19.0	0.0	271

The effect of an N1 substituent in increasing ΔG^* is clearly demonstrated. Compounds are listed in order of increasing ΔG^*, which closely parallels increasing size of the N1 substituent. Flunitrazepam (5.14) has a lower ΔG^* than other N1-Me compounds and this is probably a real effect, noted previously, brought about by the presence of the 2′-fluoro substituent in the 5-phenyl ring. A similar diminution in the barrier to inversion is caused also by a 2′-chloro substituent (cf. compounds (5.10) and (5.11)).

Consideration of the 5-phenyl ring shows [287] that the C2′, C6′, C3′ and C5′ carbon-13 resonance frequencies display the chemical shift equivalence of C2′ and C6′, and C3′ and C5′, indicating that there is rapid rotation of this ring about the C1′-C5 bond. There is, however, evidence of through-space H6 to F2′ coupling in (5.14) [287]. In bromazepam (14), the nitrogen atom of the 5-(2-pyridino) ring is considered [288] to be involved in a hydrogen bond C6-H...N2′ at room temperature. It is postulated that at *ca.* 100°C the hydro-

gen bond is ruptured and the pyridine ring rotates relatively rapidly about C1′-C5. In a very recent study [294], lanthanide-induced shifts, using $Yb(fod)_3$ and $Pr(fod)_3$ as shift reagents, were used for the resonance assignments of the ^{13}C spectra of (5.1), (5.6), (5.8) and (5.9), and the orientation of the 5-phenyl ring in these compounds was determined, expressed in terms of the dihedral angle C11-C5-C1′-C2′. There are considerable differences between the values obtained from the two shift reagents; however, in the case of compounds (5.6) and (5.8), whose crystal structures have been determined [295, 296], the agreement between the solution and solid-state values is reasonably good. For (5.6), the angles in solution are 18.1° [$Yb(fod)_3$] and 35.5° [$Pr(fod)_3$], and for (5.8) they are 20.9° and 13.4°. In the solid state, in (5.6) the dihedral angle is 25.5° and in (5.8), whose crystal structure contains two symmetry-independent molecules, this angle is 28.8° in one molecule and 43.7° in the other. In (5.9) the orientation is considered to be such that the fluoro substituent in the 2′-position comes close to the C6 hydrogen atom [294] (see also Ref. 287). Crystal structures are not available for the 2′-fluoro compounds (5.9) and (5.14). X-Ray analyses of a number of 2′-chloro derivatives show that in the solid state, although the 5-phenyl ring is almost always oriented so that the halogen 'points' in the direction of C6-C11, the H6 to chlorine separation is quite large (range 3.6–4.1 Å) (see *Figures 4.4, 4.7–4.9*). In (61.4) (see p. 202) the H6...F2′ separation is 3.5 Å.

X-RAY CRYSTALLOGRAPHY

X-Ray crystallography is undoubtedly the most powerful method known of determining molecular geometry. However, as is implicit in the name of the technique, the results apply to the molecule in the solid (crystalline) state. Crystal packing forces may affect the geometry of flexible parts of the molecule in a manner different from solvent interactions in solution. Nevertheless, X-ray crystallographic results generally agree reasonably well with those obtained in solution by NMR spectroscopy, and a sufficiently large number of X-ray analyses has now been carried out, especially of 5-phenyl-1,4-benzodiazepines of type (5), that the overall pattern of molecular geometry can be recognized. Additional confidence in the validity of the solid-state geometry is gained from the fact that in a number of cases the crystal structure contains two, and in one case three, independent molecules. Identical molecules are therefore exposed to different packing forces, so that the effect of these can be assessed. In general, only the orientation of the 5-phenyl ring is significantly affected (see *Table 4.2*).

Crystal structures of 16 benzodiazepines of type (5) have been published

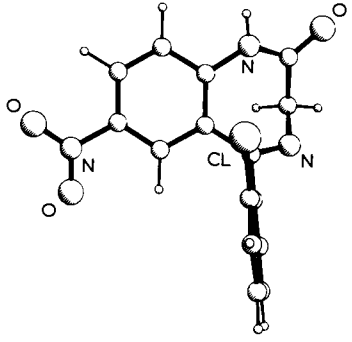


Figure 4.7. Compound (5.11).

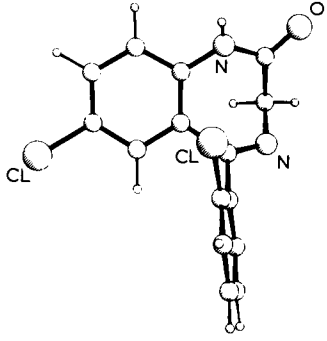


Figure 4.8. Compound (5.19).

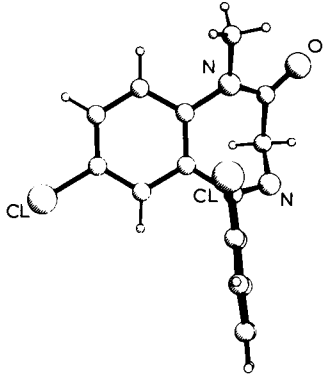


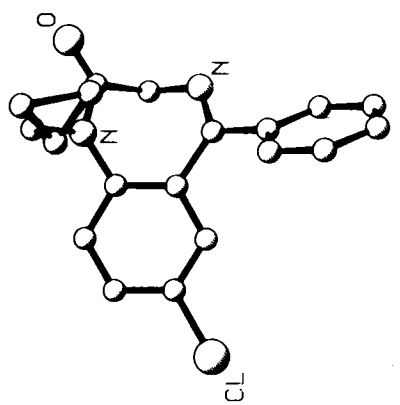
Figure 4.9. Compound (5.49).

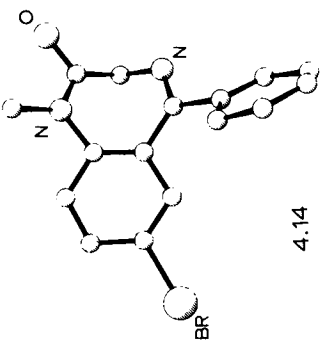
Table 4.2. GEOMETRIC PARAMETERS DERIVED FROM X-RAY CRYSTAL STRUCTURE ANALYSES OF SELECTED BENZODIAZEPINES

θ_1 is the angle between the planes of the 5-phenyl ring and the fused benzene moiety. θ_2 and θ_3 are the stern and bow angles of the boat shaped seven-membered ring. *T*(N1-C2) is the endocyclic torsion angle about N1-C2. *L*(N1-C2) and *L*(C5-C1′) are the lengths (Å) of the N1-C2 and C5-C1′ bonds. Under *T*(C5-C1′) are listed the torsion angles C11-C5-C1′-C2′ and N4-C5-C1′-C2′. Estimated standard deviations are generally < 0.01 Å for lengths and < 1° for angles; in (5.19) and (5.46) e.s.d.'s are greater by factors of *ca.* 2 and 4, respectively.

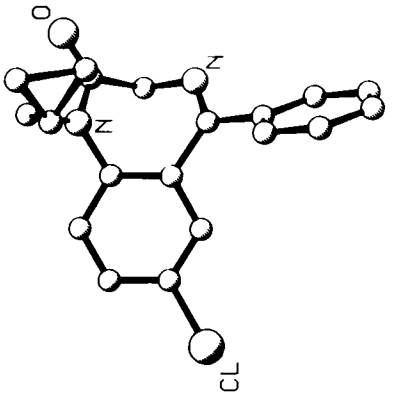
	θ_1	θ_2	θ_3	Δ	T(*N1-C2*)	L(*N1-C2*)	L(*C5-C1′*)	T(*C5-C1′*)
(5.2)	54	33	63	2.3	0.2	1.36	1.49	30, 152
	66	33	63	1.6	2.8	1.36	1.49	42, 138
(5.3)	73	35	62	3.1	3.2	1.35	1.48	52, 131
	81	33	58	2.9	4.0	1.31	1.49	58, 121
(5.4)	66	34	63	2.8	0.5	1.38	1.49	40, 142
	67	36	64	2.1	4.0	1.37	1.50	37, 144
	59	40	63	3.5	5.6	1.37	1.49	24, 160
(5.6)	55	38	58	9.3	13.4	1.37	1.49	25, 157
(5.8)	71	37	62	4.6	3.3	1.37	1.49	44, 138
	67	40	60	5.3	8.8	1.37	1.49	29, 151
(5.10)	62	32	60	1.3	0.9	1.36	1.49	35, 144
(5.11)	84	34	58	4.4	7.2	1.36	1.50	66, 116
	78	33	59	2.4	4.0	1.36	1.51	61, 123
(5.19)	80	33	59	1.7	1.0	1.34	1.48	63, 122
(5.24)	75	34	60	3.7	2.8	1.38	1.49	54, 131
(5.46)	74	36	58	2.3	2.6	1.31	1.56	38, 138
(5.47)	62	39	59	9.0	11.8	1.36	1.49	24, 156
	68	39	60	7.0	10.1	1.36	1.49	35, 146
(5.48)	66	37	61	3.8	7.2	1.36	1.49	35, 145
(5.49)	86	39	58	8.2	14.8	1.37	1.50	63, 120
(5.50)	69	32	59	4.4	6.3	1.35	1.51	48, 134
(5.51)	62	36	61	4.8	3.4	1.37	1.50	32, 151
(3.1)	67	42	58	8.1	12.4	1.32	1.47	27, 150
(3.7)	61	35	59	2.2	2.2	1.30	1.49	29, 149
	65	32	61	5.8	5.8	1.31	1.50	42, 137
(9.2)	75	31	54	3.2	3.6	1.34	1.53	48, 130
(13.6)	85	44	58	6.7	6.4	1.37	1.58	73, 159
(59)	66	29	53	2.2	2.5*	1.33*	1.51	42, 139

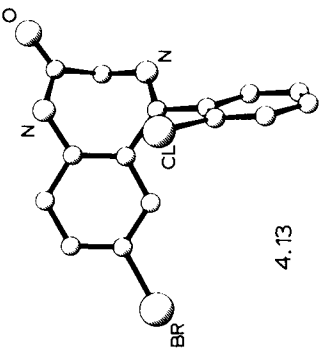
* Refers to bond C1-N2.



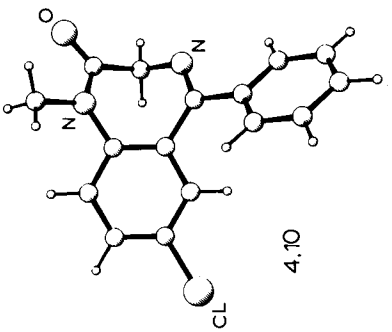


4.14

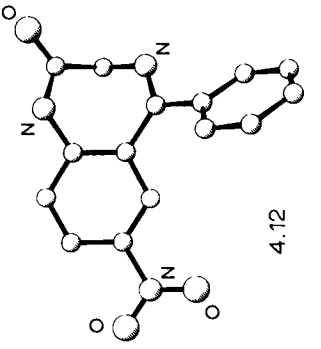




4.13



4.10



4.12

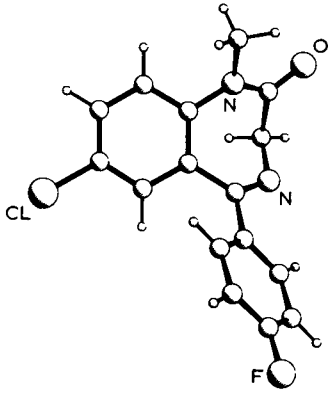


Figure 4.10. Compound (5.6).

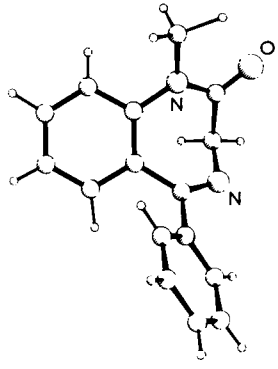


Figure 4.11. Stereoscopic view of compound (5.5).

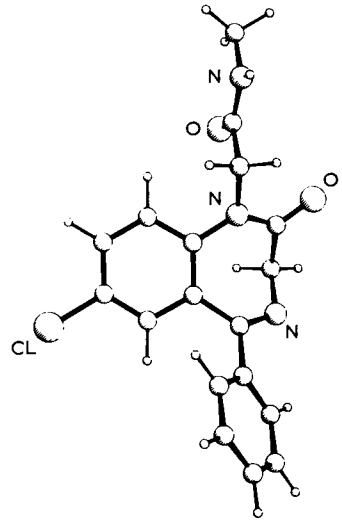


Figure 4.12. Compound (5.10).

Figure 4.13. Compound (5.24).

Figure 4.14. Compound (5.46).

Figure 4.15. Compound (5.47).

Figure 4.16. Compound (5.48).

Figure 4.17. Compound (5.51).

and computer drawings [297] of the molecules as viewed in a direction perpendicular to the plane of the fused benzene moiety are shown in *Figures 4.3* [298], *4.4* [299], *4.5* [300], *4.10* [295], *4.11* [296] (stereoscopic view), *4.12* [301], *4.7* [302], *4.1* [283], *4.8* [303], *4.13* [304], *4.14* [305], *4.15* [306], *4.16* [306], *4.9* [303], *4.6* [307] and *4.17* [308]. In certain of these diagrams, hydrogen atoms could not be shown, as their positions had not been determined in the X-ray analyses. To a first approximation, the molecular framework of all the compounds is very similar. Although the seven-membered heterocyclic ring contains only one formal double bond and one shared aromatic bond, it adopts a cycloheptatriene-like boat conformation. The third 'double' bond is the amide bond, N1-C2. This bond is affected by electron delocalization between the nitrogen lone pair and the carbonyl oxygen atom; the bond is shortened to a length about half-way between the C-N pure single- and double-bond values and the disposition of bonds at the nitrogen atom is near planar. The overall geometry of the N1-C2 bond closely resembles that of a normal double bond. Bond lengths and endocyclic torsion angles about N1-C2 are listed in *Table 4.2*.

The molecules of type (5) can be described in terms of four planes; the fused benzene (benzo) moiety together with the linked atoms N1 and C5, the central plane of the 'boat' consisting of atoms N1, C2, N4, C5, the plane forming the bow of the boat, atoms C2, C3, N4 and that of the 5-phenyl ring. The 5-phenyl ring is oriented at angles within the range 54–86° to that of the benzo plane, constituting the major geometrical differences between these molecules. The larger dihedral angles (>75°) occur in compounds with a halogen substituent at the 2′-position of the phenyl ring. Geometrical parameters are listed in *Table 4.2*. Angles between the benzo plane and that of the 5-phenyl ring are listed under θ_1. Multiple entries occur when the crystal contains more than one independent molecule. As noted previously, differences in geometrical parameters in such cases represent the effect of differing packing forces on identical molecules. The 'stern' plane, formed by atoms N1, C5, C10 and C11 can be considered (to a reasonable approximation) to coincide with the benzo plane. The stern and bow angles of the boat-shaped ring are listed under θ_2 and θ_3. Stern angles are all in the range 32–40° and bow angles in the range 58–64°.

As a means of comparing the structures of different compounds and also in relation to the NMR results, it is useful to have a measure of the deviation of the conformation of the seven-membered ring from that of 'ideal' cycloheptatriene. In an ideal cycloheptatriene boat, with mirror (C_s) symmetry, the ring torsion angles about N1-C2, N4-C5 and C10-C11 are zero and the other four torsion angles taken in pairs are equal in magnitude but of opposite sign. To obtain a quantitive measure of distortion, we have calculated a deviation

parameter, Δ, based on the asymmetry parameter of Duax, Weeks and Rohrer [309], where Δ is given by $[1/5(T1^2 + T4^2 + T6^2 + (T2 + T3)^2 + (T5 + T7)^2)]^{1/2}$ and $T1$–$T7$ are the ring torsion angles about N1-C2, C2-C3, C3-N4, N4-C5, C5-C11, C11-C10, C10-N1, respectively; in the ideal case $\Delta = 0$.

The values of Δ which were obtained are listed in *Table 4.2*. In general they are small, showing that the ring conformations are close to that of a cycloheptatriene-like boat, despite the presence of the hetero-atoms. The larger distortions (> 5°) all occur in compounds with a methyl substituent at N1, although the N1-Me substituted compounds (5.4), (5.46) and (5.48) are only slightly distorted. Compounds (5.8) and (5.51), where N1 carries the bulky cyclopropylmethyl and methylacetamido groups, have only moderately distorted rings, as has (5.50), where C3 carries a large substituent. The torsion angles about N1-C2 generally parallel the Δ values.

The distorting effect of a substituent at N1 on the solid-state geometry of the seven-membered ring is thus not in agreement with the results obtained in solution by NMR spectroscopy [280] (see p. 188), which indicate quite the opposite effect.

A halo substituent at the 2′-position of the 5-phenyl ring generally increases the biological activity and, according to the NMR results, decreases the barrier to ring inversion of the seven-membered ring (*Table 4.1*). Any electronic interaction between the 5-phenyl ring and the benzodiazepine framework would affect the length of the bond between C5, and C1′ of the phenyl ring. The C5-C1′ bond lengths are given in *Table 4.2*. The mean length for unsubstituted rings is 1.49Å, the same as for the chloro-substituted ones. The X-ray analysis of (5.46) is of relatively low accuracy and the length of 1.56 Å for this compound was not included in the averaging. There is also no correlation between the length of C5-C1′ and θ_1, the angle between the 5-phenyl ring and the benzo plane, nor between it and the C11-C5-C1′-C2′ or N4-C5-C1′-C2′ torsion angles. There is, therefore, no evidence from the X-ray results of any electron delocalization across this bond which corresponds in length to a normal single bond between trigonally hybridized carbon atoms.

The seven-membered ring of medazepam (5.12) which does not have a keto function at C2, is essentially a cycloheptadiene-like pseudo-boat [283, 284]. The torsion angles about N1-C2 are 26.5° and 29.3° in the two independent molecules in the crystal of the free base [283] and 34.8° in the hydrochloride salt [284]. The length of the N1-C2 bond, 1.46 Å, in all three cases, corresponds to a C-N single bond. In the salt, protonation occurs at the imine nitrogen N4, and this results in an increase of *ca.* 7° in the angle at N4 and a decrease of 4° in the endocyclic angle at C5. Bond lengths do not seem to be affected by protonation.

Of the fifteen benzodiazepines of type (5) listed in *Table 4.2*, twelve are considered [15] to have high or moderate CNS-depressant activity and two, (5.47) and (5.48) only slight activity [306]. No pharmacological data seem to be available for compound (5.50). It is evident from the structural parameters listed in *Table 4.2* and the overall appearance of the molecules shown in the diagrams that there is no obvious relationship between molecular geometry and activity; it would not be possible to pick out (5.47) and (5.48) as being 'different' from the other compounds. Structure-activity relationships are considered in greater detail on p. 205.

The 3H-1,4-benzodiazepine system of chlordiazepoxide (3.1) has also been examined by X-ray crystallography, but to a much more limited extent. The seven-membered ring of chlordiazepoxide hydrochloride [310] and the C3-hydroxy compound (3.7) [311] adopts a cycloheptatriene boat-like conformation, but significant distortions occur. The deviation parameter, Δ, and other selected geometrical parameters are included in *Table 4.2*. The N1-C2 bond is a formal double bond in these compounds, but is somewhat longer than a pure C-N double bond, the accepted length of which is 1.28 Å, indicating appreciable electron delocalization involving N1 and the methylamino side-chain. Protonation of (3.1) occurs at N1. Pharmacologically, (3.7) is considerably less potent than (3.1). These molecules are pictured in *Figures 4.18* and *4.19*.

The crystal structures of four 5-phenyl-1,3,4,5-tetrahydro-2H-1,4-benzodiazepin-2-ones, (61.1)–(61.4), have also been determined [272, 312, 313]. The N1-C2 bond in these compounds again has a geometry approximating to that of a double bond, similar to the situation in the benzodiazepines of type (5). The N4-C5 bond is, however, a pure single bond and the disposition of

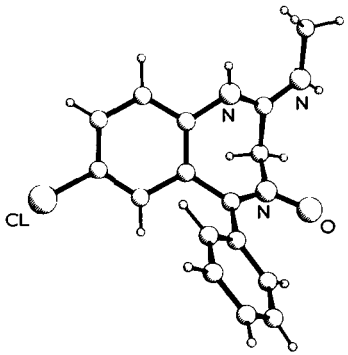


Figure 4.18. Compound (3.1).

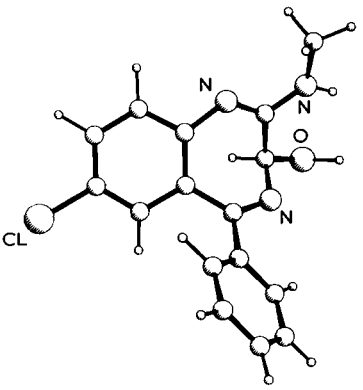
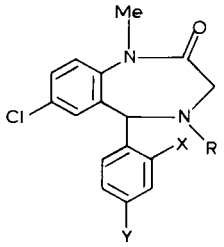


Figure 4.19. Compound (3.7).



(61)

(61.1) R = Me, X = Y = Cl
(61.2) R = $CONH_2$, X = Y = H
(61.3) R = X = Y = H,
(61.4) R = Me, X = F Y = H

bonds at N4 is pyramidal and at C5 tetrahedral, and the overall geometry of the seven-membered ring is that of a cycloheptadiene pseudo-boat. The N4 substituent is oriented pseudo-equatorial and in three of the compounds the 5-phenyl ring is also pseudo-equatorial. In the N4-carbamoyl derivative (61.2), the phenyl ring is pseudo-axial. It is considered that the axial conformation is preferred because of steric hindrance due to the bulky carbamoyl group at the adjacent N4 [312] (*Figures 4.20–4.22*). Benzodiazepines of this type have only low CNS activity.

Only two crystal structures of the important triazolo[4,3-*a*][1,4]benzodiazepines of type (9) have been reported, those of the highly active (9.2) [314] and (9.3) [315]. The conformation of the seven-membered ring in (9.2) [314] is similar to that found in the type (5) compounds and approximates closely to a cycloheptatriene boat shape with a relatively low value of 3.2° for the deviation parameter (see *Table 4.2* and *Figure 4.23*). Details of the structure of (9.3) are not available. The analysis was carried out on crystals containing both protonated and unprotonated molecules, and it is of interest that protonation occurs at N2 of the triazolo ring [315].

Some structural data are also available for *d*-face-fused benzodiazepines. The crystal structure of ketazolam (10) has been determined [25], but only in preliminary form, and geometrical parameters are not available. Full data are, however, available for the oxazolam analogue (13.6) [316] (*Figure 4.24*) and another related compound (62) [317]. The seven-membered ring of (13.6) approximates quite closely to a cycloheptatriene boat (Δ = 6.7°), despite the fact that it is chemically analogous to the cycloheptadiene-shaped rings of (61.1)–(61.4). In (62), which lacks the carbonyl function, the diazepine ring

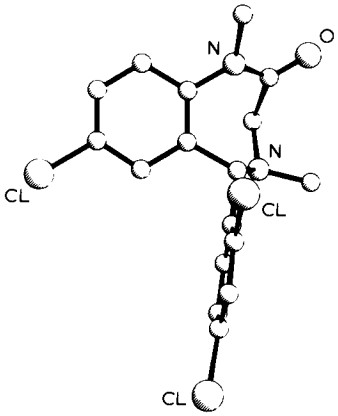


Figure 4.20. Compound (61.1).

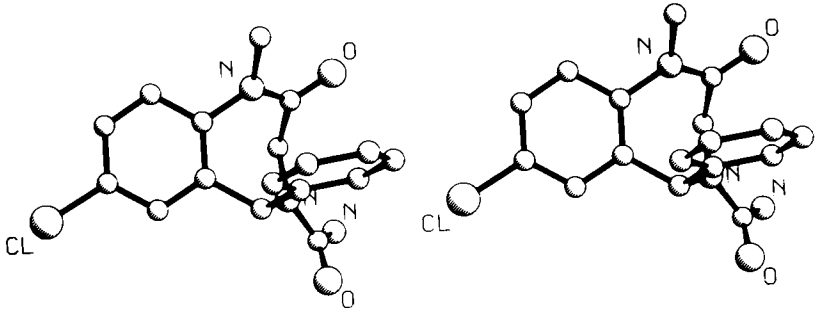


Figure 4.21. Stereoscopic view of compound (61.2).

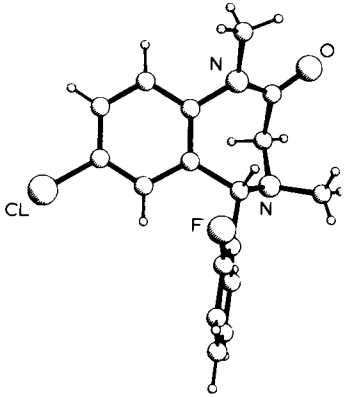


Figure 4.22. Compound (61.4).

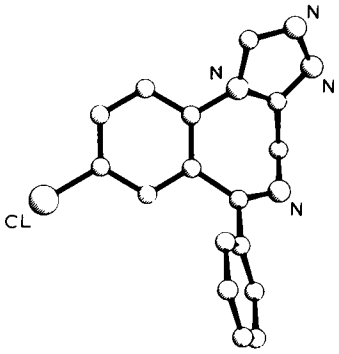


Figure 4.23. Compound (9.2).

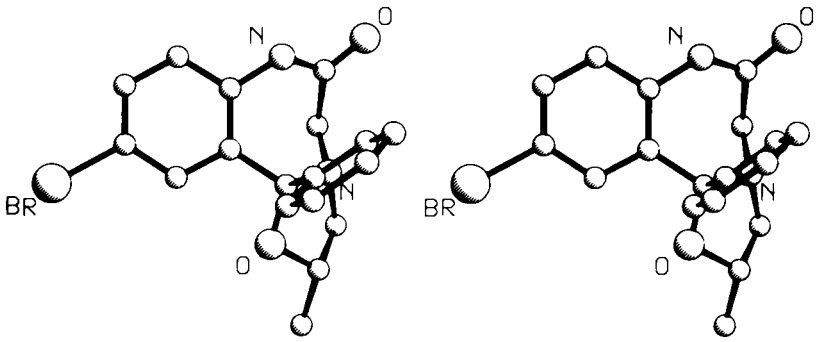
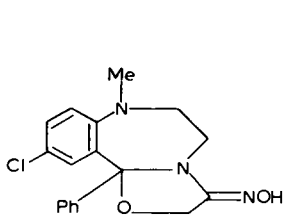
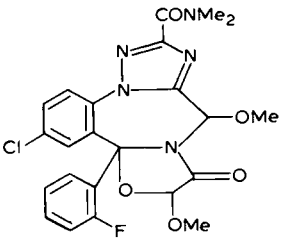


Figure 4.24. Stereoscopic view of compound (13.6).

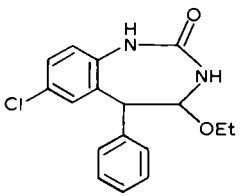
has a cycloheptene chair-like conformation. All three structures have the phenyl ring orientated pseudo-axial. A combination of *a*- and *d*-face fusion occurs in (63) [318]. Here the seven-membered ring is no longer of the cycloheptatriene type (Δ = 25.2°), but the phenyl ring is again pseudo-axial.



(62)

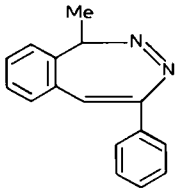


(63)

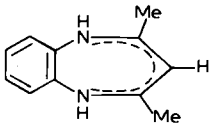


(64)

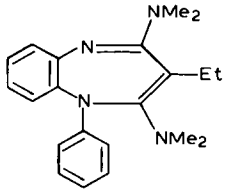
The crystal structure of the triazino[4,3-*d*][1,4]benzodiazepinetrione (30.2), which lacks a phenyl ring in the 5-position of the 1,4-benzodiazepine system, has also been determined as the hydrobromide salt [70]. The geometry of the N1-C2 bond of the 1,4-benzodiazepine system approximates to that of a single bond (length 1.44 Å, endocyclic torsion angle 34.4°) so that the seven-membered ring has a cycloheptadiene-like pseudo-boat conformation with Δ = 21.4° (*Figure 4.2*). The hydrogen atoms have not been located in this analysis, so that the site of protonation is unknown; however, the observed geometry of the N1-C2 bond is consistent with protonation at N1 as this would oppose electron-delocalization across this bond, so that it would remain essentially a single bond.



(65)



(66)



(67)

Benzodiazepines with nitrogen atoms in positions other than 1 and 4 have also been studied by X-ray crystallography. The geometry of the triazolo[5,1-*a*][2,4]benzodiazepine (59) [135] is similar to that of the triazolo[4,3-*a*][1,4]benzodiazepine, estazolam (9.2). The seven-membered ring adopts a relatively undistorted cycloheptatriene-like boat conformation (*Table 4.2* and *Figure 4.25*) with $\Delta = 2.2°$. In both molecules, this ring is slightly flatter (smaller values of θ_2 and θ_3) than in the benzodiazepines of type (5), but the difference is only very slight. A preliminary report of the crystal structures of 5-phenyl-2,3-dihydro-2,4-benzodiazepin-1-one (57.1) and the 1,3-benzodiazepine (64) has appeared [319] but details of molecular geometry are not yet available. The heterocyclic ring of the 4-phenyl-2,3-benzodiazepine (65) has a somewhat distorted cycloheptatriene-like boat shape ($\Delta = 12.8$) [320]. Here the stern plane is formed by atoms N3, C4, C5, C11, and the bow plane by C10, C1 and N2. The crystal structures of the 1,5-benzodiazepines (66) [321] and (67) [322]

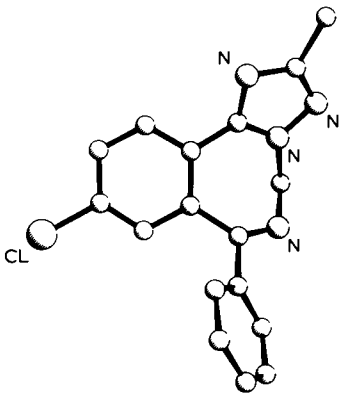


Figure 4.25. Compound (59).

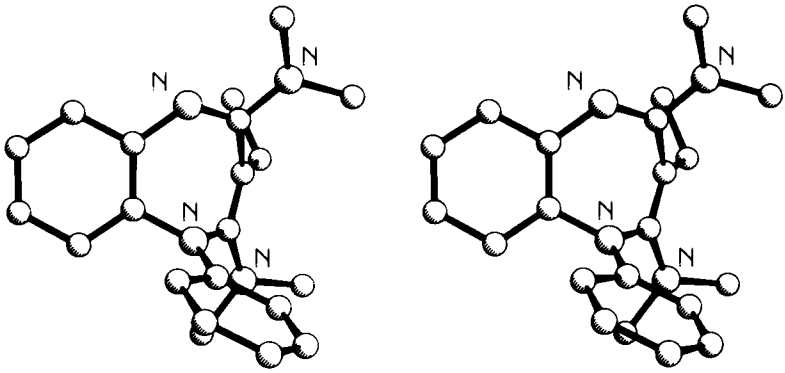


Figure 4.26. Stereoscopic view of compound (67).

have also been determined. Compound (66) in the form of the chloride salt is essentially a planar entity, electron delocalization extending from N1 via C2, C3 and C4 to N5 with the positive charge distributed over the two nitrogen atoms.

The seven-membered ring of (67) is essentially a cycloheptatriene-like boat which is, however, somewhat distorted (Δ = 11.2°); the bow plane is formed by the phenyl-bearing nitrogen atom and the two adjacent carbon atoms, and the stern plane by the N-C double bond and its two adjacent ring atoms (*Figure 4.26*).

STRUCTURE-ACTIVITY RELATIONSHIPS

The interaction of small molecules (drugs) with macromolecules (proteins) has been the subject of intense investigation over many years. The subject has been addressed at many levels and with techniques ranging from the purely theoretical to the use of more-or-less sophisticated physicochemical methods. The goal, however, has been unified, to enable correlations to be drawn between structural parameters and some measure of biological activity. The benzodiazepines have not escaped such attentions.

The benzodiazepines are extremely lipophilic molecules and they associate with plasma proteins to a very great extent (> 95% bound). Such association is not a productive phenomenon, in that the association leads to no subsequent event; it is merely a sequestration of the drug by the protein. A second type of interaction, however, does occur when the drugs of this class interact with

their CNS specific receptor site. In this case, the interaction results in a pharmacological response; the interaction is a specific recognition-site-mediated event and attempts have been made to define the pharmacophore within the benzodiazepine molecule. The aim of such studies has, of course, been to allow the prediction of compounds with greater efficacy than those currently available. There have also been additional attempts to delineate the structural variants within the benzodiazepine class which differentiate between the various aspects of the pharmacological profile of this class of drugs: anxiolytic, sedative, anti-convulsant and muscle relaxant.

The sequestration or nonspecific binding is represented by investigations of benzodiazepines bound to human serum albumin (HSA). These studies have led to some interesting, though at times conflicting, results by different groups of workers. Circular dichroism and gel filtration measurements by Müller and Wollert [323–327] indicated that the fused benzene ring of the benzodiazepine is the essential group in the binding. It was also found that both enantiomers of the chiral compound (5.45) bind to the same site on the HSA, but their affinities differ by a factor of about 40 [327]. Sjöholm, Sjödin and Roosdorp [328, 329] have also studied the benzodiazepine-HSA system by circular dichroism (CD) and have obtained essentially similar results. More recent CD and gel filtration studies [330, 331] have shown that the (*S*)-enantiomer of the chiral benzodiazepine (5.31) binds much more strongly than the (*R*)-enantiomer, in agreement with the results obtained [327] for the enantiomers of (5.45). However, contrary to the earlier conclusions [324, 327], it is considered [331] that the two nitrogen atoms of the benzodiazepine, rather than the fused benzene ring, are involved in the binding. It is also concluded that the benzodiazepines bind in their preferred boat conformation; for the C3-substituted compounds, e.g., (5.31), the conformation is primarily such that the substituent is in the quasi-equatorial orientation, in agreement with NMR and X-ray crystallographic results on uncomplexed benzodiazepines.

An NMR study of flurazepam (5.9) bound to HSA in D_2O solution has, however, led to the conclusion [332] that the complexation site of the drug molecule is primarily its heterocyclic component close to N4. The interaction is considered to be electrostatic in nature, cationic [325] binding sites on HSA interacting with the N4/C3 region of the heterocyclic ring which has been shown by CNDO/2 molecular orbital calculations to be negatively charged [273, 333]. The fused benzene ring is considered to act as a secondary hydrophobic site for binding.

Binding constants have been measured [329, 334] for a number of benzodiazepines, but there does not seem to be any significant correlation between affinity and biological activity. It has, however, been noted [330] that the more

strongly binding (*S*)-enantiomer of (5.31) also has the higher biological activity [335].

In a study carried out by Lucek and Coutinho [334], in which the percentage protein binding to human plasma proteins was determined at a fixed concentration for 52 benzodiazepine analogues, it was found that the lipophilic character of the molecule, as estimated by the Hansch substituent constant, π, was the main determinant of the propensity of the compounds to bind. However, influences of halogen substitution in the C2′ position which resulted in decreased binding, were thought to result from steric factors which restricted the free rotation of the 5-phenyl ring, thus preventing the optimal orientation of this structure to HSA binding loci [334].

The nonspecific interactions with HSA are characterized by their relatively low affinity, the Gibbs free energy of binding for one series studied [324] being in the range 5–8 kcal/mol, and their complete lack of correlation with pharmacological potency of the compounds.

In producing their pharmacological response, the benzodiazepines interact with specific glycoproteins, and some properties of this interaction have already been described. Relevant factors in understanding the molecular nature of this interaction relate to the steric and electrostatic parameters of both the ligand and receptor in isolation and the complex formed between them both. Although obviously of great interest, X-ray crystal structure analysis of the benzodiazepine-receptor complex itself has not so far proved possible. Information may be obtained regarding the receptor and drug-receptor complex indirectly by studying a series of drug molecules in various environments, by methods such as X-ray crystallography and NMR and by the calculation of electronic parameters either *de novo* or based on structural data. The resultant information may then be further analyzed in attempts to correlate the biological activity with structural elements and thus gain some impression of the pharmacophore involved in the receptor interaction.

In such studies, various measures of the biological activity of the benzodiazepines have been used. ED_{50} values for producing effects in defined whole animal test systems have the serious disadvantage that not only does the correlation depend on the receptor-ligand complex, but it depends also on the pharmacokinetic parameters essential in the arrival of the drug at the receptor. These are likely to overwhelm the analysis with the result that specific drug-receptor correlations will be lost in the noise of the data. In such studies, no attempt has so far been made to analyse these two factors separately.

The first of such studies [333], with a relatively small number of compounds, calculated both the partition coefficients and a number of electronic parameters and led the authors to conclude that, although lipophilicity was not signifi-

cantly correlated with anti-convulsant activity, the charge on N4 showed a very significant correlation. The authors suggested that a major interaction between these compounds and their receptor was afforded through hydrogen bond formation at N4. Halogen substitution at C2′ resulted in the 5-phenyl ring being rotated from the N1-C2-N4-C5 plane, resulting in decreased delocalization of the C5-N4 electrons and thus providing an increased charge density at N4, a hypothesis which is not supported by X-ray crystallographic data (see *Table 4.2*). Such substitutions are in accord with increased receptor affinity and biological efficacy.

In an NMR investigation of hydrogen bond formation between the benzodiazepines (5.1) (5.6) (5.10) (5.16) (5.42)–(5.44) and the nucleobases 1-ethyl-2,4-dihydroxy-5-methylpyrimidine (e^1Thy) and 2,4-dihydroxy-1,3-dimethylpyrimidine in deuterochloroform solution over a range of temperatures, is was observed [336, 337] that the N1-non-methylated compounds (5.1), (5.10), (5.42) and (5.43) form hydrogen bonds with their N1-H functions. The strengths of these increase with increasing electronegativity of the C7 substituent of the benzodiazepine. This correlates with increasing biological activity. The C2 carbonyl oxygen atom acts as a hydrogen bond acceptor, but the strength of the interaction is not affected by the nature of the substituent at C7. The methylated compounds (5.6), (5.16) and (5.44) interact only in this way and the interaction is weak. Standard free energy values, $\Delta G°$, for the interaction of N1-H with e^1Thy are -1.2, -1.4, -1.7 and -1.9 kcal/mol for (5.42), (5.1), (5.43) and (5.10), respectively (C7-substituents H, Cl, CF_3, NO_2). In the methylated compounds (5.6), (5.16) and (5.44), the $\Delta G°$ values for the interaction N3-H(e^1Thy)...O-C2 (benzodiazepine) are close to 0.5 kcal/mol [337].

Blair and Webb [273] carried out studies with 59 benzodiazepines and drew correlations between the total set or subsets of these with ED_{50} values obtained from inclined screen, footshock, anti-pentylenetetrazole and cat muscle relaxant activity (for more detailed description of these tests see p. 174). Electronic parameters were calculated using CNDO/2 based on the crystal structure of diazepam, making the assumption that the molecular modifications used would not affect the skeletal structure (an assumption which subsequent X-ray crystal structure data invalidate). Significant correlations were found with the above biological activity indices and the charge on the carbonyl oxygen atom of the lactam (+ve r) and the total molecular dipole moment (−ve r), the latter being superior. In no case did the introduction of the Hansch lipophilic substituent significantly improve the correlations. The generated equations, however, failed to account for the very low activity of compounds with a C4′ substituent.

Similar CNDO/2 calculations were used to attempt correlations between

various electronic parameters of 1,3-dihydro-5-phenyl-1,4-benzodiazepin-2-ones [338] and their anticonvulsant activity in mice. No significant correlation was found between their lipophilicity constants, though the p_y orbital on C10 appeared to be clearly predictive of biological activity.

Preliminary studies by Chananont, Hamor, Martin and Tate [339] have failed to confirm such a correlation using MNDO calculations based on X-ray crystallographic analysis of 15 compounds and their affinity constants for the benzodiazepine receptor. However, using stepwise multiple regression analysis, biological activity appeared to correlate with the electronic charge on C1′, dipole moment and HOMO energy. Additional calculations are required, however, to validate these suggestions with more structural determinations. Correlations were also found with certain electronic parameters obtained by CNDO/2 methods on seven benzodiazepines and anti-pentylenetetrazole activity, the most significant being with the difference in energy between LUMO and HOMO [339a].

A study carried out on 54 benzodiazepino-oxazoles using lipophilic, Hansch *F* values and three indicator variables attempted correlations with nine measures of biological activity. The *r* values varied from 0.962 to 0.799 for $n = 30$ and predictions were made from these equations relating to differentiation between different behavioural profiles, though no validation of these was given [340].

Chromatographic R_m values were obtained experimentally for 41 benzodiazepines and those obtained using a reversed-phase silicone oil system were found to correlate well with both observed and calculated log *P* values [341]. These were then used to investigate structure-activity relationships with both exploratory and conflict behaviour. It was found necessary to introduce indicator variables in order to obtain marked correlations and, although the final equations obtained in both the punished and unpunished behavioural situations appeared different in terms of their dependence on lipophilicity, the analysis did not allow firm conclusions to be drawn.

An attempt to use structural factors to differentiate parts of the pharmacological spectrum of the benzodiazepines, however, has been made (structure type 5). It was suggested that a separation could be found between punished and unpunished behaviours [342]. The procedures used required the consideration of only eight of the most active compounds from a series of 12; here the optimal requirements for anti-punishment activity were N1 methyl, C2′ halogen, with no substituent at position C3, while non-punished behaviour (indicative of sedative activity) was favoured by N1 hydrogen, C2′ chlorine and C3 hydroxyl. The conclusions from such a small series must be viewed, however, with considerable caution.

Although the number of studies with the benzodiazepines has not been extensive, it is clear that little consensus opinion can be drawn from them. The limitations of most of the studies mentioned above are considerable. Metabolic factors must clearly be taken into account in whole animal studies, and inclusion of compounds which are degraded to a common metabolite would necessarily be eliminated from correlations. Pharmacokinetic parameters differ markedly within the series and some account must be taken of these differences. Such approaches therefore are unlikely to provide clear indications of structural requirements at the receptor itself. The problem can be overcome. Instead of using whole animal measures of biological activity, correlations can be attempted between structural parameters and the affinity constants for the CNS-specific receptor [339]. Recently, one study has appeared using the novel distance geometry approach of Crippen [343].

The analysis uses the experimentally determined free energies of binding of the benzodiazepine series. Each of the compounds was then described in terms of the Cartesian co-ordinates of each atom, though some were later eliminated, these being obtained from published crystal structures in certain cases or developed from those where structural data were not available. Matrices were then constructed representing the molecular shape of each ligand, by sampling all possible dihedral angles about rotatable bonds, and commonalities of structure points were sought using the 18 most strongly binding ligands. This resulted in five common points to the ligands in this subset. Subsequently, energetic barriers were built into the model in order to explain the lower affinities of the remaining ligands, and the model was iteratively refined. In this manner, a model was produced representing the pharmacophore of the benzodiazepine structure and it also included representatives of non-benzodiazepine compounds which exhibit high affinities for the benzodiazepine receptor from a total of five chemical classes. This is a considerable achievement although, as the author points out, it is possible that other combinations of structural commonalities may serve as well. Following calculation of the binding energies for all the structures used in the analysis, the root-mean-square deviation from the observed free energies of binding was 1.1 kcal/mol.

Using a less sophisticated, though no less valid approach, Camerman and Camerman [344, 345] have sought commonalities between the X-ray crystallographic structures of a number of anticonvulsant drugs including phenytoin, the barbiturates and the benzodiazepines. They suggest that the pharmocophore may involve two hydrophobic groups together with two electron donor groups, represented by the two aromatic rings, C2 oxygen and N4 of the benzodiazepines.

Several reservations must be placed on attempts to define structure-activity relationships using structural data obtained in this way. In all such attempts to define structural commonalities, the basic assumption is that the chemical structures being studied interact with a unique receptor site in the same defined manner. This may not be the case. It is clear that while the barbiturates and the benzodiazepines facilitate GABA-mediated transmission, by some effect on chloride flux, the receptors through which these two drug classes produce their effects are not the same [346]. It is difficult to envisage what meaning can be placed on such structural commonalities when the molecular mechanism of action of the compounds is different.

Simple observations of structure, however, make it clear that the 5-phenyl substituent is of paramount importance in producing the activity profile of the benzodiazepines. The compounds Ro15-1788 [269] and 1,3-dihydro-5-methyl-2H-1,4-benzodiazepin-2-one [347] both lack this instrument and, while the first antagonizes the actions of the benzodiazepine, the latter is an overt convulsant.

Structural data obtained on ligands in isolation represent the spatial and electronic parameters which are important in the recognition of the ligand by receptor. In the case of antagonists, the measurement of K_d, from binding studies, will represent the free energy involved in this interaction. However, in the case of agonists, the situation is more complex. Not only must the ligand bind to the receptor, but presumably it deforms the receptor in some way in order to initiate the effector process. This requires energy which is obtained from the energy of the initial binding interaction; in the case of agonists, therefore, the measured K_d is the net energy resultant from these two processes, i.e., the initial binding and the deformation of receptor structure [348]. Attempts to correlate structural data with affinity for the receptor, as measured by K_d, would be simpler for antagonists, as here only a single process is involved, namely, that of receptor recognition.

As the sophistication of such approaches increases, attempts must also be made to look at the deformation of the ligands. It is here with the relatively rigid structure of the benzodiazepine molecule that we may hope for greater success in the future for, in the final analysis, we must hope that the numbers produced by such structure-activity approaches are interpretable in terms of mechanisms and not simply in terms of numbers!

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REFERENCES

1. L.H. Sternbach, Prog. Drug Res., 22 (1978) 229.
2. L.H. Sternbach, J. Med. Chem., 22 (1979) 1.
3. G. Zbinden and L.O. Randall, Adv. Pharmacol. Chemother., 5 (1967) 213.
4. L.H. Sternbach and E. Reeder, J. Org. Chem., 26 (1961) 1111.
5. L.H. Sternbach, U.S. Pat 2, 893, 992 (1959); Chem. Abstr., 54 (1960) 597c.
6. L.H. Sternbach and E. Reeder, J. Org. Chem., 26 (1961) 4936.
7. E. Costa and P. Greengard, eds., Mechanism of Action of Benzodiazepines, (Raven Press, New York, 1975).
8. M. Lader, ed., Arzneim.-Forsch., 30 (1980) 851.
9. R.W. Olsen, Annu. Rev. Pharmacol. Toxicol., 22 (1982) 245.
10. J.F. Tallman, S.M. Paul, P. Skolnick and D.W. Gallager, Science, 207 (1980) 274.
11. S.C. Bell and S.J. Childress, J. Org. Chem., 27 (1962) 1691.
12. S.C. Bell, U.S. Pat. 3, 176, 009 (1965); Chem. Abstr., 62 (1965) 16281c.
13. S.C. Bell, R.J. McCaully, C. Gochman, S.J. Childress and M.I. Gluckman, J. Med. Chem., 11 (1968) 457.
14. S.J. Childress and M.I. Gluckman, J. Pharm. Sci., 53 (1964) 577.
15. L.O. Randall, W. Schallek, L.H. Sternbach and R.Y. Ning, Psychopharmacol. Agents, 3 (1974) 175.
16. L.H. Sternbach, R.I. Fryer, W. Metlesics, E. Reeder, G. Sach, G. Saucy and A. Stempel, J. Org. Chem., 27 (1962) 3788.
17. J. Schmitt, P. Comoy, M. Suquet, G. Collet, J. le Meur, T. Clim, M. Brunaud, J. Mercier, J. Salle and G. Siou, Chim. Ther., 4 (1969) 239.
18. F.H. McMillan and I. Pattison, Fr. Pat. 1,394,287 (1965); Chem. Abstr., 63 (1965) 8387b.
19. H.M. Wuest, U.S. Pat. 3,192,200 (1965); Chem. Abstr., 63 (1965) 11592c.
20. L.H. Sternbach, G.A. Archer, J.V. Earley, R.I. Fryer, E. Reeder, N. Wasyliw, L.O. Randall and R. Banziger, J. Med. Chem., 8 (1965) 815.
21. L.H. Sternbach, R.I. Fryer, O. Keller, W. Metlesics, G. Sach and N. Steiger, J. Med. Chem., 6 (1963) 261.
22. G.A. Archer and L.H. Sternbach, J. Org. Chem., 29 (1964) 231.
23. MIMS (Monthly Index of Medical Specialties), (Medical Publications, London, June 1982).
24. J.B. Hester, Jr., A.D. Rudzik and B.V. Kamdar, J. Med. Chem., 14 (1971) 1078.

25. J. Szmuszkovicz, C.G. Chidester, D.J. Duchamp, F.A. MacKellar and G. Slomp, Tetrahedron Lett., (1971) 3665.
26. K.H. Hauptmann, K.H. Weber, K. Zeile, P. Danneberg and R. Griesemann, South Afr. Pat. 6,800,803 (1968); Chem. Abstr., 70 (1969) 106579.
27. S. Rossi, O. Pirola and R. Maggi, Chim. Ind. (Milan), 51 (1969) 479.
28. K.H. Weber, A. Bauer and K.H. Hauptmann, Justus Liebigs Ann. Chem., 756 (1972) 128.
29. United States Pharmacopeia Dispensing Information, 1981, 115.
30. F. Tenconi, R. Tagliabue and L. Molteni, Ger. Offen. 2,339,790 (1974); Chem. Abstr., 80 (1974) 133492.
31. G.Ferrari and C. Casagrande, Ger. Offen. 2,142,181 (1972); Chem., Abstr., 76 (1972) 140908.
32. K. Meguro and Y. Kuwada, Tetrahedron Lett., (1970) 4039.
33. T. Miyadera, A. Terada, M. Fukunaga, Y. Kawano, T. Kamioka, C. Tamura, H. Takagi and R. Tachikawa, J. Med. Chem. 14 (1971) 520.
34. R.I. Fryer, R.A. Schmidt and L.H. Sternbach, J. Pharm. Sci., 53 (1964) 264.
35. J. Schmitt, P. Comoy, M. Suquet, J. Boitard, J. le Meur, J.J. Basselier, M. Brunaud and J. Salle, Chim. Ther., 2 (1967) 171, 254.
36. R.B. Moffett and B.V. Kamdar, J. Heterocycl. Chem., 16 (1979) 793.
37. J.B. Hester, Jr., A.D. Rudzik and P.F. von Voigtlander, J. Med. Chem., 23 (1980) 392.
38. J.B. Hester, Jr., A.D. Rudzik and P.F. von Voigtlander, J. Med. Chem., 23 (1980) 643.
39. R.I. Fryer and A. Walser, U.S. Pat. 4,032,535 (1977); Chem. Abstr., 87 (1977) 102388.
40. J.B. Hester, Jr., A.D. Rudzik and P.F. von Voigtlander, J. Med. Chem., 23 (1980) 402.
41. J.B. Hester, P.F. von Voigtlander and G.N. Evenson, J. Med. Chem., 23 (1980) 873.
42. M. Gall, B.V. Kamdar and R.J. Collins, J. Med. Chem., 21 (1978) 1290.
43. J.B. Hester, Jr. and P.F. von Voigtlander, J. Med. Chem., 22 (1979) 1390.
44. J.B. Hester, Jr., C.G. Chidester and J. Szmuszkovicz, J. Org. Chem., 44 (1979) 2688.
45. A. Walser and G. Zenchoff, J. Med. Chem., 20 (1977) 1694.
46. A. Walser and G. Zenchoff, J. Heterocycl. Chem., 15 (1978) 161.
47. J.P. Maffrand, G. Ferrand and F. Eloy, Tetrahedron Lett., (1973) 3449.
48. T. Hara, K. Itoh and N. Itoh, J. Heterocycl. Chem., 13 (1976) 1233.
49. M. Gall and B.V. Kamdar, J. Org. Chem., 46 (1981) 1575.
50. I.R. Ager, G.W. Danswan, D.R. Harrison, D.P. Kay, P.D. Kennewell and J.B. Taylor, J. Med. Chem., 20 (1977) 1035.
51. E.E. Garcia, J.G. Riley and R.I. Fryer, J. Org. Chem., 33 (1968) 1359.
52. T. Hara, Y. Kayama, T. Mori, K. Itoh, H. Fujimori, T. Sunami, Y. Hashimoto and S. Ishimoto, J. Med. Chem., 21 (1978) 263.
53. R.B. Moffett, G.N. Evenson and P.F. von Voigtlander, J. Heterocycl. Chem., 14 (1977) 1231.
54. R.G. Smith, R.A. Lucas and J.W.F. Wasley, J. Med. Chem., 23 (1980) 952.
55. D.L. Coffen, R.I. Fryer, D.A. Katonak and F. Wong, J. Org. Chem., 40 (1975) 894.
56. N.W. Gilman, B.C. Holland and R.I. Fryer, J. Heterocycl. Chem., 14 (1977) 1163.

57. A. Walser, L.E. Benjamin, Sr., T. Flynn, C. Mason, R. Schwartz and R.I. Fryer, J. Org. Chem., 43 (1978) 936.
58. A. Walser, R.F. Lauer and R.I. Fryer, J. Heterocycl. Chem., 15 (1978) 855.
59. A. Walser, T. Flynn and R.I. Fryer, J. Heterocycl. Chem., 15 (1978) 577.
60. R.I. Fryer, J. Blount, E. Reeder, E.J. Trybulski and A. Walser, J. Org. Chem., 43 (1978) 4480.
61. A. Walser and T. Flynn, J. Heterocycl. Chem., 17 (1980) 1697.
62. N.W. Gilman and R.I. Fryer, J. Heterocycl. Chem., 14 (1977) 1171.
63. H. Natsugari, K. Meguro and Y. Kuwada, Chem. Pharm. Bull., 27 (1979) 2927.
64. P.N. Samuelson, J.G. Reves, N.J. Kouchioukos, L.R. Smith and K.M. Dole, Anesth. Analg. (Cleveland), 60 (1981) 802.
65. M.E. Derieg, J.V. Earley, R.I. Fryer and L.H. Sternbach, U.S. Pat. 3,965,151 (1976); Chem. Abstr., 85 (1976) 160183.
66. T. Mitsushima and S. Ueki, Nipon Yakurigaku Zasshi, 74 (1978) 959; Chem. Abstr., 90 (1979) 180083.
67. T. Kamioka, I. Nakamaya, T. Hara and H. Tagaki, Arzneim.-Forsch., 28 (1978) 838.
68. N.P. Peet and S. Sunder, J. Heterocycl. Chem., 14 (1977) 561.
69. R. Madronero and S. Vega, J. Heterocycl. Chem., 15 (1978) 1127.
70. P.C. Wade, B.R. Vogt, B. Toeplitz, M.S. Puar and J.Z. Gougoutas, J. Org. Chem., 44 (1979) 88.
71. H. Breuer, Tetrahedron Lett., (1976) 1935.
72. S. Raines, S.Y. Chai and F.P. Palopoli, J. Heterocycl. Chem., 13 (1976) 711.
73. R. Littell and D.S. Allen, Jr., J. Med. Chem., 8 (1965) 722.
74. W. von Bebenburg and H. Offermanns, Ger. Offen. 2,259,471 (1973); Chem. Abstr., 79 (1973) 66412.
75. B. Saleta, J. Grunberger, L. Linzmayer and R. Stadler, Arzneim.-Forsch., 30 (1980) 513.
76. L. Fontanella, L. Mariani and G. Tarzia, Ger Offen. 2,511,599 (1975); Chem. Abstr., 84 (1976) 5013.
77. L. Fontanella, L. Mariani, G. Tarzia and N. Corsico, Eur. J. Med. Chem., 11 (1976) 217.
78. H.A. de Wald, I.C. Nordin, Y.J. L'Italien and R.F. Parcell, J. Med. Chem., 16 (1973) 1346.
79. H.A. de Wald, S. Lobbestael and D.E. Butler, J. Med. Chem., 20 (1977) 1562.
80. H.A. de Wald, S. Lobbestael and B.P.H. Poschel, J. Med. Chem., 24 (1981) 982.
81. R. Jaunin, Helv. Chim. Acta, 57 (1974) 1934.
82. M. Nakanishi, T. Tahara, K. Araki, M. Shiroki, T. Tsumagari and Y. Takigawa, J. Med. Chem., 16 (1973) 214.
83. O. Hromatka, D. Binder, C.R. Noe, P. Stanetty and W. Veit, Monatsh. Chem., 104 (1973) 715, and references therein.
84. O. Hromatka, D. Binder, P. Stanetty and G. Marischler, Monatsh. Chem., 107 (1976) 233.
85. O. Hromatka, D. Binder and G. Pixner, Monatsh. Chem. 104 (1973) 1348, and references therein.
86. K. Hirai, H. Sugimoto and T. Ishiba, J. Org. Chem., 45 (1980) 253.
87. O. Hromatka, D. Binder and K. Eichinger, Monatsh. Chem., 104 (1973) 1513, 1599.
88. K.H. Weber, A. Bauer, A. Langbein and H. Daniel, Justus Liebigs Ann. Chem., (1978) 1257.

89. T. Tahara, K. Araki, M. Shiroki, H. Matsuo and T. Munakata, Arzneim.-Forsch., 28 (1978) 1153.
90. R.I. Fryer, J.V. Earley and A. Walser, J. Heterocycl. Chem., 15 (1978) 619.
91. H. Fujimori, K. Kayama, T. Hara, K. Itoh and T. Sunami, J. Heterocycl. Chem., 14 (1977) 235.
92. M. Tanaka, H. Isozaki and K. Inanago, Jap. J. Pharmacol., 27 (1977) 517.
93. G. Cesco, S. Giannico, I. Fabbrucci, L. Scaggiante and N. Montanaro, Arnzeim.-Forsch., 27 (1977) 146, 436.
94. M. Steinman, J.G. Topliss, R. Alekel, Y.S. Wong and E.E. York, J. Med. Chem., 16 (1973) 1354.
95. E. Wolf, H. Kohl and G. Haertfelder, Ger. Offen. 2,022,503 (1971); Chem. Abstr., 76 (1972) 72570.
96. S. Allen and I. Oswald, Br. J. Clin. Pharmacol., 3 (1976) 165.
97. A.N. Nicholson, B.M. Stone and C.H. Clarke, Br. J. Clin. Pharmacol., 3 (1976) 533.
98. H. Yamamoto et al., South Afr. Pat. 68,06,061 (1969) Chem Abstr., 72 (1970) 90541.
99. T. Sukamoto, K. Ito and T. Nose, Jap. J. Pharmacol., 28 (1978) 33P.
100. A.V. Bogatskii, S.A. Andronati, U.I. Vikhlyaev, T.A. Klygul, O.P. Rudenko and V.V. Zakusov, U.S.S.R. Pat. 484,873 (1975); Chem. Abstr., 83 (1975) 209429.
101. A. Walser, G. Zenchoff and R.I. Fryer, J. Med. Chem., 19 (1976) 1378.
102. W.A. Khan and P. Singh, Org. Prep. Proc. Int., 10 (1978) 105.
103. T. Kovac, F. Kajfez, V. Sunjic, N. Blazevic and D. Kolbah, J. Med. Chem., 22 (1979) 1093.
104. S. Ebel and H. Schütz, Arzneim.-Forsch., 29 (1979) 1317.
105. H. Natsugari, K. Meguro and Y. Kuwada, Chem. Pharm. Bull., 27 (1979) 2084.
106. M. Ogata, H. Matsumoto and K. Hirose, J. Med. Chem., 20 (1977) 776.
107. V. Sunjic, J. Kuftinec and F. Kajfez, Arzneim.-Forsch., 25 (1975) 340.
108. M. Zinic, D. Kolbah, N. Blazevic, F. Kajfez and V. Sunjic, J. Heterocycl. Chem., 14 (1977) 1225.
109. R.Y. Ning, R.I. Fryer and B.C. Sluboski, J. Org. Chem., 42 (1977) 3301.
110. V. Sunjic, M. Oklobdzija, A. Lisini, A. Sega, F. Kajfez, D. Srzic and L. Klasinc, Tetrahedron, 35 (1979) 2531.
111. R.I. Fryer, J.V. Earley, N.W. Gilman and W. Zally, J. Heterocycl. Chem., 13 (1976) 433.
112. H. Natsugari, K. Meguro and Y. Kuwada, Chem. Pharm. Bull, 27 (1979) 2608.
113. F. Gatta, M.R. del Giudice, L. di Simone and G. Settimj, J. Heterocycl. Chem., 17 (1980) 865.
114. G.A. Archer, R.I. Kalish, R.Y. Ning, B.C. Sluboski, A. Stempel, T.V. Steppe and L.H. Sternbach, J. Med. Chem., 20 (1977) 1312.
115. R. Kalish, E. Broger, G.F. Field, T. Anton, T.V. Steppe and L.H. Sternbach, J. Heterocycl. Chem., 12 (1975) 49.
116. C. Corral, R. Madromero and S. Vega, J. Heterocycl. Chem., 14 (1977) 985.
117. S. Inaba, M. Akatsu, T. Hirohashi and H. Yamamoto, Chem. Pharm. Bull., 24 (1976) 1076.
118. J.H. Gogerty , R.G. Griot, D. Habeck, L.C. Iorio and W.J. Houlihan, J. Med. Chem., 20 (1977) 952.
119. O. Bub, Ger. Offen. 1,913,536 (1970); Chem. Abstr., 73 (1970) 120691.
120. A. Bauer, K.H. Weber and M. Unruh, Arch. Pharm., 305 (1972) 557.

121. J. Husser, J. Seffen and G.K. Wolf, Arzneim.-Forsch., 25 (1975) 1650.
122. G. Roma, A. Ermili and A. Balbi, Farmaco, Ed. Sci., 32 (1977) 81.
123. G. Roma, A. Balbi and A. Ermili, Farmaco, Ed. Sci., 32 (1977) 393.
124. G. Roma, E. Vigevani, A. Balbi and A. Ermili, Farmaco, Ed. Sci., 34 (1979) 62.
125. Z.F. Solomko, V.L. Pikalov, P.A. Sharbatyan and V.S. Tkachenko, Vopr. Khim. Khim. Tekhnol., 46 (1977) 12; Chem. Abstr., 88 (1978) 105280.
126. A. Ushirogochi, Y. Tominaga, Y. Matsuda and G. Kobayshi, Heterocycles, 14 (1980) 7.
127. A. Bauer, K.H. Weber, P. Danneberg and F.J. Kuhn, Ger. Offen. 2,318,673 (1974); Chem. Abstr., 82 (1974) 57747.
128. R.B. Moffett, B.V. Kamdar and P.F. von Voigtlander, J. Med. Chem., 19 (1976) 192.
129. B.S. Meldrum and R.W. Horton, Psychopharmacology, 60 (1979) 277.
130. A.W. Chow, R.J. Gyurik and R.C. Parish, J. Heterocycl. Chem., 13 (1976) 163.
131. T. Hara, H. Fujimori, Y. Kayama, T. Mori, K. Itoh and Y. Hashimoto, Chem. Pharm. Bull., 25 (1977) 2584.
132. G. Rackur and I. Hoffman, Eur. Pat. Appl. 24,038 (1981); Chem. Abstr. 95 (1981) 81034.
133. U. Golik, Tetrahedron Lett., (1975) 1327; J. Heterocycl. Chem., 12 (1975) 903.
134. R.I. Fryer and J.V. Earley, J. Heterocycl. Chem. 14 (1977) 1435.
135. P.C. Wade, T.P. Kissick, B.R. Vogt and B. Toeplitz, J. Org. Chem., 44 (1979) 84.
136. J. Körösi and T. Lang, Chem. Ber. 107 (1974) 3883.
137. H.L. Goldberg and R.J. Finnerty, Am. J. Psychiat., 136 (1979) 196.
138. H. Ohnishi, C. Ito, K. Suzuki, T. Niho, M. Shimora and K. Yamaguchi, Nippon Yakurigaku Zasshi, 78 (1981) 139; Chem. Abstr., 95 (1981) 180868.
139. V. Saano, A. Urtti and M.M. Airaksinen, Pharmacol. Res. Commun., 13 (1981) 75.
140. J.R. Boissier and P. Simon, Anesth. Analg. (Cleveland), 21 (1964) 455.
141. L. de Repentigny, G.K. Hanasono and G.L. Plaa, Can. J. Physiol. Pharmacol., 54 (1976) 671.
142. D.M. Chambers and G.C. Jefferson, Br. J. Pharmacol., 60 (1977) 393.
143. C. Stumpf, G. Gogolak, S. Huck and A. Andics, Anaesthesist, 25 (1976) 579.
144. D.M. Chambers, G.C. Jefferson and C.A. Ruddick, Eur. J. Pharmacol., 50 (1978) 103.
145. S. Hodesson, S.T. Rich, J.O. Washington and L. Apt., Lab. Animal Care, 15 (1965) 336.
146. L. Gyermek, Life Sci., 14 (1974) 1433.
147. C. Stumpf, G. Gogolak, S. Huck and A. Andics, Anaesthesist, 24 (1975) 264.
148. C. Stumpf, R. Jindra, S. Huck and H. Ewers, Anaesthesia, 28 (1979) 3.
149. W. Haefely, L. Pieri, P. Polc and R. Schaffner, in: Handbook of Experimental Pharmacology, Vol. 55/II, eds. F. Hoffmeister and G. Stille (Springer-Verlag, Berlin, 1981).
150. L.O. Randall, W. Schallek, G.A. Heise, E.F. Keith and R.E. Bagdon, J. Pharmacol. Exp. Ther., 129 (1960) 163.
151. E.A. Swinyard and A.W. Castellion, J. Pharmacol Exp. Ther., 151 (1966) 369.
152. J.E. Blum, W. Haefely, M. Jalfre, P. Polc and K. Schärer, Arzneim.-Forsch., 23 (1973) 377.
153. M.I. Gluckman, Curr. Ther. Res., 7 (1965) 721.

154. A.D. Rudzik, J.B. Hester, Jr., A.H. Tang, R.N. Straw and W. Friis, in: The Benzodiazepines, eds. S. Garattini, E. Mussini and L.O. Randall (Raven Press, New York, 1973) p. 285.
155. R. Nakajima, C. Hattori and Y. Nagawa, Jap. J. Pharmacol., 21 (1971) 489.
156. W. Schlosser, E. Zavatsky, E. Kappel and E.B. Zigg, Pharmacologist, 15 (1973) 162.
157. W. Haefely, A. Kulcsar, H. Möhler, L. Pieri, P. Polc and R. Schaffner, in: Mechanism of Action of Benzodiazepines, eds. E. Costa and P. Greengard (Raven Press, New York, 1975) p. 131.
158. R. Dingledine, L.L. Iversen and E. Breuker, Eur. J. Pharmacol., 47 (1978) 19.
159. W.E. Stone and M.J. David, Epilepsia, 19 (1978) 361.
160. W.D. Matthews and G.P. McCafferty, Neuropharmacology, 18 (1979) 885.
161. W.R. Buckett, Br. J. Pharmacol., 68 (1980) 177P.
162. C.C. Mao, A. Guidotti and E. Costa, Naunyn Schmiedebergs Arch. Pharmacol., 289 (1975) 369.
163. R.W. Horton, B.S. Meldrum, M.C.B. Sawaya and J.D. Stephenson, Eur. J. Pharmacol., 40 (1976) 101.
164. P. Soubrie and P. Simon, Neuropharmacology, 17 (1978) 121.
165. J.D. Wood, M.P. Russel, E. Kyrylo and J.D. Newstead, J. Neurochem., 33 (1979) 61.
166. V.V. Markovich and R.U. Ostrovskaya, Byull. Eksp. Biol. Med. U.S.S.R., 84 (1977) 1429.
167. E.A. Swinyard, in: Experimental Models of Epilepsy, eds. D.P. Purpura, J.K. Penry, D.B. Tower, D.M. Woodbury and R.D. Walter (Raven Press, New York, 1972) p. 433.
168. C.J.E. Niemegeers and P.J. Lewi, in: Industrial Pharmacology III, eds. S. Fielding and H. Lal (Futura Publishing Co., New York, 1979) p. 141.
169. J.F. Reinhard and J.F. Reinhard, Jr., in: Anticonvulsants, ed. J.A. Vida (Academic Press, New York, 1977) p. 57.
170. R. Racine, K. Livingston and A. Joaquin, Electroenceph. Clin. Neurophysiol., 38 (1975) 355.
171. R. Racine, W.M. Burnham and K. Livingston, Electroenceph. Clin. Neurophysiol., 47 (1979) 204.
172. D. Ashton and A. Wauquier, Psychopharmacology, 65 (1979) 7.
173. T.E. Albertson, S.L. Peterson and L.G. Stark, Neuropharmacology, 19 (1980) 643.
174. J.C. Millichap, Epilepsia, 10 (1969) 315.
175. L.O. Randall and B. Kappel, in Ref. 154, p. 27.
176. S.N. Pradhan and N.N. De, Br. J. Pharmacol., 8 (1953) 399.
177. S. Courvoisier, J. Clin. Exp. Psychopath., 17 (1956) 25.
178. N.W. Dunham and T.S. Miya, J. Am. Pharm. Assoc., 46 (1957) 208.
179. J.R. Boissier, J. Tardy and J.C. Diverres, Med. Exp., 3 (1960) 81.
180. T.W. Robbins, in: Handbook of Psychopharmacology, eds. L.L. Iversen, S.D. Iversen and S.H. Snyder (Plenum Press, New York) 7 (1977) 37.
181. J.R. Boissier and P. Simon, Therapie, 17 (1962) 1225.
182. N.A. Nolan and M.W. Parkes, Psychopharmacologia, 29 (1978) 277.
183. S.D. Iversen, Arzneim.-Forsch., 30 (1980) 862.
184. I. Geller and J. Seifter, Psychopharmacologia, 1 (1960) 482.
185. I. Geller, J.T. Kulak and J. Seifter, Psychopharmacologia, 3 (1962) 374.

186. D.L. Margules and L. Stein, Psychopharmacologia, 13 (1968) 74.
187. J.B. Vogel, B. Beer and D.E. Clody, Psychopharmacologia, 21 (1971) 1.
188. S.E. File and J.R.G. Hyde, Br. J. Pharmacol., 62 (1978) 19.
189. J.A. Gray, in Ref. 180, Vol. 8, p. 433.
190. K.E. Moyer, Commun. Behav. Biol., 2 (1968) 65.
191. L.O. Randall, G.A. Heise, W. Schallek, R.E. Bagdon, R. Banziger, A. Boris, R.A. Moe and W.B. Abrams, Curr. Ther. Res., 3 (1961) 405.
192. L.L. Scheckel and E. Boff, in: Use of Non-human Primates in Drug Evaluation, ed. H. Vagtborg (University of Texas Press, Austin, 1968) p. 301.
193. R.E. Tedeschi, D.H. Tedeschi, A. Mucha, L. Cook, P.A. Mattic and E.J. Fellows, J. Pharm. Exp. Ther., 125 (1959) 28.
194. D.H.Tedeschi, P.J. Fowler, R.B. Miller and E. Macko, in: Aggressive Behaviour, eds. S. Garattini and E.B. Sigg (Excerpta Medica, Amsterdam, 1969) p. 245.
195. L.H. Sternbach, L.O. Randall and S.R. Gustafson, Psychopharmacol. Agents, 1 (1964) 137.
196. J.B. Malick, Pharmacol. Biochem. Behav., 8 (1978) 497.
197. B.L. Baxter, Life Sci., 3 (1964) 531.
198. J.B. Malick, Arch. Int. Pharmacodyn., 186 (1970) 137.
199. W.H. Funderburk, M.H. Foxwell and M.W. Hakala, Neuropharmacology, 9 (1970) 1.
200. J.B. Malick, R.D. Sofia and M.E. Goldberg, Arch. Int. Pharmacodyn. 181 (1969) 459.
201. H. Schutz, Benzodiazepines: A Handbook (Springer Verlag, Berlin, 1982) p. 4.
202. J.H. Kanto, R. Sellman, M. Haataja and P. Hurme, Int. J. Clin. Pharmacol. Biopharm., 16 (1978) 258.
203. G.K. Woo, S.J. Kolis and M.A. Schwartz, Pharmacologist, 19 (1977) 164.
204. H. Kitagawa, Y. Esumi, S. Kurosawa, S. Sekine and T. Yokoshima, Xenobiotica, 9 (1979) 415.
205. M. Gall, J.B. Hester, Jr., A.D. Rudzik and R.A. Lahti, J. Med. Chem., 19 (1976) 1057.
206. M. Gall, R.A. Lahti, A.D. Rudzik, D.J. Duchamp, C. Chidester and T. Scahill, J. Med. Chem., 21 (1978) 542.
207. K. Hirai, T. Ishiba, H. Sugimoto, K. Sasakura, T. Fujishita, T. Toyoda, H. Joyama, H. Hatakeyama and K. Hirose, J. Med. Chem., 23 (1980) 764.
208. K. Hirai, T. Ishiba, H. Sugimoto, T. Fujishita, Y. Tsukinoki and K. Hirose, J. Med. Chem., 24 (1981) 20.
209. R.A. Lahti and M. Gall, J. Med. Chem., 19 (1976) 1064.
210. H. Schutz in Ref. 201, p. 205.
211. R.I. Shader and D.J. Greenblat, Am. J. Psychiat., 134 (1977) 652.
212. S.A. Kaplan, M.L. Jack, K. Alexander and R.E. Weinfield, J. Pharm. Sci., 62 (1973) 1789.
213. C.M. Metzler, H. Ko, M.E. Royer, W. Veldkamp and O.I. Linet, Clin. Pharm. Ther., 21 (1977) 111.
214. T.R. Browne, N. Engl. J. Med., 299 (1978) 812.
215. R.S. Warner, Psychosomatics, 6 (1965) 347.
216. S.E. File, Psychopharmacology, 73 (1981) 240.
217. A.N. Nicholson, Practitioner, 223 (1979) 479.

218. A.N. Nicholson, B.M. Stone and P.A. Pascoe, Br. J. Clin. Pharmacol., 10 (1980) 459.
219. D.J. Greenblatt and R.I. Shader, Benzodiazepines in Clinical Practice (Raven Press, New York, 1974).
220. T.A. Ban, W.T. Brown, T. Da Silva, M.A. Gagnon, C.T. Lamont, H.E. Lemann, F.W. Lowy, J. Ruedy and E.M. Sellers, Can. Pharm. J., (1981) 301.
221. J. Kanto, E. Iisalo, V. Lehtinen and J. Salminen, Psychopharmacologia, 36 (1974) 123.
222. J. Marks, The Benzodiazepines: Use, Overuse, Misuse and Abuse (MTP, Lancaster, 1978).
223. N.M. Davis, S. Brookes, J.A. Gray and J.N.P. Rawlins, Q.J. Exp. Psychol., 33B (1981) 227.
224. J.A. Gray, personal communication.
225. R.F. Schmidt, E. Vogel and M. Zimmermann, Naunyn Schmiedebergs Arch. Pharmacol., 258 (1967) 69.
226. P. Polc, H. Möhler and W. Haefely, Naunyn Schmiedebergs Arch. Pharmacol., 284 (1974) 319.
227. W.E. Haefely, Br. J. Psychiatr., 133 (1978) 231.
228. E. Costa, A. Guidotti and G. Toffano, Br. J. Psychiatr. 133 (1978) 239.
229. R.F. Squires and C. Braestrup, Nature (London), 266 (1977) 732.
230. H. Möhler and T. Okada, Science, 198 (1977) 849.
231. S.H. Snyder and J.P. Bennett, Annu. Rev. Physiol., 38 (1976) 153.
232. H. Möhler and T. Okada, Br. J. Psychiatr., 133 (1978) 261.
233. R.C. Speth, R.W. Johnson, J. Regan, T. Reisine, R.M. Kobayashi, N. Bresolin, W.R. Roeske and H.I. Yamamura, Fed. Proc., 39 (1980) 3032.
234. C. Braestrup, M. Nielsen, G. Biggio and R.F. Squires, Neurosci. Lett., 13 (1979) 219.
235. C.R. Mackerer, R.L. Kochman, B.A. Bierschenk and S.S. Bremner, J. Pharmacol. Exp. Ther. 206 (1978) 405.
236. C. Braestrup, C. Nissen, R.F. Squires and A. Schousboe, Neurosci. Lett., 9 (1978) 45.
237. J.P. Fry and S. McHanwell, J. Physiol., 325 (1982) 32P.
238. W.E. Müller, U. Schläfer and U. Wollert, Neurosci. Lett., 9 (1978) 239.
239. C. Braestrup and R.F. Squires, Br. J. Psychiatr., 133 (1978) 249.
240. C. Braestrup, R. Albrechtsen and R.F. Squires, Nature (London), 269 (1977) 702.
241. H. Möhler, T. Okada, J. Ulrich and P. Heitz, Life Sci., 22 (1978) 985.
242. R.C. Speth, C.J. Wastek, P.C. Johnson and H.I. Yamamura, Life Sci., 22 (1978) 859.
243. R.F. Squires, D.I. Benson, C. Braestrup, J. Coupet, C.A. Klepner, V. Myers and B. Beer, Pharmacol. Biochem. Behav., 10 (1979) 825.
244. M. Nielsen and C. Braestrup, Nature (London), 286 (1980) 606.
245. A.S. Lippa, D.J. Critchett, M.C. Sano, C.A. Klepner, F.N. Greenblatt, J. Coupet and B. Beer, Pharmacol. Biochem. Behav., 10 (1979) 831.
246. U. Quast and H. Mahlmann, Eur. Soc. Neurochem., Abstr. (1981) S189.
247. T.H. Chiu, D.M. Dryden and H.C. Rosenberg, Mol. Pharmacol. 21 (1982) 57.
248. A. Doble, L.L. Iversen and I.L. Martin, Br. J. Pharmacol., 75 (1982) 42P.
249. I.L. Martin and A. Doble, J. Neurochem., submitted.

250. J.D. Hirsch, R.L. Kochman and P.R. Sumner, Mol. Pharmacol., 21 (1982) 618.
251. C.L. Brown and I.L. Martin, Br. J. Pharmacol., in press.
252. C. Braestrup and M. Nielsen, Brain Res., 147 (1978) 170.
253. J.M. Candy and I.L. Martin, J. Neurochem., 32 (1979) 655.
254. J.F. Tallman, J.W. Thomas and D.W. Gallager, Nature (London), 274 (1978) 383.
255. G.J. Wastek, R.C. Speth, T.D. Reisine and H.I. Yamamura, Eur. J. Pharmacol., 50 (1978) 445.
256. I.L. Martin and J.M. Candy, Neuropharmacology, 17 (1978) 993.
257. M. Karobath and G. Sperk, Proc. Natl. Acad. Sci. U.S.A., 76 (1979) 1004.
258. T. Costa, D. Rodbard and C. Pert, Nature (London), 277 (1979) 315.
259. J.M. Candy and I.L. Martin, Nature (London), 280 (1979) 172.
260. T. Costa, L. Russell, C.B. Pert and D. Rodbard, Mol. Pharmacol., 20 (1981) 470.
261. R.E. Study and J.L. Barker, Proc. Natl. Acad. Sci. U.S.A., 78 (1981) 7180.
262. J. Hughes, T.W. Smith, H.W. Kosterlitz, L.A. Fothergill and H.R. Morris, Nature (London), 258 (1975) 577.
263. M. Massotti and A. Guidotti, Life Sci., 27 (1980) 847.
264. A. Guidotti, B. Ebstein and E. Costa, Soc. Neurosci. Abstr. (1981) 634.
265. L.G. Davies and R.K. Cohen, Biochem. Biophys. Res. Commun., 92 (1980) 141.
266. J.H. Woolf and J.C. Nixon, Biochemistry, 20 (1981) 4263.
267. W. Hunkeler, H. Möhler, L. Pieri, P. Polc, E.P. Bonetti, R. Cumin, R. Schaffner and W. Haefely, Nature (London), 290 (1981) 514.
268. A.J. Czernik, B. Petrack, C. Tsai, R.F. Granat, R.K. Rinehart, H.J. Kalinsky, R.A. Lovell and W.D. Cash, Pharmacologist, 23 (1981) 160.
269. A.J. Czernik, B. Petrack, H.J. Kalinsky, S. Psychoyos, W.D. Cash, C. Tsai, R.K. Rinehart, F.R. Granat, R.A. Lovell, D.E. Brundish and R. Wade, Life Sci., 30 (1982) 363.
270. L.H. Sternbach, L.O. Randall, R. Banziger and H. Lehr, in: Drugs Affecting the Central Nervous System, Vol. 2, ed. A. Burger (Marcel Dekker, New York, 1968) p. 237.
271. P. Linscheid and J.M. Lehn, Bull. Soc. Chim. Fr., (1967) 992.
272. J. Karle and I.L. Karle, J. Am. Chem. Soc., 89 (1967) 804.
273. T. Blair and G.A. Webb, J. Med. Chem., 20 (1977) 1206.
274. J.E. Anderson, Q. Rev. Chem. Soc., 19 (1965) 426.
275. P. Nuhn and W. Bley, Pharmazie, 22 (1967) 532.
276. W. Bley, P. Nuhn and G. Benndorf, Arch. Pharm., 301 (1968) 444.
277. M. Sarrazin, M. Bourdeaux-Pontier, C. Briand and E.J. Vincent, Org. Magn. Reson., 7 (1975) 89.
278. W. Sadée, Arch. Pharm., 302 (1969) 769.
279. M. Raban, E.H. Carlson, J. Szmuszkovicz, G. Slomp, C.G. Chidester and D.J. Duchamp, Tetrahedron Lett. (1975) 139.
280. G. Romeo, M.C. Aversa, P. Giannetto, M.G. Vigorita and P. Ficarra, Org. Magn. Reson., 12 (1979) 593.
281. M.C. Aversa, P. Giannetto, G. Romeo, P. Ficarra and M.G. Vigorita, Chim. Ind. (Milan), 61 (1979) 155.
282. G. Romeo, M.C. Aversa, P. Giannetto, P. Ficarra and M.G. Vigorita, Org. Magn. Reson., 15 (1981) 33.
283. G. Gilli, V. Bertolasi, M. Sacerdoti and P.A. Borea, Acta Crystallogr., B34 (1978) 3793.

284. P. Chananont, T.A. Hamor and I.L. Martin, Acta Crystallogr., B36 (1980) 898.
285. M.C. Aversa, G. Romeo, P. Giannetto, P. Ficarra and M.G. Vigorita, J. Heterocycl. Chem., 17 (1980) 551.
286. M.C. Aversa, P. Giannetto, G. Romeo, P. Ficarra and M.G. Vigorita, Org. Magn. Reson., 15 (1981) 394.
287. R. Haran and J.P. Tuchagues, J. Heterocycl. Chem., 17 (1980) 1483.
288. M. Sarrazin, R. Faure, C. Aubert and E.J. Vincent, J. Chim. Phys. Phys.-Chim. Biol., 77 (1980) 91.
289. A. Patra, A.K. Mukhopadhyay, A.K. Mitra and A.K. Acharyya, Org. Magn. Reson., 15 (1981) 99.
290. K.A. Kovar, D. Linden and E. Breitmaier, Arch. Pharm., 314 (1981) 186.
291. S.P. Singh, S.S. Parmar, S.A. Farnum and V.I. Stenberg, J. Heterocycl. Chem., 15 (1978) 1083.
292. V. Šunjić, A. Lisini, A. Sega, T. Kovač, F. Kajfež and B. Ruscić, J. Heterocycl. Chem., 16 (1979) 757.
293. W. Sadée, H.J. Schwandt and K.H. Beyer, Arch. Pharm., 306 (1973) 751.
294. H.H. Paul, H. Sapper, W. Lohmann and H.O. Kalinowski, Org. Magn. Reson., 19 (1982) 49.
295. A. Camerman and N. Camerman, J. Am. Chem. Soc., 94 (1972) 268.
296. G. Brachtel and M. Jansen, Cryst. Struct. Commun., 10 (1981) 669.
297. W.D.S. Motherwell, PLUTO 78. Program for plotting molecular and crystal structures. Report 'Cambridge Crystallographic Files', University of Manchester Regional Computer Centre, (Manchester, 1981).
298. G. Gilli, V. Bertolasi, M. Sacerdoti and P.A. Borea, Acta Crystallogr., B34 (1978) 2826.
299. G. Bandoli and D.A. Clemente, J. Chem. Soc., Perkin 2, (1976) 413.
300. Z. Galdecki and M.L. Glowka, Acta Crystallogr., B36 (1980) 3044.
301. G. Gilli, V. Bertolasi, M. Sacerdoti and P.A. Borea, Acta Crystallogr., B33 (1977) 2664.
302. P. Chananont, T.A. Hamor and I.L. Martin, Cryst. Struct. Commun. 8 (1979) 393.
303. P. Chananont, T.A. Hamor and I.L. Martin, Acta Crystallogr., B37 (1981) 1371.
304. A.A. Karapetyan, V.G. Andrianov, Y.T. Struchkov, A.V. Bogatskii, S.A. Andronati and T.I. Korotenko, Bioorg. Khim., 5 (1979) 1684.
305. R.F. Dunphy and H. Lynton, Can. J. Chem., 49 (1971) 3401.
306. L.H. Sternbach, F.D. Sancilio and J.F. Blount, J. Med. Chem., 17 (1974) 374.
307. R.I. Fryer, J.V. Earley and J.F. Blount, J. Org. Chem., 42 (1977) 2212.
308. P. Chananont, T.A. Hamor and I.L. Martin, Acta Crystallogr., B36 (1980) 2115.
309. W.L. Duax, C.M. Weeks and D.C. Rohrer, Topics Stereochem., 9 (1976) 271.
310. C. Hernstadt, D. Mootz, H. Wunderlich and H. Mohrle, J. Chem. Soc., Perkin 2 (1979) 735.
311. P. Chananont, T.A. Hamor and I.L. Martin, Acta Crystallogr., B36 (1980) 1238.
312. M. Czugler, A. Kalman, J. Rohricht, M. Low, L. Urogdi and L. Kisfaludy, Tetrahedron Lett., (1977) 917.
313. P. Chananont, T.A. Hamor and I.L. Martin, Acta Crystallogr., B36 (1980) 1690.
314. K. Kamiya, Y. Wada and M. Nishikawa, Chem. Pharm. Bull., 21 (1973) 1520.
315. J.B. Hester, Jr., D.J. Duchamp and C.G. Chidester, Tetrahedron Lett., (1971) 1609.

316. S. Sato, N. Sakurai, T. Miyadera, C. Tamura and R. Tachikawa, Chem. Pharm. Bull., 19 (1971) 2501.
317. R. Jaunin, W.E. Oberhänsli and J. Hellerbach, Helv. Chim. Acta, 55 (1972) 2975.
318. R. Heckendorn and A.R. Gagneux, Helv. Chim. Acta, 61 (1978) 848.
319. D. Mastropaolo, A. Camerman, N. Camerman and L. Chan, Abstr., Am. Cryst. Assoc. Vol. 7, No. 1 (1979) 22.
320. R.O. Gould and S.E.B. Gould, J. Chem. Soc., Perkin 2, (1974) 1075.
321. J.C. Speakman and F.B. Wilson, Acta Crystallogr., B32 (1976) 622.
322. J. Galloy, J.P. de Clercq and M. van Meerssche, Cryst. Struct. Commun., 8 (1979) 981.
323. W. Müller and U. Wollert, Naunyn Schmiedebergs Arch. Pharmacol., 278 (1973) 301.
324. W. Müller and U. Wollert, Naunyn Schmiedebergs Arch. Pharmacol., 280 (1973) 229.
325. W. Müller and U. Wollert, Naunyn Schmiedebergs Arch. Pharmacol., 283 (1974) 67.
326. W. Müller and U. Wollert, Res. Commun. Chem. Pathol. Pharmacol., 9 (1974) 413.
327. W. Müller and U. Wollert, Mol. Pharmacol., 11 (1975) 52.
328. I. Sjöholm and T. Sjödin, Biochem. Pharmacol., 21 (1972) 3041.
329. T. Sjödin, N. Roosdorp and I. Sjöholm, Bichem. Pharmacol., 25 (1976) 2131.
330. T. Alebić-Kolbah, F. Kajfež, S. Rendić, V. Šunjić, A. Konowal and G. Snatzke, Biochem. Pharmacol., 28 (1979) 2457.
331. A. Konowal, G. Snatzke, T. Alebić-Kolbah, F. Kajfež, S. Rendić and V. Šunjić, Biochem. Pharmacol., 28 (1979) 3109.
332. M. Sarrazin, J.C. Sari, M. Bourdeaux-Pontier and C. Briand, Mol. Pharmacol., 15 (1979) 71.
333. M. Sarrazin, M. Bourdeaux-Pontier and C. Briand, Ann. Phys. Biol. Med., 9 (1976) 211.
334. R.W. Lucek and C.B. Coutinho, Mol. Pharmacol., 12 (1976) 612.
335. V. Šunjić, J. Kuftineć and F. Kajfež, Arzneim.-Forsch., 25 (1975) 340.
336. H.H. Paul, H. Sapper and W. Lohmann, Z. Naturforsch., 33c (1978) 870.
337. H.H. Paul, H. Sapper and W. Lohmann, Biochem. Pharmacol., 29 (1980) 137.
338. R.W. Lucek, W.A. Garland and W. Dairman, Fed. Proc., 38 (1979) 541.
339. P. Chananont, T.A. Hamor, I.L. Martin and H. Tate, unpublished data.
339a. G. Gilli, P.A. Borea, V. Bertolasi and M. Sacerdoti, Abstr., Eur. Cryst. Meet., 4 (1977) 38.
340. M. Yoshimoto, T. Kamioka, T. Miyadera, S. Kobayashi, H. Takagi and R. Tachikawa, Chem. Pharm. Bull., 25 (1977) 1378.
341. G.L. Biagi, A.M. Barbaro, M.C. Guerra, M. Babbini, M. Gaiardi and M. Bartoletti, J. Med. Chem., 23 (1980) 193.
342. M. Babbini, M. Gaiardi and M. Bartoletti, Life Sci., 25 (1979) 15.
343. G.M. Crippen, Mol. Pharmacol., 22 (1982) 11.
344. A. Camerman and N. Camerman, in: Antiepileptic Drugs: Mechanisms of Action, eds. G.H. Glaser, J.K. Penry and D.M. Woodbury (Raven Press, New York, 1980) p. 223.
345. A. Camerman and N. Camerman, Acta Crystallogr., B37 (1981) 1677.
346. R.W. Olsen, J. Neurochem., 37 (1981) 1.
347. R.A. O'Brien and N.M. Spirt, Life Sci., 26 (1980) 1441.

348. T.J. Franklin, Biochem. Pharmacol., 29 (1980) 853.
349. F.H. Allen, S. Bellard, M.D. Brice, B.A. Cartwright, A. Doubleday, H. Higgs, T. Hummelink, B.G. Hummelink-Peters, O. Kennard, W.D.S. Motherwell, J.R. Rodgers and D.G. Watson, Acta Crystallogr., B35 (1979) 2331.

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5 The Present Status of Chelating Agents in Medicine

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INTRODUCTION

Chelating agents have a curious place in medical science. Although the clinical need to treat the toxic effects of metal ions is relatively rare, awareness and knowledge of the subject is widespread. This is because of the ubiquitious involvement of metal ions in biological systems and their fundamental role in biochemistry. So the physiological consequences of administering compounds which alter their distribution *in vivo* touch upon an extraordinarily diverse

variety of scientific interests. In addition to nutritionists attempting to ensure adequate dietary supplies of essential elements, toxicologists trying to prevent or treat the harmful effects of metals, and physicians using radionuclides as a diagnostic aid, there are many with a less direct but nevertheless pertinent concern with chelating agents.

From a long list, it is worthwhile considering a few examples. First, because it is probably the most widely recognised inorganic aspect of medicine, one might mention that the role of iron in oxygen-transport by the blood gives rise to a multitude of disorders, the treatment of which ultimately necessitates chelation therapy to reduce iron stores. This involves many clinical disciplines. Secondly, there is the less direct example provided by Wilson's disease: this rare complication of copper metabolism has implications for geneticists, neurologists and ophthalmologists as well as those who are primarily concerned with the molecular mechanisms of the disease and its treatment. Finally, as a good illustration of the way heavy metal ions (and consequently chelating agents) may command very broad medical attention, there is the recently renewed debate about the sociological and psychological impact of lead from petrol.

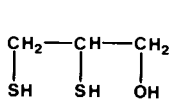
The fact that so many medical specializations have an interest in trace elements is a profound reflection on their integrated and intrinsic participation in the chemical processes of life. There can thus be little doubt that medical opportunities of great promise await the development of methods to manipulate therapeutically the concentrations of metal ions *in vivo*. There is already much evidence that such approaches will be fruitful in the fight against many different pathologies, some of which are very common indeed. For example, the effect of copper in reducing the inflammation associated with rheumatoid arthritis [1, 2], the role of trace elements in the mechanisms of the immune response system [3], their involvement in the action of certain antibacterial and antiviral drugs [4], the suggestion that surplus iron may be a fundamental cause of heart disease [5] and that metal complexes may be used to control infections [6] and cancerous growth [7] are all matters of topical research interest.

Whilst it seems likely that such approaches to inorganic medicinal chemistry will attract increasing attention, research into conventional chelation therapy will not correspondingly diminish. On the contrary, the need for reliable experimental data and a sound theoretical explanation of how administered agents alter metal ion distributions *in vivo* will become more imperative than ever. So, although the present review is largely concerned with methods of treating heavy metal intoxication, many of the ideas discussed are relevant to the broader aspects outlined above. A main objective is to establish the com-

mon ground between different approaches to chelation therapy rather than to be over-concerned with the details of individual metal toxicity and its treatment. A second objective is to examine some other medical applications of chelating agents which have become prominent in the last few years.

CHEMICAL PRINCIPLES

Almost without exception, the chelating agents in general clinical use today had been introduced by the mid-1960's. The first was 2,3-dimercaptopropanol (BAL, 1) which was developed during the Second World War [8] to treat arsenical gas poisoning. Next, ethylenediaminetetraacetic acid (EDTA, 2), which had been patented in 1935 by Munz, was administered to humans to remove lead some 15 years later [9]. Then, through the 1950's and the early part of the next decade, there followed a period of intense research into the properties of chelating agents from a chemical and physiological point of view.



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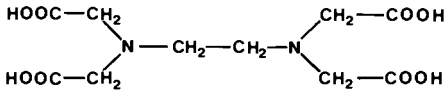

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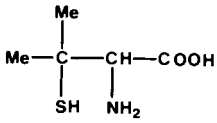

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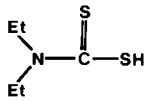

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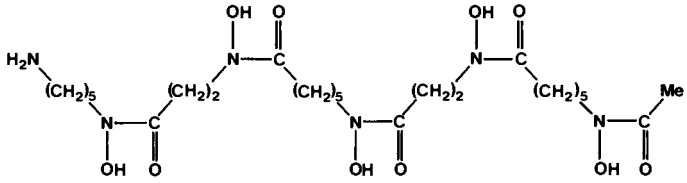

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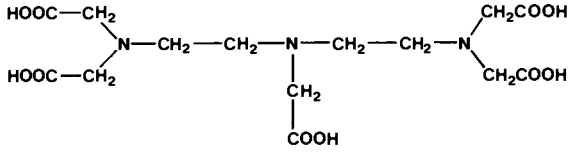

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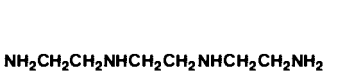
This resulted in the introduction of penicillamine (PEN, 3) by Walshe in 1954 [10], the identification of diethyldithiocarbamic acid (DDC, 4) as a specific treatment for nickel carbonyl poisoning in 1958 [11] and the discovery of desferrioxamine (DFOA, 5) as the iron complex in 1960 [12]. During this period, diethylenetriaminepentaacetic acid (DTPA, 6) was also established as a possible alternative to EDTA, particularly for radionuclide decorporation [13].

The increased scientific understanding of chelation therapy which this activity produced naturally led to a widespread mood of optimism. It must have seemed that, if the rapid progress which was being made simply continued, there would soon be available both drugs and therapeutic regimens of such efficacy that most of the difficulties then associated with the treatment of heavy metal intoxication would be vanquished. Perhaps it is not surprising that Utopia failed to materialize, but it is, nevertheless, a most striking fact that an extraordinary decline in the rate of progress occurred. With the exception of 2,2,2-triethylenetetramine (TRIEN, 7) [14] and 2,3-dimercaptopropanesulphonic acid (DMPS, 8) [15], both of which have yet to be established as drugs of choice, no new chelating agents have been accepted into the clinic (or even seem likely to be) for nearly 20 years.



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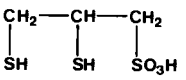

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There are possibly a number of reasons for this. However, it has certainly not been through any lack of effort: the substantial literature which exists on the subject and which continues to be produced, testifies to the great endeavour that has in fact been made. For example, in 1972 the United States Congress specifically authorized funds of over 5,000,000 dollars for research into Cooley's anaemia, the major priority being identified as the development of more effective iron-chelating agents to offset transfusional iron overload [16, 17]. Yet, the clinical advances achieved by this and other recent programmes have only been of a methodological nature.

Neither can the lack of progress be due to insufficient appreciation of the problem. The ideal properties of chelating agents are well known and are listed in almost every review of the subject. These principles were, in fact, clearly defined by the early pioneers but little further insight has accrued since.

In chelation therapy, it is desirable to administer selective agents which bind the target metal ion very powerfully whilst not interfering with the

metabolism of vulnerable essential elements. It is also clear that an ideal agent should penetrate into the body compartment where the offending metal ion has been largely deposited. In addition, the drug must be cheap, non-toxic, resistant to metabolic degradation and conveniently (i.e., orally) administered. Using these criteria, most compounds which are chemically capable of binding metal ions are readily eliminated as possible therapeutic agents. Moreover, none of the present medical chelators satisfies all these demanding conditions. It seems that a much greater understanding of the complexities of metal binding in a biological context is needed to break the present deadlock.

LIGANDS, COMPLEXES AND CHELATING AGENTS

A complex is formed by association between a metal atom or ion and a ligand – a species which may be a polar molecule or an anion that donates electrons to the metal centre. In general, metal complexes are stable and independent molecular entities which can be characterized in solution and/or the solid state.

The concept of complexation of metal ions was introduced by Werner in 1893 [18]. He described the bonding in terms of two valences, a 'principal' valency, now termed the oxidation number or oxidation state, and an 'auxiliary' valency, now termed the co-ordination number. Co-ordination numbers are simply the number of donor groups attached to the central metal ion of the complex. Cations in lower oxidation states (+1, +2, +3) commonly display co-ordination numbers of 4 or 6, but any value from 2 to 12 can occur.

The term 'chelate', from the Greek word *chele* (meaning crab's claw) was introduced in 1920 [19] to describe those complexes in which the ligand bonds through at least two donor groups so that a ring system is formed. Ligands which have the potential to form such rings are called chelating agents. They are often associated with additional complex stability, particularly when the rings are five-membered [20]. The word denticity is used to describe how many donor groups the chelating agent has available for metal ion binding; many of the clinically important chelating agents are multidentate, meaning 'many-toothed'.

Whilst all the metals can form complexes and the number of complexing agents is extensive, the atoms which are usually found to donate electrons to the metal are relatively few. For the most part, they are confined to the elements of Groups V and VI in the Periodic Table, with nitrogen, oxygen and sulphur being the best examples. The nature of, and the reasons for, the specific interactions between metals and these donor atoms is discussed subsequently in some detail.

In aqueous solution, metal ions by themselves are always highly solvated.

Hydrolysis occurs when the co-ordinated water molecules successively lose protons by dissociation. The effect is to replace bound water by hydroxyl ions, leading to a concomitant reduction in the electrical charge on the cation. When the charge has been reduced to zero, the neutral metal hydroxide tends to precipitate. This may be prevented in the presence of stronger complexing agents which are able to displace water or hydroxide as the ligand.

The ability of chelating agents to compete for aquated metal ions in this way may be expressed quantitatively as the equilibrium constant for the reaction of the metal ion, M^{m+}, and the ligand, L^{n-}, to form the complex, $ML^{(m-n)+}$. In general, the true thermodynamic equilibrium constant (applicable at infinite dilution of the reacting species) is not easily determined and most investigators report a stoichiometric or concentration constant, K, where

$$K = \frac{[ML^{(m-n)+}]}{[M^{m+}][L^{n-}]}$$

These practical constants incorporate activity coefficients which are less than unity and generally held invariant during experiments by the addition of high, constant concentrations of background electrolyte(s). Sodium perchlorate and potassium nitrate are the two which are most commonly employed because they are the least likely to interact with the metal and ligand under investigation [21]. This inertness facilitates thermodynamic interpretations of the equilibrium constants, but is of less value when the purpose is to calculate complex concentrations in biological fluids [22]. For this reason, an increasing number of investigators now employ sodium chloride as the background electrolyte in formation constant determinations.

Whenever the co-ordination number of a metal ion is at least twice the effective denticity of the ligand, an ML_2 type complex may be formed. Such step-wise co-ordination often extends to other stoichiometries such as ML_3 and ML_4. In these cases, it is convenient to define the equilibria in terms of overall formation constants, β, which relate the concentration of each species to the free concentration of its components raised to some appropriate power. Omitting charges for the sake of clarity, this can be written as

$$[ML] = \beta_1[M][L]$$
$$[ML_2] = \beta_2[M][L]^2$$
$$[ML_3] = \beta_3[M][L]^3 \text{ etc.}$$

Note that $\beta_1 = K$ (the first stepwise equilibrium constant).

In fact, relatively few metal-ligand systems confine themselves solely to the formation of these ML, ML_2 and ML_3 complexes. Most include other species such as the protonated complex MLH and the hydroxy complex ML(OH). In addition, the formation of polynuclear complexes of the type M_2L_2 or M_2L_3 is commonplace. The recognition of complexes other than the simple, stepwise species is fundamental to modern approaches in aqueous inorganic chemistry.

Accordingly, it is necessary to formulate the equilibrium concentration of various complex species as a general expression such as

$$S_j = \beta_j \Pi_i X_i^{k(i,j)}$$

where S_j is the concentration of complex species, j, β_j is the corresponding formation constant, X_i is the free concentration of the ith component and $k(i, J)$ is the stoichiometric coefficient of component i in complex j.

It is important to appreciate that the formation constant, β_j, should not be used as a direct indication of the extent to which the metal-ligand complex exists in aqueous solution. The comparison of formation constants is a very common error occurring in the literature. How misleading it can be is shown by the many spurious examples used to support the concept of the 'chelate effect' [23]. This effect refers to the enhancement of complex stability which is supposed to be conferred by the formation of chelate rings. Whilst there is no doubt that the ring closure which completes the co-ordination of, say, a bidentate ligand such as ethylenediamine makes a significant contribution to the complex's stability, it is fallacious to illustrate this by comparing its formation constant with that of the corresponding unidentate ligand (which, in this case, is ammonia). For one thing, the formation constants so compared often have different units. This mistake is made even in the most respected of textbooks [24].

While formation constants do reflect an intrinsic strength of metal-ligand binding, many other factors contribute towards the final distribution of complexes. In aqueous solution, the equilibria always involve some competition with hydroxide ions, as has already been mentioned. Even more important in most cases, is the fact that most complexing reactions involve the displacement of protons from the ligand by the metal ion. Both of these considerations make complex distributions generally dependent on the pH of the solution. It is possible to judge the effect of all the competing interactions only in the very simplest and restricted of circumstances.

In general, the overall outcome of equilibrium in such multicomponent systems must be determined mathematically. This is done by solving the set

of so-called mass balance equations in which the total concentration of each component is made up of the sum of contributions from each of the constituent species [25]. Thus,

$$T_i = X_i + \sum_j S_j k(i,j)$$

where T_i is the total concentration of the ith component.

By substituting for the concentration of each complex species, S_j, these mass balance relationships can be expanded into a set of non-linear equations expressing each total concentration in terms of the free concentrations of each component. Hence, one obtains a set of n equations in n unknowns, where n is the number of components.

For many years, the non-linear nature of such systems made the general solution of these equations difficult. However, with the advent of high-speed computers, numerical techniques appeared which tackle the problem by successive improvement of some initial estimate of the solution. A number of computer programmes which do this in the context of entirely generalized equilibria are now available [26]. So, with the growing availability of computing facilities, the mathematical part of the problem is no longer much of an obstacle.

In consequence, exact speciation distributions can be calculated for any multicomponent system provided only that the formation constants of all the complexes occurring in solution are well characterized. Since comprehensive tabulations of formation constants have been compiled [27, 28], it is possible to carry out such calculations for many thousands of binary and ternary combinations of metal and ligand. This forms the basis of the subsequent discussion on computer simulation models of metal complex distributions in biological fluids.

SELECTIVITY

Improved selectivity has probably been the objective pursued most ardently in the quest for better chelating agents. There are two evident disadvantages with compounds such as EDTA which strongly sequester a variety of metal ions. There is, of course, the danger that side-effects caused by interference with the metabolism of essential trace elements may limit their usefulness [29]. In addition, any interaction with metals that occur in relative abundance *in vivo* (e.g., calcium ions) will clearly act to suppress their avidity for toxic target ions [30].

The chemical basis for designing selective chelating agents is now well estab-

lished. The factors which favour co-ordination of one metal ion over another are straightforward and so here it is necessary to present only a brief summary.

In 1958, Ahrland, Chatt and Davies [31] showed that there is a characteristic variation in the stability of complexes which depends on where the metal and ligand are located in the Periodic Table. The majority of metals in their common oxidation states form their most stable complexes with ligands at the head of Groups V, VI and VII (i.e., nitrogen, oxygen and fluorine). The alkali and alkali earth metals, the earlier transition elements and zinc belong to this group. On the other hand, a number of other metals do not. They include the transition elements from Group VI onwards, cadmium, mercury and the heavy metals, thallium, lead, bismuth and polonium. The latter group form their most stable complexes when the ligand donor atoms come from the second row in the Periodic Table (i.e. phosphorus, sulphur or chlorine). It is also noteworthy that the first set of metals displays an order of affinity for the halide ions ($F \gg Cl > Br > I$) which is reversed by the second set.

The relative strength of bonding between a particular metal ion and a single electron donor site on a ligand depends on the electronic character of the atoms concerned. Those combinations in which both atoms have a similar preference for either ionic or covalent bonding are favoured [32]. It can be readily appreciated that acceptors which form bonds that tend to be predominantly ionic will associate most strongly with donors of an ionic rather than a covalent disposition, and *vice versa*. A weaker link may be anticipated between atoms that are not so well matched.

These facts have been embodied in two common classifications of metals and ligands according to which Lewis acids (electron acceptors) and Lewis bases (electron donors) are subdivided into two main groups. These have been called class (a) and class (b) [31, 32] or 'hard' and 'soft' [33, 34]. Small, highly charged atoms that are not easily polarized belong to the class (a) or 'hard' category. These are inclined to bond in a mainly ionic way. On the other hand, larger, more polarizable atoms fall into the class (b) or 'soft' category and they form bonds which have much more covalent character.

The predictive usefulness of these concepts depends on an ability to divide both metals and ligands into the two different groups. This has been done quantitatively by largely empirical methods. In particular, Pearson's concept [33] of 'hard' and 'soft' acids and bases (HSAB) has been widely recognized as having considerable practical application. Yet some troublesome limitations have become evident. A large group of metals, amongst which are many of relevance to chelation therapy, cannot satisfactorily be described as either 'hard' or 'soft' and, hence, have been termed 'intermediate'.

The term 'intermediate' is something of a misnomer since, rather than lying

between the two extremes, they exhibit an ambivalent character. In spite of attempts to quantify the HSAB approach [35], little further has emerged to provide more theoretical insight into how chelating agent selectivity might be improved. This can possibly be attributed to uncertainties in respect of certain fundamental definitions [32].

Nevertheless, in accord with the HSAB philosophy, certain rules of thumb can be established. There is no doubt that 'soft' metals such as mercury, silver, cadmium and gold favour interaction with 'soft' sulphur donor atoms, whereas, at the other extreme, the complexation of calcium, magnesium, ferric and plutonium ions is likely to be dominated by 'hard' oxygen donors. Metal ions such as Cu(II), Ni(II) and Zn(II) will bond strongly to both 'hard' and 'soft' donors; indeed, they often appear to prefer a mixture of both kinds.

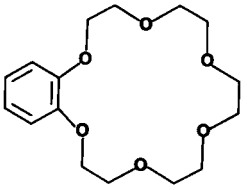
To discriminate between HSAB 'intermediate' metal ions, other theoretical considerations thus need to be invoked. Foremost in this regard are the thermodynamic consequences of partly filled *d*-orbitals. Moving across the transition series, the first-row metals exhibit two important trends. First, there is a decrease in ionic radius and thus an increase in charge/radius ratio which favours stronger co-ordination. Second, there is the phenomenon known as *d*-orbital splitting, which permits certain metal ions, most notably Cu(II) and Ni(II), to adopt configurations which stabilize complex formation [24]. These factors lie behind the general observation that the equilibrium constants of analogous complexes of divalent first-row transition metal ions from manganese to copper increase monotonically. Since the *d*-orbitals of Zn(II) are completely filled, this ion does not enjoy such an advantage and hence the corresponding formation constants of Zn(II) are generally smaller than those of Cu(II) and Ni(II). There are surprisingly few exceptions to this order, known as the Irving-Williams order of stability [36]; those that there are usually involve special modes of bonding.

In consequence, differences in metal ion selectivity between ligands depend largely on the fact that certain donor atoms enhance *d*-orbital splitting more than others. In particular, nitrogen generally exhibits a greater effect than oxygen. So ligands which contain many nitrogen donor sites or which have shortened nitrogen-metal interatomic distances display a marked preference for Cu(II) and, to a lesser extent, Ni(II).

Another possible means of establishing selectivity is to exploit the ionic size differences between various metal ions. Chelating sites on ligands with limited flexibility have a natural preference for those cations which fit most comfortably into their molecular architecture. For example, macrocyclic molecules can be synthesized such that their ion-binding cavities span a range of sizes and, since only small differences in the diameters of the cavity and the co-ordinating

cation can be tolerated, extraordinary specificity for almost any chosen metal can be accomplished [37].

This attribute is demonstrated particularly well by chelating agents known as ionophores because they selectively increase the cation permeability of biological membranes. The synthetic ionophores encompass the two-dimen-



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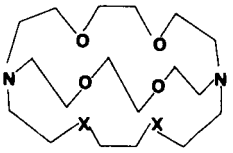

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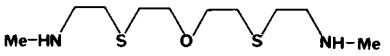


(11)

sional crown ethers such as benzo-18-crown-6 (9), the three-dimensional cryptands (10) and the acyclic podands (11), the latter being sufficiently flexible to wrap themselves around the central cation [38–41]. On the other hand, there are two basic groups of naturally occurring ionophores. These are (i) the cyclodepsipeptides, such as valinomycin, which characteristically incorporate D-amino acids, and (ii) the cyclic and acyclic polyethers, such as nonactin and nigericin, which possess pyran and furan moieties as well as carboxylic, phenolic and occasionally ketoenolic groups [39, 40].

Most of these ionophores are noted for their ability to complex and discriminate between the alkali metal ions. However, some of them exhibit considerable specificity for other metals. Ionophores lasalocid A (X-573A) and A23187 are highly selective for Ca(II) over Mg(II). They have consequently been used extensively as probes for Ca(II) in physiological research (e.g., Ref. 42). Selectivity for divalent metal ions has also been reported for a variety of cyclic peptides [43–45].

The ideas outlined in this section rationalize, satisfactorily, the observed preferences of ligands for particular metal ions and suggest how better chelating therapeutical selectivity might be accomplished. However, there are a number of matters to be discussed in following sections leading to the conclusion that improved selectivity, in itself, is only one of many factors which need to be considered.

COMPUTER SIMULATIONS

All organic compounds containing heteroatoms that have lone pairs of electrons to donate are potentially capable of interacting with metal ions. Accordingly, the number of possible ligands in any biological system is legion and every co-ordinating partnership which is established, prevails at the expense of other combinations. It is in this competitive context that the ability of chelating agents to bind metal ions *in vivo* needs to be considered. The affinities which are observed when a given metal and ligand interact in isolation may be substantially altered in the presence of other metals and ligands [24]. There is the example of EDTA, which forms more stable complexes with Hg(II) than with Zn(II) yet removes zinc from the body without affecting mercury [46].

Although there were some earlier approaches to the question of which type of ligand would predominate in association with particular metal ions in biological fluids, Perrin was the first to realize that a quantitative calculation based upon multiple thermodynamic equilibria was required to provide a definitive answer [47]. Such calculations must be based upon the equilibrium constants of all the competing reactions and the total amounts of each metal and ligand available for distribution amongst the various chemical species.

Under these circumstances, the outcome clearly depends on a multitude of factors, none of which in itself can ever be held wholly responsible for the behaviour of the system. Accordingly, attempts to improve chelation therapy which focus on only one property of the agents at any time are almost certain to be frustrated. Moreover, overcoming the complexities of multiple equilibria in biofluids is only one part of the problem. The multicompartmental nature of biological systems is another which greatly exacerbates the difficulties. If progress is to be made, much greater attention must be paid to the interrelationship between all these factors than has been given hitherto.

Attempts to investigate metal complex formation in biological systems by the mathematical approaches outlined earlier are faced with a number of specific complications. Foremost amongst these are (i) the lack of quantitative thermodynamic data concerning associations between metal ions and biological macromolecules such as proteins and (ii) the uncertainties which still surround the composition of many biofluids, both in respect of constituents and concentrations. Nevertheless, as described later in this section, certain techniques which overcome some of these difficulties have been developed. Since all of these depend on computers to assess the representative concentrations of various chemical species in their biological environment, they have collectively become known as computer simulations.

The first computer simulations of metal ion equilibria in blood plasma were based on a relatively small number of low-molecular-weight components [48–50]. In this respect, they clearly suffered from a major limitation, since it was well known that the transport proteins, albumin and transferrin, normally bind an overwhelming percentage of the transition metal ions present in plasma that exist in an exchangeable or labile form.

However, these early models served a very useful purpose. Apart from being the first step upon which subsequent developments would be based, they also provided, for the first time, an invaluable insight into how equilibrium processes dominate the biological role of the low-molecular-weight ion fraction.

Almost contemporaneously, a number of reports had been published concerning various experimental attempts to identify the predominant low-molecular-weight complexes in plasma or serum [51–58]. As these were not generally in accord with the computer simulations, controversy about differing approaches developed which still remains unresolved and which has, in fact, been extended to several biological contexts other than the original one concerning blood plasma [59–61].

As scientific knowledge always depends on the extent of agreement between experimental observations and the theoretical understanding of them, it is important to examine the reasons for these discrepancies and to seek ways of resolving them. In this sense, computer simulation models should be regarded as an embodiment of thermodynamic theory. They provide an exact description of the equilibrium position of the defined system within the accuracy of the formation constants used in the calculations. This means that for biological purposes, the precision of most results is more than adequate. So, computer simulations are clearly a valuable tool for investigating the metal-ligand equilibria that operate in biological fluids. They are especially useful for checking conclusions from biochemical experiments in which it is assumed that equilibrium is maintained, for helping to interpret the results of physiological experiments in which the mechanism is often obscure and for examining systems in which species concentrations occur below the current limits of analytical detection. In all respects, these attributes complement experimental approaches rather than compete with them.

Very much the same can be said when the limitations of computer simulations come to be considered. An objection often raised is that it is difficult, if not impossible, to define adequately all the interactions which might occur in complicated biological mixtures. Certainly, failure to include any significant complex may render the computer model invalid. Yet, as outlined below, it does not necessarily do so, depending on the way the calculations are per-

formed and the kind of information required from them. Experience suggests that when experimental conclusions and those based on the computer simulations are in disagreement, *both* approaches need to be examined for weaknesses which might reasonably lead to the discrepancy. A corollary is that taking areas of agreement for granted greatly underestimates their significance. The insight into the physiological role of metal-ligand equilibria which computer simulations provide should be assessed in this light.

Soon after the first computer simulations of multicomponent equilibria in blood plasma appeared, ways of improving them began to be developed. Perrin soon extended his original model [48] to include Ca(II) and Mg(II) in addition to the metal ions, Cu(II) and Zn(II) [62, 63]. An attempt was also made to take account of the metal binding by albumin, but this was not particularly successful. (The new model predicted negligible copper-albumin complex formation and it computed a calcium distribution between albumin and globulin in disagreement with direct measurement [64].)

It is difficult to include metal-protein binding in computer simulations because at present the complexes cannot be satisfactorily characterized in a thermodynamic sense. However, several approaches have now been devised which permit reliable calculations to be performed, albeit under certain restricted circumstances. These depend on the fact that the metal-protein interactions in question can be omitted from the computer simulations, without adversely affecting their results, provided that the free metal ion concentrations (rather than the totals) are supplied. Although these free concentrations are not generally known with confidence, estimates from a variety of sources can be made and, in any case, a series of simulations can be performed such that a range of free metal ion concentrations is covered.

By such means, it has been shown that the percentage distribution of transition metal ions amongst the naturally occurring low-molecular-weight ligands in blood plasma is independent of the exact extent of metal-protein binding [22, 65]. Moreover, since the protein-containing biofluids tend to buffer the free metal ion concentrations, it was later shown that the effects of administered chelating agents relative to one another could also be assessed. This information is conveniently represented by a factor, known as the Plasma Mobilizing Index (PMI), which quantifies the ability of chelating agents to compete for labile metal ions in the biofluid [66].

In effect, PMI is defined as the factor by which the size of the low-molecular-weight fraction of the target metal ion is increased by the administration of the chelating agent. This increase initially occurs only at the expense of metal-protein complexes. Since the free metal ion concentration remains constant, a quantitative knowledge of the metal-protein binding is not needed for

the PMI calculation. It thus provides a measure of chelating agent effectiveness at equilibrium which depends on the formation constants of the chelating agent itself and, to a lesser extent, on the formation constants and concentrations of the endogenous, low-molecular-weight constituents of plasma. In most cases, it can be regarded as a very reliable indication of the relative affinity of various administered agents for particular metal ions in the biofluid.

Such information is especially valuable when it comes to understanding the physiological effects of chelating agents. In particular, the polyaminocarboxylic acids cause urinary excretions of heavy metals which, without noticeable exception, follow the order displayed by their PMI curves [66]. This means that their ability to sequester the metal in competition with the natural components of plasma is the dominant factor determining their therapeutic activity. On the other hand, the administration of sulphydryl-containing ligands often leads to smaller urinary excretion of metals than a simple interpretation of the PMI curves might suggest (F. Planas-Bohne, private communication). This is partly because the ligands interact strongly with plasma protein and partly because they can penetrate into cells. Both factors mean that they are effectively reduced in concentration. Since PMI curves are a function of ligand concentration, it is of course possible to take such effects into account provided they can be accurately quantified.

A most important feature of the PMI calculations is the negative evidence which they often supply. Although many pharmaceuticals are capable of binding metal ions on their own, they are rarely powerful enough to do so in the competitive environment of blood plasma. An interesting general role for computer simulations which has thus emerged has been to dispel suggestions that chemical effects demonstrated *in vitro* also have significance *in vivo* (e.g., Refs. 67, 68).

BIOLOGICAL ASPECTS

The study of transition metal ion physiology has received fresh impetus in recent years because of the interest in their essential biological roles and concern with their potentially toxic effects as environmental contaminants. It is, however, evident that, as it has been in the past, progress will be determined in future more by the academic framework in which research is conducted than by the actual complexities of the subject. This is because sufficient insight can be achieved only by a more comprehensive, interdisciplinary approach than has been adopted hitherto. A thorough understanding of present problems in chelation therapy (at both molecular and cellular levels) requires the joint perspective of chemists, biochemists, physiologists and physicians [69].

This view is supported by the confusion which has commonly plagued detailed investigations into transition element metabolism. Often, experiments have been based on traditional methods used for low-molecular-weight organic compounds or metal ions such as sodium. These are not always applicable to transition metal ions. In particular, the results may be entirely spurious whenever the effects of complexation are neglected in the design, execution or interpretation of such research. For example, early work on the mechanisms of intestinal iron absorption concluded that active transport processes were involved [70, 71]; however, complex formation within the mucosal cell was not adequately appreciated [72] and, only by the most careful experimental work, has it now been demonstrated that movement across the membrane occurs by passive diffusion [73, 74].

Accordingly, this section outlines some of the effects of metal ion complexation in biological systems and deals with their implications for chelation therapy.

METAL TOXICITY

It is now well established that the toxicity of metal ions can be ascribed to their interference in certain critical biochemical processes, the most prominent of these involving intracellular enzyme and membrane systems [75]. Co-ordination of transition metal ions to donor groups on biological macromolecules (particularly those belonging to the cysteine and histidine residues of proteins) is primarily responsible. This may affect molecular conformations and/or interfere with the binding of natural substrates. Thus, all heavy metal ions are liable to poison membranes and inhibit enzymes (especially those depending on sulphydryl functional groups). Disruption of oxidative phosphorylation biochemistry is often the critical effect and prenatal or neonatal life is especially susceptible [76], underlying the general importance of metal ions in growth processes.

However, the discovery that such lethal elements as arsenic might also be physiologically essential [77] makes it clear that no genuine distinction can be drawn between toxic and beneficial metals. This was recognized in the early years of this century by Bertrand [78], who observed that all the trace elements are poisonous if ingested in sufficient amount. Subsequently, Venchikov [79] has described the general effect of increasing metal ion concentrations on physiological well-being as, first, having a metabolic function (if any) and then a pharmaco-toxicological action. This biphasic response always terminates in death as the consequence of excessive exposure to the metal. The main difference between elements thus arises because some do not have any natural physiological role.

These ideas provide a framework in which the practical effects and, hence, the treatment of metal toxicity can be considered. The transition elements fall into two categories, depending on whether they have a major biological function or not.

For those metals like iron, copper, zinc and manganese that do, there are generally very efficient homeostatic control mechanisms. These serve both to protect against assimilation of excessive amounts of the element and to mitigate the effects of unusually high levels which, nevertheless, might accumulate *in vivo*. Thus, such elements display toxic symptoms only when their homeostatic processes fail or are temporarily swamped. Zinc and manganese are, accordingly, amongst the least toxic of metals and relatively massive doses of iron and copper need to be ingested before any permanent damage is done.

The toxic effects in man arising from various kinds of exposure to different metals is shown in *Table 5.1*. A clear distinction may be made between the effects of acute, subclinical and chronic poisoning. With the essential elements

Table 5.1. THE TOXICITY OF METALS

Data taken mainly from Refs. 80 and 733.

B = haematogical complications
C = carcinogenic risk
D = dermatitis
E = encephalopathy
G = gastroenteritis
H = cardiac involvement
K = kidney failure
L = liver necrosis
M = mental implications
N = neurological disturbance
O = osteomalacia
P = pulmonary complication
S = shock

Element	*Acute effects*	*Chronic effects*
Al	N	O
As	G, N	N, J, L, C, M
Cd	G, P, K	P, K, L, O
Cr	G	D, C
Cu	B, L	N, L
Fe	G, S, L	H, C, L
Hg	G, K, P*, N**	G, N, M
Mn	P*	N, M
Ni	N***	D, C, M***
Pb	G, H, S	G, H, K, N, E
Zn	N*, G*, P*, K*	–

* Vapour or fume.
** Methyl mercury.
*** Nickel carbonyl.

there is obviously no parallel to the subclinical toxicity of other heavy metals and chronic overload only develops in certain exceptional circumstances [80]. These may be as a consequence of a genetic defect in trace element metabolism (as in Wilson's disease [81]) or because the natural homeostatic control processes have been unnaturally overwhelmed (as in transfusion siderosis [82], so-called Bantu siderosis [83], or in the syndrome of prolonged manganese poisoning in Chilean mineworkers, locura manganica [84]).

With the non-essential elements, however, the pattern in *Table 5.1* reflects the degree of exposure rather than any fundamental distinction in response to the different kinds of toxicity. Chronic and acute poisoning thus represent two extreme outcomes, with subclinical toxicity covering the middle ground. Children with pica who consume lead-based paints [85], welders using cadmium-containing flux [86], and victims of mercury poisoning [87], may all present with acute toxicity symptoms. Yet, subclinical and, ultimately, chronic poisoning may equally well develop if exposure at sufficiently low levels occurs over an extended period [88–92]. Episodes of acute poisoning tend to attract the public's attention, but the long-term, subtle effects of exposure to polluting metals such as lead, cadmium and nickel are probably of far greater significance.

The non-essential elements, particularly those associated with pollution such as lead, mercury, cadmium and plutonium, tend to accumulate in the body in specific tissues and may persist in their detrimental effects long after the period of assimilation and give rise to irreversible biochemical damage at relatively low levels of exposure. These problems are in some ways worsened by physiological mechanisms used to detoxify the metal, since these may substantially increase the half-life of the element *in vivo*. Cadmium binding by metallothionein [93], the incorporation of lead into bone [94] and the immobilization of plutonium in erythropoietic marrow [95] are three examples in which the metal is initially removed but may later be released to cause further damage.

METAL COMPLEXES IN BIOFLUIDS

Since good health requires that the essential transition metal ions are all maintained at optimum levels, the need for efficient homeostatic mechanisms to regulate internal concentrations is evident. It is important that each metal ion occurs in free concentrations high enough to sustain those complexes with a physiological role yet not so high as to interfere with donor sites that must remain free for other interactions.

Very little concrete information about the actual free concentrations occur-

ring within biological fluids is available and that which there is tends to be inconsistent. Using ion-selective electrodes, values for Ca(II) of about 10^{-3} M in plasma and 10^{-7} M in cells have been measured [64, 96–98] and non-protein-bound Mg(II) levels in plasma can also be determined [99], but there are no comparable experimental techniques which could, at present, be applied to the transition elements. Estimates of the free metal ion concentrations in plasma can be obtained only from metal-protein binding considerations [22], as indicated above. There have been great improvements in the methods used to determine the binding constants necessary for this (e.g., Ref. 100). However, no corresponding calculations can as yet be made for cell cytoplasm where the redox potential and the predominant complexes are less certain. Williams has estimated that the concentrations of Mn(II) are likely to be around 10^{-6} M in vesicular spaces and considerably smaller in the cells' bulk solution [101]. Complex formation by low-molecular-weight metabolites generally means that none of the first row transition elements is likely to occur above this limit. Yet, this conclusion undermines the implications of numerous studies in which enzyme activation by millimolar concentrations of Mn(II) has been demonstrated [102–105], and could alter much of our present perception of the biochemical role of Mn(II) (J. Chesters, personal communication).

Regardless of the absolute free concentrations which do prevail, the relative values must be such that they counterbalance the general effect of the Irving-Williams order of stability. Free concentrations of Cu(II) are certain to be lower than those of Zn(II) or Mn(II). Otherwise, for example, many zinc metalloenzymes would be poisoned by the stronger binding of copper. Interestingly, this does not require as sensitive a balancing act as may at first appear. It happens naturally whenever two or more transition metal ions are placed in solution with an excess of a strong complexing agent. Indeed, this is an inevitable outcome of the relative stability of the metal complexes formed by the complexing agent as predicted by the Irving-Williams order. In practice, the specific metal binding sites on transport and storage proteins effectively serve this purpose.

Metal-containing protein species can, broadly speaking, be classified into two groups according to the way in which the metal ion is incorporated. First, there are the complexes formed by proteins which pre-exist in the biofluid and which have binding sites that co-ordinate the metal ion as and when it becomes available. The second group includes all those metalloproteins which have their metal ions incorporated as part of their biochemical synthesis; significant quantities of apoprotein do not exist prior to metal ion binding.

Often the second type of metalloprotein has a specific biological role that needs to be fulfilled regardless of the local concentrations of free metal ion or

its associated low-molecular-weight complexes. For this reason, the metal ion is frequently bound in a thermodynamically irreversible way and cannot be extracted without disrupting the entire molecular structure.

In contrast, many metal-protein interactions are thermodynamically reversible. This means that the extent of binding is determined by equilibrium considerations and, in particular, depends on the surrounding concentration of free metal ion. As the free metal ion level rises, so does the concentration of the metal-protein complex, until the binding site(s) on the protein become(s) fully saturated. Conversely, as the free metal ion concentration falls, the metal-protein complex tends to dissociate.

These generalizations are fundamental to an appreciation of the mechanism of any chelation therapy. In biological systems, chelating agents must sequester transition metal ions in the context of this competitive equilibrium. The other relatively high concentrations of metal ions bound tightly within some metalloproteins are not available until biodegradation of the whole molecule releases the metal into the low-molecular-weight equilibrium pool [106].

Matters are further complicated by the various kinetic parameters which may be associated with thermodynamically reversible binding. When association and dissociation are both rapid processes, the interaction is labile and the metal ion is said to be 'exchangeable'. This does not mean that the binding is necessarily weak, since rapid metal ion exchange is possible even when the interaction is as avid and as specific as that of serum albumin for Cu(II) [107]. Under such circumstances, chelating agents, if sufficiently powerful, can remove the metal from the protein directly [108, 109].

However, when the dissociation process involves a kinetically slow step (possibly as a consequence of binding deep within the protein structure), equilibrium may not be established within the physiological time-scale available to the chelating agent. If the rate at which the exchange reaction takes place is slower than the rate of excretion or metabolic degradation of the chelating agent, then, no matter how tenaciously the metal may be bound, the mobilization achieved may be negligible.

The ability of a chelating agent to promote heavy metal decorporation thus profoundly depends upon the type of metal-protein complex from which the metal needs to be removed. In general, high affinity for the target ion is a necessary but, in itself, insufficient condition for effectiveness. For example, there are many siderophores (compounds produced by micro-organisms to mobilize iron from the environment) such as DFOA which have the thermodynamic potential to remove Fe(III) from transferrin but which are unable to do so in practice. The complexes they form with Fe(III) are so stable that they exist without dissociation in the presence of apotransferrin [110]; yet no

discernible metal ion exchange from Fe(III)-transferrin to siderophore takes place. This kinetic obstacle prevents every one of the established iron-chelating therapeuticals from tapping the large pool of metal ion transported by transferrin in plasma. To overcome this single impediment would improve the therapy of iron overload beyond present recognition. Clearly, not enough is known about transferrin's uptake and release of iron, in spite of continued work on old questions (cf. Ref. 111) such as synergistic anion binding [112] and the functional heterogeneity of binding sites [113–118] as well as the development of some interesting new approaches [119–122].

The transition metal storage proteins within cells represent another substantial reservoir of metal ions which could serve as the target for chelating therapeuticals. They possess an intriguing set of properties which combine a certain degree of thermodynamically reversible binding with the induction of synthesis as a response to increasing metal ion concentration [123–126]. In this respect, there are distinct parallels between the two most prominent metal storage proteins, ferritin and metallothionein. The primary purpose of both would seem to be concerned with the homeostasis of essential elements [127, 128]: ferritin acts for iron [129] and metallothionein for zinc [130] and perhaps copper [131]. Yet both readily incorporate polluting heavy metals, sometimes even at the expense of the natural cation [132–135]. Metallothionein, containing an extraordinary percentage of thiol functional groups [136], avidly sequesters HSAB 'soft' metals such as Cd(II) and Hg(II), whilst 'harder' ions like Pu(IV) are incorporated by ferritin.

The ability to synthesize these storage proteins appears to be a common feature, shared by a wide variety of tissues [137, 138]. The apoproteins do not exist in appreciable amounts, so protein synthesis is induced when necessary. It is still unclear whether the apoprotein, once synthesized, simply binds the excess metal or whether there are specific mechanisms for inserting the ions during synthesis. On the other hand, it is known that there are two distinct ways in which the metal ions can be released. Since the biological half-lives of the metalloproteins in question are, under normal circumstances, relatively short, the whole metal content is liberated when the molecule is degraded. Metal ions can also be removed, albeit to a limited extent, by competition from powerful chelating agents [139, 140]. So, intracellular release can occur from the intact molecule. However, in response to metal overload, this lability tends to be lost and turnover rates become greatly reduced.

Together, these properties of ferritin and metallothionein permit them to act as a very efficient homeostatic mechanism. A steady state is set up involving a labile metal ion pool which is buffered by the synthesis and degradation of the metalloprotein. Such a system has considerable capacity because protein

synthesis can continue to assimilate metal ions until the influx abates or the cell is ultimately overwhelmed. The system can also respond to falling metal ion concentrations, initially by dissociation and subsequently (over periods of more than a day or so) through biological turnover without resynthesis.

This picture suggests that transition metals are likely to be more readily available to chelating agents within cells than without. Neither the kinetic nor the equilibrium barrier is likely to be as severe as is the case when the metal must be obtained from plasma transport proteins. So, the metal ion cycle which takes place within cells between the low-molecular-weight pool and metalloprotein storage forms ought to be a stage at which target metal ions are most vulnerable to sequestration. Yet, it seems that few, if any, of the conventional chelating therapeuticals take advantage of this. The reason lies in the need imposed on the agent and its complexes to traverse biological membranes.

MULTICOMPARTMENTAL DISTRIBUTIONS

A great deal of experimental research has been done with the purpose of discovering how both metal ions and chelating therapeuticals distribute themselves *in vivo* after administration. Most often the work has entailed tracing radioactive material that has been injected or given orally to animals. From this, a well-defined pattern has emerged from which certain valuable generalizations can be drawn.

Essential transition metal ions appearing in the plasma as a result of intravenous injection or absorption from the gastrointestinal tract or discharge of the lymphatic system (following intraperitoneal and subcutaneous injection) are generally removed, often very efficiently, by the liver [141–143]. Redistribution to other organs may subsequently occur depending on the particular metal, but excretion to the bile is one typical pathway that is followed. The principal exception to this rule is provided by iron which, when specifically bound to transferrin, is not primarily routed to the liver but instead goes to erythropoietic marrow [144].

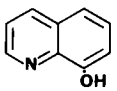
Although the toxic heavy metals tend to integrate into the homeostatic mechanisms of chemically similar elements, they are generally unable to duplicate all of the specific interactions involved. So their distribution differs, especially in respect of uptake by blood cells (mainly erythrocytes) [145, 146]. The appearance of heavy metals in, and the damage they cause to, other organs, is thus, usually, a secondary effect and, as such, is accentuated in chronic rather than acute exposure.

In one respect at least it is possible to understand in general terms the

observed distribution of metals following toxic exposures. Organometallic and carbonyl complexes diffuse rapidly across most membranes separating biological compartments because they tend to be electrically neutral and lipophilic in nature. Examples include ferrocene [147], nickel carbonyl [148], methyl mercury [149] and tetraethyl lead [150]. Exposure to these species produces an entirely different metal distribution amongst body organs compared with the patterns arising from administration of the metal as an ordinary complex or as a salt. They are even able to penetrate the most protective of all membranes, the blood-brain barrier.

Lipophilicity also dominates the compartmental distribution of chelating agents. As few, if any, of these compounds participate in the active physiological mechanisms available to metabolites such as the naturally occurring amino acids, their transport *in vivo* depends upon nonspecific processes, the most important of which is passive diffusion. So, electrically neutral and nonpolar molecules tend to be best absorbed across the gastrointestinal tract and achieve a wide distribution amongst various tissues. On the other hand, agents which exist in solution as highly charged species cannot diffuse through biological membranes and are thus confined to the body compartment into which they are administered. Whilst there are certain biological mechanisms, such as endocytosis, which sometimes bypass these restrictions, they are not of much practical significance. The principle that distribution is determined by the charge on and polarity of the chelating agent molecule is very well established and accounts almost entirely for a substantial body of relevant experimental fact.

To be effective when taken by mouth, a therapeutic chelator must pass through the gastrointestinal epithelium. In man, the pH of the gastrointestinal tract rises from pH 2 in the stomach, through pH 6 in the small intestine, to pH 8 in the colon. If the drug remains fully ionized over the whole of this pH range, it cannot passively diffuse through lipid cell membranes and, hence, does not penetrate the epithelium. On the other hand, if at some stage the drug occurs in an unionized or partially ionized form, the neutral component can cross into plasma. The partitioning of the drug between the plasma and the gastrointestinal tract is thus determined by the acid dissociation constants of the drug. Uptake of an ionizing compound thus generally occurs if the acidic group(s) protonate at pH > 3 and the basic group(s) deprotonate at pH < 8 [151].



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Much less information is available concerning the body compartmental distribution of labile metal complexes but, from the reliable evidence that there is, it can be inferred that the same principles apply. For example, in the classic study of the antibacterial properties of 8-hydroxyquinoline (HQ, 12), it was shown that the activity was due to the passive diffusion of the neutral bis-complex through the cell membrane [152]. Similarly, a correlation has been demonstrated between the antibacterial activity of crown ethers and their alkali metal ion transport efficiency [153]. It has also been shown that the more lipophilic the complex formed by Fe(III), the greater is the amount of metal which is delivered to reticulocytes, presumably by diffusion through the cell membrane [154]. Further work demonstrates that the lipophilicity of injected complexes determines their route of excretion: polar complexes are confined to extracellular space until they are excreted by glomerular filtration into the urine, whereas less polar species tend to be taken up by the liver and emerge in the bile [155, 156]. Every one of the established chelating agents discussed in the next section appears to behave in the same kind of way.

Similar conclusions can be drawn from knowledge about the behaviour of ionophores in biological systems. Their antibiotic properties are due to their ability to transport cations through lipid membranes by encasing the metal within a bulky organic structure of low net polarity. Support can also be inferred from the physiological behaviour of radionuclide complexes used for diagnostic imaging of body organs and the fact that complexation facilitates heavy metal ion transfer through the placenta [157, 158].

Conversely, there is overwhelming evidence that metal ions and their complexes which carry a significant electrical charge or are highly polar have a greatly restricted ability to move from one body compartment to another unless there are specific transport mechanisms for doing so. It is unlikely that such mechanisms are available to transition metal ions (except, possibly, in a few special circumstances). So, charged transition metal complexes are generally confined to the biofluid in which they are formed, or into which they are administered. This is a major obstacle in most chelation therapies.

There is no doubt that these factors play a fundamental part in determining the effectiveness of particular chelating agent regimens. There is a general rule that the sooner chelation therapy is commenced, the better. A marked and sudden deterioration in efficacy of chelating agents is always observed some time after exposure to the toxic metal [159]. This corresponds to the stage at which the metal becomes dispersed into the tissues and is thus no longer accessible to the direct action of the chelating agent in plasma. Unfortunately, the period during which the chelator can intercept the metal ion in plasma is often relatively short. Given this and, as mentioned previously, that the metal

may not be as firmly bound to proteins within cells as without, it would seem highly desirable to develop agents which can specifically enter intracellular space.

A great deal of effort has, in fact, been devoted towards attaining this objective. Esterification of polar functional groups was an obvious starting point [160, 161]. Other methods of getting chelating agents inside cells are somewhat more promising. Delivery using liposome-encapsulation [162, 163] or red cell ghosts [164–166] has received a great deal of recent attention. However, so far, neither can be said to have truly fulfilled its potential in the clinic. The reason may well be that the rationale behind all these efforts has tended to be oversimplified: if all the consequences of the changes introduced are not taken into account, the gain from one desirable characteristic may be obtained only at the expense of losing others.

CURRENT PERSPECTIVES IN CHELATION THERAPY

The discussion so far has focussed on those fundamental principles which govern the behaviour of chelating agents in biological systems. In this section, the established chelating agents are reviewed to show how their various physiological properties have been or can be exploited therapeutically.

The same basic principles must be taken into account by those attempting to design better agents and/or better drug regimens. In particular, a comprehensive overview of the whole subject is required, otherwise the chances of finding improvements, either of agent or of method, are not much higher than they would be in a random screen of new compounds. The evident failure of the latter approach, which fairly describes the often directionless search of the last two decades, demonstrates how unprofitable this route is likely to be. Accordingly, those areas in current chelation therapies which seem most amenable to future development are given special emphasis.

THE MUTUAL EXCLUSIVENESS OF IDEAL PROPERTIES

The single, most salutory lesson which can be learned from experience in chelation therapy research is that the properties so often listed [167–172] as requirements for an ideal chelating agent are, probably always, going to be mutually exclusive. There are sound chemical reasons for believing that attempts to combine all of the most desirable features into a single agent must intrinsically result in some self-contradiction.

The conundrum assumes its worst proportions when it comes to the need

for an ideal agent to compete effectively against naturally occurring ligands such as the proteins of metal transport and storage. This requires the agent to possess several very powerful electron donor sites. However, such functional groups are inclined to make the molecule polar and, more often than not, anionic. So, most chelating compounds tend to have difficulty traversing biological membranes and many cannot do so at all. The compounds EDTA and DTPA illustrate the handicap this confers: they are poorly absorbed from the gastrointestinal tract [173] and are thus ineffective when given orally; moreover, even when administered by injection, they are almost entirely confined to extracellular space [174] and are therefore unable to come into direct contact with toxic metal deposits in tissues.

Another aspect of the contradictory nature of ideal chelating properties is kinetic in origin. Whenever chelating activity depends on a large number of binding groups, sequestration tends to be a slow molecular process. This is particularly so when the metal must be acquired from another ligand, such as a protein, which itself utilizes many co-ordinating groups and which may hold the ion deep within its polypeptide structure (*vide supra*). This is why DFOA does not acquire Fe(III) from transferrin: the binding sites which bestow on it a thermodynamic ability to do so are an inseparable part of the kinetic handicap which thwarts it. Similarly, the macrocyclic tetramines do not induce a significant cupriuresis in spite of very high copper-binding formation constants [175].

A further ambivalence is associated with chelating agent selectivity. The more powerful a chelating agent, the more likely it is to bind a variety of metals *in vivo* and, hence, the more likely it is to interfere with the metabolism of one or other essential trace element. This accounts for many of the more prominent side-effects of compounds such as BAL, PEN and the polyaminocarboxylic acids (*vide infra*). The initial exacerbation of heavy metal toxicity which is sometimes observed following the administration of chelating agents (e.g., Refs. 176–178) almost certainly manifests a complexation of essential metals that are vulnerable to displacement from their biological binding sites by the toxic ion.

In general, this problem is difficult and may be impossible to resolve by simple chemical modifications to the structure of chelating agents with the aim of making them more selective. The chemical features which confer selectivity also automatically restrict the nature of the compound in a way which, experience reveals, often imposes disadvantages in other respects. One example is the toxicity commonly associated with ionophores which excludes their clinical use. Another is provided by DFOA: this compound is so selective for iron that no side-effects have ever been attributed to interactions with other metal ions;

however, the hydroxamic acid functional groups are also at the root of DFOA's major clinical limitation, namely, rapid metabolic degradation (e.g., Ref. 179).

In all of the above cases, a problem arises because the chelating agent needs to achieve powerful metal ion binding. Another essential requirement which tends to be incompatible with other desirable properties is the need to penetrate intracellular space in order to sequester toxic metals deposited either in the bones or in the soft tissues. It is clearly inefficient for an agent to rely on natural mobilization processes to carry the target metal ion back into plasma before it can be complexed. However, attempts to get the agent to penetrate into tissues have engendered some profound complications.

The need to possess good solubility in water (for transport through aqueous media such as blood plasma) and good solubility in lipophilic environments (for tissue penetration and oral absorption) tend, from a chemical point of view, to be incompatible. Furthermore, if structures with detergent-like properties are synthesized in an attempt to solve this problem, it is more than likely that they will have disruptive effects on cell membranes.

Indeed, all attempts to improve cellular permeability by modifying the structure of chelating agents to make them more lipophilic are liable to introduce new manifestations of toxicity. Esterification of DTPA is a good illustration in which the compound might be acting as a non-ionic surfactant. There is also the possibility of detrimental effects whenever powerful chelating agents that interact with essential trace elements are introduced into cells. Finally, there is the danger that, having mobilized a toxic metal, lipophilic chelating agents may bring about its redistribution to other tissues (e.g., Ref. 180).

Simple modifications to the structures of chelating agents with the intention of making them more lipophilic so that they can diffuse into cells are hence unlikely to have overall advantages. Greater consideration needs to be given to the consequences of the electrical charge which resides on the ligand and the complex species both inside and outside the cell. In fact, very few chelating agents can be expected to promote a spontaneous efflux of toxic metal ions by entering the cell themselves. This is because any ligand which, being electrically neutral, diffuses into a cell will usually form a charged complex that cannot readily diffuse back out. Here again, there is a conflict between two basic requirements which make the properties of an ideal chelating agent much more elusive than is commonly supposed.

THERAPEUTIC CHELATING AGENTS

BAL

It is, perhaps, surprising that BAL, which has been in service for about 40 years, has not been superceded as the recommended drug for the toxicity of some half-a-dozen elements. It is the chelating agent of choice for arsenic, gold and inorganic mercury poisoning, it is a part of the currently accepted strategy for treating lead poisoning and it has been suggested as the antidote for antimony, bismuth, chromium, selenium and tellurium poisoning, although in the last two cases its use must be regarded as dubious.

BAL is a clear, slightly yellow liquid with a most obnoxious, garlic-like odour so powerful that its smell permeates through the tissues of patients being treated with it. As it has limited solubility in water and is prone to decompose, it is administered by parenteral injection of a 10% solution in peanut oil with 20% benzyl benzoate added as a stabilizer. The treatment is painful and nauseating. Patients need to be placed on parenteral fluids to minimize vomiting [85]. High doses also cause fever, marked elevation of blood pressure, convulsions and even coma. It may only be serendipitous that no fatalities have occurred.

In addition to the above-mentioned disadvantages, BAL is positively contraindicated in certain kinds of metal poisoning. In particular, it should not generally be used to treat patients who have been chronically exposed to cadmium [181]. There is also a grave risk associated with its use against alkyl mercury poisoning [182, 183]. The reason for this is clear. It forms complexes which are not only excreted from plasma but also readily diffuse back into tissues. In cadmium poisoning, this damages the kidneys and in the case of organic mercury, metal transport to the brain is facilitated.

Since BAL is also known to be metabolically degraded at a rapid rate, one may well wonder why this agent has retained its prominent position for so long. The answer is two-fold: adjacent sulphydryl groups make it a very avid co-ordinator of HSAB 'soft' metals and, at physiological pH, all of its functional groups are electrically neutral, so it can penetrate tissues in which the toxic metal has accumulated. The fact that, in the presence of high ligand concentrations, an electrically charged and hence water-soluble bis-complex forms with divalent metal ions may be an additional attribute of some significance.

Thus, the features which give BAL its therapeutic efficacy are also responsible for its clinical drawbacks. Clearly, other dithiols with similar chelating properties but with ionized side-groups to make them water-soluble are

unlikely to displace BAL as a leader in the league of chelating agents, even if they are much less toxic. Alternatives such as DMPS certainly have a role to play in the treatment of poisoning by HSAB 'soft' metals because it is desirable to trap in plasma any of the metal ion which has not yet been distributed into the tissues. However, BAL's ability to complex the metal ion after it has moved across and, hence, become shielded by a biological membrane is a massive advantage indeed.

The polyaminocarboxylic acids

Amongst the chelating compounds used either for chemical or medicinal purposes, EDTA stands out as the archetypal metal-binding agent. It has accordingly been tested as a therapeutic sequestrant in almost every conceivable kind of metal poisoning. One wonders if any other substance has been employed so hopefully in so many medical contexts on the basis of its reputation alone!

In fact, apart from some specialized applications which utilize its ability to bind calcium, EDTA can be called a drug of choice in only one instance: this is as the main antidote currently recommended for acute lead intoxication.

This failure to match investigators' expectations may be attributed to the fact that the affinity which EDTA has for almost all metal ions is greatly diminished in the very competitive complexing environment of biological media. The crux of the matter is that, compared with other medicinal chelating agents, EDTA just does not have sufficient selectivity to offset the relatively high concentrations of Ca(II) in blood plasma. Thus, DTPA and DFOA are generally more effective for HSAB 'hard' metals whilst BAL and PEN are the preferred treatment for 'soft' metals. The ambivalent HSAB character of lead may indeed be the reason why it emerges as the only target metal ion of clinical significance for EDTA.

With many metals, DTPA enjoys a considerable advantage over EDTA which cannot be due solely to the greater degree of 'hard' character conferred by its additional carboxylate functional groups. DTPA has proved unquestionably superior to EDTA in the decorporation of many radionuclides (*vide infra*). This is a consequence of the greater atomic radii of the lanthanides and actinides compared with the first-row transition metals. Since DTPA is octadentate and EDTA is only hexadentate, the former agent is superior with metal ions having co-ordination numbers of 8 or more.

In view of their wide application, much work has been done to investigate the metabolism of the polyaminocarboxylic acid chelating agents. Absorption from the gastrointestinal tract is restricted to about 5% of the dose and this has precluded oral administration [173]. Intramuscularly or parenterally

injected, however, the compounds are assimilated rapidly and completely. They appear unchanged in the urine almost immediately and are just about quantitatively excreted by glomerular filtration at the kidneys, with half-lives in plasma of a little less than 1 h [174].

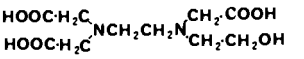
In their bodily distribution, they remain almost exclusively in extracellular water, unable to enter the red blood cells or other tissues. Only a few percent of the amount administered finally emerges in the faeces.

This distribution pattern accounts for the strikingly limited toxicity of these agents. Although their potential side-effects (especially those of EDTA) are widely documented, they are much less toxic than most compounds in the pharmacopeia and, with the possible exception of DFOA, they have given rise to fewer clinical complications than any alternative chelating agent. No problems with DTPA have ever been reported in man [184]. Damage to renal tubules [185–187] is the only really serious hazard to be considered, but highly concentrated infusions of EDTA have been known to cause thrombophlebitis and, following massive doses, an acute febrile systemic reaction with myalgia, headache and fever can develop [188]. As with all chelating agents, there is also the danger of teratogenesis [189–192].

It seems probable that even these effects are due to the binding of essential metal ions. It is noteworthy that when the agents are injected intraperitoneally as the Ca(II) salts, EDTA (with an LD_{50} value in mice of 17.4 mmol kg^{-1}) is less toxic than DTPA (in which the comparable LD_{50} is 12.5 mmol kg^{-1}) [193]. Moreover, Zn(II) and Mn(II) are convincingly implicated by computer simulation studies [66], cell culture [194] and animal experiments as susceptible metal ions. Urinary excretion of Zn(II) and Mn(II) is significantly enhanced by both polyaminocarboxylic acids [195–197]. Furthermore, Planas-Bohne and Olinger [198] have shown that Mn(II) losses correlate well with the lethality which DTPA produces in mice. Cantilena and Klaassen [199] have recently compared the excretion of endogenous metal ions from mice injected with a variety of chelating agents. Nitrilotriacetic acid (NTA) had only a slight effect on magnesium and none of them significantly increased the excretion of calcium. On the other hand, both DTPA and EDTA promoted substantial losses of iron, manganese and zinc.

Other convincing evidence is provided by studies in which the toxicity of Zn-DTPA has been compared with its Ca(II) counterpart [200]. Ca-DTPA is much the more toxic. However, the cumulative effect of a dose of Ca-DTPA which has been subdivided and administered at short intervals is not nearly as severe as when it is given all at once. This implies that the induced biochemical lesion is at least partly reversible. With Zn-DTPA, there is, significantly, no such dependence of toxicity on the treatment schedule.

From the evidence presented above, it seems reasonable to conclude that chelation therapy with polyaminocarboxylic acids can be extended safely to doses higher than the currently accepted maximum of about 4 g per day [201]. This would, of course, depend on the positive demonstration that EDTA and DTPA infusates supplemented with a mixture of appropriate metal ions were, as expected, substantially less toxic than the solutions of calcium sodium salts presently administered. Calculations concerning trace element supplements for the fluids used in total parenteral nutrition [202] can also be applied to chelating agent infusions [203]. Such an approach promises to greatly improve various aspects of the treatment for lead and plutonium poisoning.



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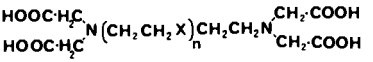
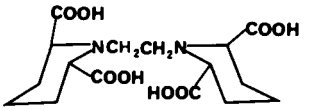


n=2; X = NCH_2COOH
n=1; X = O
X = S

(14)



(15)

Other polyaminocarboxylic acids of diverse structure have been examined intermittently for various medicinal properties. *N*-Hydroxyethylethylenediaminetriacetic acid (HEDTA, 13) was amongst the first agents tested for iron removal [204]. Similarly, evidence has accumulated to suggest that triethylenetetraaminehexaacetic acid (TTHA, 14, $n = 2$, X = NCH_2COOH) and its analogues (14, $n = 1$, X = O or S) might be very effective chelating agents for a whole range of metal ions [205]. However, in clinical applications, these agents have tended to be eclipsed by EDTA and DTPA. As a result, novel structures [206] such as (15) have yet to be tested, even in animals.

Penicillamine

Penicillamine (PEN) was first identified and named as an amino acid hydrolysis product of penicillin [207]. Walshe subsequently demonstrated that the compound was excreted in the urine of patients receiving the antibiotic [208] and soon afterwards he went on to show that, because of its striking ability to

promote copper excretion, it was very effective in the treatment of Wilson's disease [10, 209]. His discoveries transformed the prognosis of Wilson's disease sufferers who until then had had to endure injections of BAL to achieve only marginal benefit [209]. The new agent was both stable and soluble in aqueous solution, it was administered orally, and it routinely produced 20-fold increases in the urinary output of copper. About 1.5 g daily, for life, is the usual dose.

PEN has since become something of a wonder drug. Walshe's early expectations [10] that it could be used as a chelating agent for other heavy metals such as lead, mercury and gold [210] and that it would be effective against cystinuria [211, 212] have been fulfilled. The compound has also assumed wide application in the control of rheumatoid arthritis [213–218] and it may exhibit some benefit in the treatment of progressive systemic sclerosis [219], primary biliary cirrhosis [220–222] and keloids [223] (although these claims are controversial [224–226]). These ubiquitious medicinal properties have meant that PEN has been subjected to much more scientific scrutiny than would have been the case had it been useful only as a chelating agent. Its metabolism and pharmacokinetics have been thoroughly elucidated [227–229]. There has been a spate of investigations into its clinical toxicity [230–235]. Taken together, these may create the impression that the drug is excessively toxic, but this needs to be kept in perspective. As always, the risks of medication must be weighed against the benefits. In this case, the balance often favours PEN. First of all, the drug is much less toxic in patients with Wilson's disease than in others [178], probably because its powerful effects on copper metabolism do less damage when the metal ion is present in such abundance (reduction of PEN levels by copper chelation seems most unlikely). Secondly, before the introduction of TRIEN, there was no realistic alternative in Wilson's disease treatment. Thirdly, many of the earlier reports of PEN hypersensitivity stemmed from cases treated with racemic mixtures synthesized from DL-valine [236]; since PEN does not have the pronounced anti-pyridoxine effects of the L-isomer, this is the only form of the compound administered nowadays and, as a result, the frequency of complications has fallen to about one-third of former levels.

Nevertheless, current therapy using PEN is not ideal. About 10% of patients with Wilson's disease develop an absolute intolerance (this may be compared with a figure of 60% of those receiving the drug for any reason, who exhibit some adverse reaction [231]). Some still consider pyridoxine supplements as necessary to prevent PEN-induced neuropathy [237]. The most serious side-effects include nephrotic syndrome, autoimmune disturbance and bone marrow depression [234, 238, 239]. Visual dysfunction due to induced loss of zinc is another potential complication [240]. So it seems certain that the less toxic TRIEN will be prescribed instead of PEN [241] in an increasing number of cases in future.

Desferrioxamine

Desferrioxamine (DFOA) enjoys the distinction of being by far the most selective of all the chelating agents in clinical use. Whilst the formation constant of its iron complex ($\log \beta = 31$) is modest alongside reported values for other siderophores such as enterobactin ($\log \beta = 52$), the binding to iron is many orders of magnitude greater than to the other essential transition metals, copper ($\log \beta = 14$), zinc ($\log \beta = 10$), manganese ($\log \beta = 8$), magnesium ($\log \beta = 4$) and calcium ($\log \beta = 2$) [27].

DFOA is a trihydroxamic derivative isolated from *Streptomyces pilosus*. The compound is one of dozens of different siderophores produced by various fungi and bacteria to enhance iron bioavailability. This is a common evolutionary necessity because the metal ion, which exists in the environment almost exclusively as very insoluble polymeric hydroxides [106], tends to be a rate-limiting nutrient for growth. The siderophores illustrate how nature can, almost with abandon, synthesize required substances with the most stringently specific properties.

So, as a chelating agent, DFOA does not mobilize or enhance the excretion of any of the above-mentioned essential elements [242]. No doubt, this extraordinary selectivity is one of the reasons why it is relatively so free from toxicity [243, 244]. Another, as Neilands has pointed out [245], is that naturally occurring substances are generally likely to be less inherently toxic than synthetic ones. Apart from a few patients who are allergic to the compound itself (or to some impurity that persists through the isolation procedure), there are few side-effects. Very rapid infusions have been known to cause hypertension and histamine-like responses [172] but with the slow subcutaneous procedure which is almost universally adopted nowadays, serious complications do not arise. Doses of 10 mg kg^{-1} h^{-1} are commonly administered for extended periods without any signs of toxicity [246, 247].

Since the first reported observations on the use of DFOA in the treatment of iron overload in 1962 [248–250], about a thousand publications concerning the drug have appeared in the literature. It has revolutionized the treatment of acute iron poisoning which previously had a mortality rate of over 50% (Fe(II) sulphate used to be second only to aspirin as a cause of childhood poisoning in the United Kingdom and over 1000 cases are still reported annually in the United States). In addition, it was soon recognized as a very potent and safe iron-chelating agent in a series of clinical trials for chronic overload [242, 251–253]. However, it also became apparent that it suffered from some serious disadvantages. In fact, by the end of the decade, its future as an iron-chelating drug was unclear [254], an uncertainty which changed only after it

was demonstrated [246, 255–258] that high doses and a sustained regimen could achieve a negative iron balance in patients with transfusional siderosis. Nevertheless, even today, several handicaps restrict its clinical usefulness. Foremost amongst these is that DFOA is not nearly cost-effective enough. Very prolonged treatment is required to decrease iron stores [259–261]. The other major difficulty arises because its inconvenient mode of administration (now involving slow subcutaneous infusion [259]) strains patient compliance (especially amongst older children [261, 262]) and makes treatment more problematical in many other, less important, ways.

The cost of DFOA treatment is substantial. Budgets for single patients covering medication only can be as high as £2500 p.a. for life [171]. This is prohibitively high in many of the less affluent regions of the world where the need most frequently arises. Even in those countries where there are such resources to be spent, the monies appropriated often have to be weighed against the funding of other life-saving health services. So there is considerable financial motivation to find an iron-chelating agent cheaper than DFOA, provided that it is at least as effective.

None of the other siderophores seems a likely candidate. At the root of the cost-effectiveness problem is the very short period during which DFOA resides in plasma. Although its disappearance does not follow any simple pattern of decay kinetics, it has been shown that in humans, the concentration in plasma falls to half its initial value in less than 10 min [179]. In addition to the removal by filtration at the kidneys, there is rapid enzymatic degradation and distribution into intracellular space [263, 264]. Other hydroxamic acids which retain the effectiveness of DFOA would probably suffer from the same drawbacks.

CLINICAL CONSIDERATIONS

Transfusional siderosis

Chronic iron overload can occur in a variety of circumstances, but it invariably arises because there is no physiological mechanism which can increase excretion so as to reduce excessive internal amounts of the metal [265]. Normal homeostasis is maintained by regulating gastrointestinal absorption and by the use of efficient storage facilities to buffer fluctuations of supply. Evolution has relied heavily on the generalization that iron bioavailability is normally quite limited.

Accordingly, serious iron imbalance develops whenever the absorption process is defective, bypassed or overwhelmed for any prolonged period. This results in parenchymal accumulation of the metal and, subsequently, damage

to a wide variety of tissues. Cardiac failure is the commonest cause of death in these conditions.

There are two clearly identified pathologies associated with excessive iron absorption. In idiopathic haemochromatosis, a genetically determined disorder, the regulatory function of the epithelium is known to be impaired, although the precise nature of the lesion remains unclear. The other manifestation of uncontrolled iron absorption occurs through the unremitting consumption of certain alcoholic drinks of high iron content. In Southern Africa and to a lesser extent elsewhere in the continent, a home-made 'beer' brewed in iron pots is responsible for the syndrome known as Bantu siderosis [83, 254, 266]. A similar iron overload is also commonly found in alcoholics who depend mainly on red wine [83]. The remedy in all these conditions is to remove iron from the patients in sufficiently large quantities to deplete their pathologically enlarged deposits. Fortunately, phlebotomy (venesection) is a convenient and effective way of achieving this [267].

On the other hand, this straightforward solution is clearly impractical when the overload is caused by the repeated transfusion of blood employed in the management of various refractory anaemias. Regular blood transfusion is the only successful way of dealing with chronic aplastic anaemia or homozygous β-thalassemia. However, each transfusion imposes an additional load of iron (amounting to about 3 g per year) which the body cannot eliminate. So, iron stores rapidly enlarge and the characteristic pattern of damage develops. The symptoms include impaired growth (especially at puberty), as well as myocardial and hepatic malfunction. Siderosis rather than the anaemia itself is responsible for the premature death of patients with these transfusion-requiring conditions. Only chelation therapy can therefore improve their prognosis. Modell has reviewed recent advances in this area [268].

Although EDTA was used to remove iron from a patient with haemochromatosis as early as 1953 [269], it eliminated only 10 mg of the metal per day and is thus clearly inferior to phlebotomy. A similar lack of effectiveness was noted for dihydroxyethylglycine, HEDTA and BAL [270]. Parenteral administration of DTPA proved more promising [248, 271], but initial enthusiasm was dampened by its side-effects including pain at the site of injection, intermittent diarrhoea and an increased excretion of other trace elements. However, the discovery that DFOA was at least as effective as DTPA but markedly less problematical, soon ended the use of other chelating agents in the treatment of iron overload conditions. It is, nevertheless, worth remembering that there is not much solid evidence concerning the extent to which DFOA treatment actually prolongs life [171].

Recognition of the therapeutic potential of naturally occurring iron

chelators of microbial origin, in fact, goes back to 1952 when ferrichrome was isolated from the smut fungus, *Ustilago sphaerogena* [272]. Later, it was shown that siderophore yields could be greatly enhanced when the producing organisms were grown under iron-deficient conditions [273]. Most of these siderophores are highly specific for Fe(III) (although there are some reports of Fe(II) chelators [274, 275]). Accordingly, research into iron chelation therapy has always been intimately entwined with investigations into the microbial biochemistry of iron. A full discussion of microbial iron chelators, however, is not within the province of the present work; the interested reader is referred to reviews by Nielands [276], Emery [274] and Raymond and Carrano [277]. Nevertheless, some consideration of siderophores other than DFOA as potential iron-chelating therapeutic agents is outlined in a following section.

There has been a very determined research drive to unravel DFOA's mechanism of action [179, 278–281]. In part, this has been motivated by the belief that the benefits of chelation therapy may depend on the actual source of the iron. Deposits in the reticuloendothelial system appear to be much less harmful than those in parenchymal tissues. The old controversy over which of these kinds of cell are depleted of iron by the drug [282–287] has not been resolved. However, the weight of evidence now suggests that the question itself is not as meaningful as has often been assumed. Iron in the body is distributed between compartments in a pseudo-equilibrium or steady state governed largely by the requirements of heamoglobin turnover and the size of iron stores in various tissues [288]. So iron distribution between reticuloendothelial and parenchymal cells subsequent to DFOA administration is unlikely to depend on the actual site of chelation for very long.

A major component maintaining the pseudo-equilibrium of iron metabolism is the labile iron pool occurring within cells [289–294] and mediated by low-molecular weight complexes [295]. Since it is well known that DFOA does not derive iron from transferrin in blood plasma [296], this labile fraction, which is intermediate between ferritin and transferrin, has long been regarded as the actual source of chelated iron [297]. Presumably, any diminution in size of the labile pool can be replenished by iron released from ferritin, since it is known from studies of patients undergoing phlebotomy that iron held by this metalloprotein is normally accessible [298]. However, the labile pool can be depleted by agressive chelation protocols and the effect of chelating agents is then markedly reduced [299].

Other aspects of the interaction between DFOA and ferritin are far from resolved, in spite of much experimental effort. One of the main problems has been the marked differences found between various animal models and be-

tween *in vitro* and *in vivo* findings. However, in spite of these difficulties, a basic set of facts has been established. There is no longer any doubt that, administered by slow subcutaneous perfusion, DFOA can bring transfusional iron overload patients into iron balance. Iron clearance is enhanced by simultaneous, oral administration of ascorbic acid [300], no doubt because the rate of iron release from hepatocytes is inversely related to oxygen levels [293].

Between 10 and 40% of the iron excreted appears in the faeces. In general, the amount of chelatable iron is proportional to total iron stores, this quantity usually being dominated by the metal held in parenchymal cells of the liver [283, 301]. Iron bound to ferritin is most readily accessible and, when so removed, a compensating redistribution of iron from haemosiderin occurs. Furthermore, it is clear that iron stores in the body are heterogeneous with respect to their potential mobilizability [302, 303]; iron freshly stored in ferritin is more readily released than that in long-term deposits and iron in parenchymal cells appears more directly accessible than that of reticuloendothial origin.

Comparison of distributions of DFOA with ferrioxamine, its iron complex, is most revealing. Both substances appear in the urine shortly after intravenous injection, but their distribution volumes are significantly different [263]. Ferrioxamine is confined to extracellular water, whereas DFOA is not. This implies that the drug is able to penetrate into iron-storage tissues. The fact that the faeces contain a significant proportion of the iron excreted as a result of DFOA administration means that some complexation has to occur within the hepatocyte and that the complex so formed can be shunted directly into the bile. This is what one might expect on the basis of respective liposolubilities of related compounds [304]. It is consistent with this to find that the higher dosage of DFOA, the greater is the fraction of iron which appears in the faeces as opposed to the urine [305].

There is less agreement over DFOA's mode of operation when it comes to the iron that it is excreted in the urine. Many of the published opinions tacitly assume that the drug acts at only one location. Just because some complexation is known to take place within parenchymal cells does not mean that this occurs exclusively. The simplest and most plausible explanation for the urinary iron is that it is derived by complexation in plasma or at the plasma membrane in competition with transferrin [106]. This has been demonstrated experimentally in rats [306]. It seems highly unlikely that ferrioxamine formed within cells would diffuse back into plasma at the rate and in the quantity that any alternative explanation would require.

Further telling evidence in support of the above explanation comes from studies of DTPA as an iron-chelating agent [271, 296, 307, 308]. This com-

pound is also capable of inducing a marked excretion of iron in the urine which, one might reasonably assume, was not complexed intracellularly and which would also be available to DFOA in plasma. Furthermore, both agents show a characteristic delay before achieving optimum iron excretion. Tubular reabsorption of the complex [179, 263] can account for only part of this effect. An additional reason for the delay in peak excretion might be that the net rate of iron release to the labile pool increases for some period after chelation has commenced. Destabilization of ferritin as iron is mobilized and/or the progressive suppression of ferritin synthesis could be responsible.

The concept that chelating agents compete with transferrin for iron returning to plasma from the labile pool within cells is also attractive for other reasons. The effect of ascorbic acid on redox potential would pe predicted to enlarge the labile iron pool and could thus be responsible for the greater efficacy of chelating agents in patients supplemented with ascorbic acid. One would also expect the maximum effectiveness of DFOA to be achieved at low doses because of the limited amount of iron in the labile pool which is available at any one time. Moreover, the reason why chelation therapy is truly effective only in cases of iron overload may well be that until transferrin is fully saturated [308], it acts as a specific receptor for iron from hepatocytes [309]. Interspecies differences may be very significant in this respect. However, it will be difficult to come to more definite conclusions until the speculations outlined above have been placed on a more quantitative footing.

Wilson's disease

Wilson's disease, named after the clinician who first described the condition [310], is a rare, autosomal recessively inherited disorder, now known to arise from a defect in copper metabolism. Although Wilson himself was unaware of its aetiology, he recognized that the characteristic neurological symptoms were always accompanied by a 'profound degree of cirrhosis of the liver'. The fundamental involvement of this organ and its connection with copper metabolism soon became apparent. The finding of unusually high copper concentrations in the liver and blood [311] led to the conclusion that this metal might be responsible for the histological damage done as the disease progresses [312]. This feeling was confirmed when BAL was shown to increase urinary copper output [313, 314] and that this treatment could yield some clinical benefit [315, 316].

Today it is clear that the build-up of copper in the liver and brain is a secondary effect, arising from some, as yet unidentified, physiological malfunction. There have been various suggestions as to the primary biochemical

defect. It may concern the excretion mechanism which normally transfers the metal into the bile [317]. Abnormalities of structure or metabolism of ceruloplasmin, the plasma copper-containing protein, are other possibilities and some investigators have even proposed that the metallothionein in the liver of patients with Wilson's disease has an abnormally high affinity for copper, but these mechanisms now seem unlikely.

Whatever the fundamental cause, it is evident that treatment requires the removal of any excess copper that has accumulated and subsequent maintenance of a satisfactory copper balance. In most cases, this can be achieved by long-term PEN [10, 209] or TRIEN [14, 241, 318] chelation therapy. Occasionally, however, there are cases in which neither agent is well tolerated. As a last resort, such patients must be kept on potassium sulphide or ammonium thiomolybdate to suppress copper absorption from the gastrointestinal tract. A novel suggestion with the same objective involves oral zinc sulphate as a long-term treatment [319].

Unfortunately, as there are relatively so few sufferers of Wilson's disease, there is little practical incentive to improve the current chelation therapies. Even though it is possible to suggest compounds which ought to be more effective and less toxic than PEN and TRIEN, the latter are sufficiently satisfactory to prevent any others from being tried experimentally in human beings. The ethics of this dilemma are discused later, but here it suffices to say that further improvement in the long-term management of Wilson's disease appears improbable at the moment.

On the other hand, Danks has recently raised the problem of treating teenagers with previously undiagnosed Wilson's disease who suddenly present with acute liver failure [320]. More than half these cases die, despite the most strenuous efforts to remove copper excesses from the circulation. So there is a real need to find a chelating agent or other treatment which is effective rapidly enough to cope with the hepatic and haemolytic crisis associated with this acute copper poisoning. Consideration of how the current drugs act in Wilson's disease is an obvious starting point.

Much controversy has surrounded the question of how PEN promotes such a dramatic increase in urinary copper excretion. The normal rate can be increased 100-fold. In his early investigations, Walshe devoted much effort to discovering its mechanism of action [321]. He showed that in Wilson's disease (but not in normal controls), the loss of copper from plasma could be closely correlated with the amount that appeared in the urine. From this it was concluded that the overall effect of PEN was to render copper more available at the glomerulus. What was less clear, and remains so, is exactly how it does this.

The obvious explanation that copper bound to plasma proteins is strongly

complexed by the administered ligand and is thus mobilized into the low-molecular-weight fraction, is faced with two awkward difficulties. It is well known that Cu(II) is reduced by the sulphydryl functional group of PEN [67]. So, explanations involving a Cu(II)-PEN complex are untenable. Moreover, it is difficult to comprehend why PEN might complex Cu(I) *in vivo* very much better than the naturally occurring amino acid, cysteine.

May and Williams have recently summarized the many conflicting conclusions on this subject which have appeared in the literature over the years [322]. Too often, investigators have failed to assess their experimental results realistically in terms of the biological situation. In particular, the effect of (i) the relative concentration of ligand to free metal ion and (ii) the redox buffer capacity of the biofluid are two factors that have commonly been neglected. Many reports concerning the mixed valence, polynuclear copper-PEN complex (e.g. Ref. 323) illustrate how this kind of oversight can cause a great deal of research effort to be misdirected (c.f. Ref 324).

The problem of PEN's mechanism of action still remains to be resolved. One possibility is the effect of PEN on metallothionein. Perhaps the drug interferes with metallothionein biosynthesis (maybe by erroneous incorporation instead of cysteine?). It does not appear to extract the metal from the metallothionein complex (I. Bremner, personal communication). The suggested influence of PEN on ceruloplasmin turnover [325] has also been excluded [326].

Another possibility is that PEN forms strong Cu(I) complexes in blood plasma. Compared with cysteine, it has the advantage of being less susceptible to oxidation. So it is probable that higher levels of the reduced compound are sustained in the biofluid. The fact that PEN appears to disperse plasma copper into tissues [321] is in accord with this explanation. However, to be as effective as it is, PEN would still need to bind Cu(I) much more powerfully than cysteine. This seems unlikely but, as the formation constants have not all been experimentally determined, there is presently no way of telling for certain.

Further research in this area will not only serve to improve the chelation therapy for copper intoxication but will also provide a better understanding of many facets of normal copper metabolism. In this respect, the study of Wilson's disease has already made a major contribution, out of all proportion to the few patients involved.

Lead toxicity

Without doubt, lead toxicity represents the most serious problem in occupational medicine that is associated with poisoning by the heavy metals [327,

328]. There are still over 100 cases of acute poisoning reported annually in the United Kingdom. Moreover, many members of the general public are exposed to undue amounts of lead in a way that has no counterpart amongst other non-essential elements. It has been estimated that upwards of 200,000 tons of this metal are released annually in the emissions of internal combustion engines, some 10,000 tons being deposited in the United Kingdom [329]. Common sources other than traffic fumes include lead plumbing (especially in areas with soft water) and lead-based building materials such as paint and putty (especially from older housing). In addition to these, there is a variety of other sources which, although less common, can have very serious consequences in specific situations. These include poisoning from lead-glazed earthenware (particularly when the stored consumable is acidic in nature [330]) and illicit stills with lead-soldered joints used to produce 'moonshine' (motor car radiators are sometimes used as condensers). Workers in lead foundries (and their families living close by) [331, 332] are another group at high risk, as are those artists and hobbyists who are exposed to specialized lead-based paints [333].

Accordingly, almost everyone living in modern, western industrialized societies is exposed to unnaturally high background levels of lead which, in the opinion of some at least, are having detrimental effects on a demographic scale. This is ironic, since lead poisoning has been implicated as a factor in the fall of the Roman Empire [334].

There has recently been a massive screening project to determine the effects of lead on children living in the United States [334]. This has led to much greater awareness of lead poisoning there than, say, for example, in the United Kingdom. About 1% of the children screened [335] exhibit some overt symptoms of lead toxicity! Another 5% have assimilated excessive amounts of the metal but remain asymptomatic. Nearly a million newborn children are at risk of intoxication by lead acquired from their mothers [336].

Airborne lead is a major factor responsible for the pervasive nature of this contamination, but lead in paint was identified as the most important source in cases of high exposure. Data from the United Kingdom suggest that adults living in cities who are not specially exposed, derive most of their lead from food and water, but about 10–20% stems from vehicle exhausts [337]. Various reports suggest that, if anything, this is a conservative estimate [338, 339].

It is clear from the above considerations that the distinction between acute and chronic lead poisoning is likely to become blurred. Relatively small intakes may induce a crisis of lead toxicity in those with a long history of subacute lead exposure. This is well illustrated by the fact that childhood lead poisoning manifests itself most frequently in summer when elevated vitamin D levels, associated with increased solar radiation, tend to enhance lead uptake and

bodily redistribution [340]. Similarly, the treatment of acute cases with chelating agents may relieve the toxic symptoms, but long-term deposits can be remedied only by time and the removal of the lead source.

Perrin estimates that when the amount of lead consumed exceeds 1 mg per day, clinical symptoms are likely to develop [329]. Severe and permanent brain damage occurs in up to 50% of those who develop lead encephalopathy [85]. Otherwise, the most prominent complications are haematological; up to 95% of assimilated lead is deposited in the red blood cells where it interferes with haem biosynthesis by blocking the incorporation of iron into protoporphyrins [341]. It is known that the synthesis of porphobilinogen (catalysed by δ-aminolevulinic acid dehydratase) is the most sensitive step in this respect. As a result, lead poisoning causes a marked change in the metabolism of the porphyrins, particularly in the amount excreted [85, 145]. This forms the basis of various diagnostic tests. A recent study concerned with the cost-effectiveness of screening free erythrocyte protoporphyrin levels [342] has been heavily criticized (Ref. 343 and subsequent correspondence). Another feature of lead poisoning is the development of intranuclear inclusion bodies, primarily in the liver and kidneys.

Ever since Beyers and Lord [344] first suggested that lead poisoning could also affect behaviour and learning ability, this has been the subject of heated debate. The fear is that lead, at very much lower concentrations than are presently deemed to be toxic in humans, might be responsible for insidious but widespread psychological damage [345–351]. This belief was originally based on, and has subsequently been supported by, the repeated demonstration of such effects in animals [352–357]. Moreover, there has been a steady accumulation of claims that children of low socioeconomic status are particularly vulnerable [350]. Learning ability may be retarded and it has even been suggested that, by inducing hyperactive behaviour, lead may also cause a predisposition towards delinquent behaviour [358]. However, the DHSS Working Party on Lead in the Environment (also known as the Lawther Working Party) [337] concluded that there was no evidence in the literature (surveyed up until about the end of 1979) to demonstrate an unequivocal causual relationship between increasing body lead burden and either educational attainment or hyperactivity. They noted the considerable difficulties in conducting this kind of epidemiological research: poor choice of controls (to eliminate variations in extraneous social, genetic and environmental factors) and the lack of other strict requirements needed to establish a cause and effect relationship were emphasised.

In spite of this, the Lawther Working Party recommended a programme for progressive reduction of the amount of lead released into the air. To this end,

it is now Government intention in the United Kingdom to reduce the lead content of petrol from 0.4 g/l to 0.15 g/l by the end of 1985 [359]. Whether this is sufficient in view of the findings made since the Lawther Working Party report, supporting the belief that lead-associated intellectual deficit is a serious problem, is uncertain.

The management of lead poisoning has remained essentially unchanged for nearly 20 years. In the majority of cases, chelation therapy is not warranted: provided the cause of the lead poisoning has been identified and eradicated, careful surveillance is often all that is required. The United States National Institute for Occupational Safety and Health has drawn attention to the misuse of EDTA or PEN for the purpose of lowering blood levels in chronic cases of occupationally exposed workers [360]. In such treatment, chelating agents are not likely to be effective and may even harm the subject by increasing lead absorption.

On the other hand, when acute lead poisoning is diagnosed, a well-established protocol is recommended [361]. This involves prompt institution of chelation therapy using a combination of BAL and EDTA [177] in addition to various other supportive measures. Although still without the approval of the United States Food and Drug Administration, oral PEN is often used as a follow-up regimen, sometimes for periods as long as 6 months [362].

The combination of BAL and EDTA is markedly more effective than either agent on its own. It has greatly reduced the mortality rate associated with the encephalopathy of acute plumbism. However, there are some interesting uncertainties about the rationale behind this approach. Chisolm's argument [177] that BAL serves to increase the ratio of chelating agent to lead is not strictly relevant because in blood plasma EDTA invariably occurs in considerable excess over the concentrations of the metal iron or its low-molecular-weight complexes. Furthermore, there is much evidence to suggest that increasing the concentration of EDTA (regardless of possible toxic consequences) would not, in fact, be as effective on a molar basis as the BAL supplement. It may be concluded that the observed advantages of using BAL stem primarily from its ability to release lead from erythrocytes in a way that EDTA alone cannot [363].

This leaves some cause for concern over the presently recommended procedure for treating acute lead intoxication. As BAL and the 1:1 complex it forms with lead are both electrically neutral species at physiological pH, there is the worrying possibility that initial treatment with this chelating agent may cause some undesirable redistribution of the toxic metal. The complex formed by EDTA is much less likely to transport chelated lead anywhere except into the urine. So it is disturbing to note that BAL is routinely administered prior to

EDTA. The reason is that the condition of patients with acute lead encephalopathy often deteriorates when EDTA is given first. This effect of EDTA is almost certainly due to the depletion of essential metals, probably zinc, which has been noted earlier. Accordingly, it may be possible to improve current therapy by supplementing the EDTA infusates with appropriate amounts of zinc.

Another such area of concern is the use of PEN in lead poisoning. This agent has been used to good effect for many years [85, 327, 364–368] but if PEN is administered orally whilst lead remains in the gastrointestinal tract, it may increase uptake of the metal [361]. Similarly, PEN should not be used in cases with very high blood lead concentrations, because this can result in a rapid deterioration of the patient's condition, presumably because lead is transported from plasma into various tissues. However, Marcus has recently reviewed [362] the experiences of many researchers as well as his own and he concludes that PEN can indeed play a useful role. Nevertheless, it would generally seem unwise to administer PEN before patients had received an initial course of EDTA. In this respect, surprisingly little experimental work appears to have been done to investigate how effective a combination therapy of PEN with EDTA might be.

Cadmium toxicity

No essential biological function has been established for cadmium [369], but its adverse effects have been extensively documented. As well as impairing renal function, the metal has been shown to interfere with metabolism in general [370]. It is a carcinogen [371] and a teratogen [76] and it can lead to the development of hypertension [372–374]. Although the kidney is the critical organ in chronic cadmium toxicity [375], sub-acute pulmonary exposure leads to emphysema or other respiratory complications and long-term oral intake is almost certainly responsible for the notorious Japanese 'itai-itai' disease (an excrutiating condition characterized by bone-brittleness which may be mediated by the effect of cadmium on the kidneys). In contrast, the consternation caused by the discovery of abnormally high levels of cadmium in the soil near Shipham in the United Kingdom has proved largely unfounded [376], serving to emphasize the importance of bioavailability in these situations.

There have probably been fewer than 100 deaths which can be directly attributed to cadmium toxicity during this century, yet the implications of this metal for public health are a matter of serious concern. The incidence of serious industrial exposures to cadmium fumes (usually in welders) is at least 10-times as frequent as deaths. Moreover, as mammalian species excrete cad-

mium very slowly – the half-life in man is between 10 and 30 years for the liver and kidneys, respectively – there is a steady accumulation of cadmium in all those living in industrialized environments. It has been estimated that the average renal cadmium concentrations in United States citizens at the age of 50 are approximately one-third of the level associated with renal dysfunction in industrially exposed workers [377].

In consequence, there has been considerable research to find suitable agents to counteract cadmium poisoning in man. The rewards of this effort, however, have not been very satisfactory. No chelating agent can presently be unreservedly recommended for treating patients with cadmium overload. Although BAL and, sometimes, EDTA or PEN have been suggested [378–383], they may aggravate the nephrotoxicity of the metal ion [384]. On the other hand, the treatment of acute cadmium intoxication (almost invariably arising from accidental inhalation of cadmium oxide fume) is not entirely hopeless. As MacFarland has pointed out [385], the suggestion that BAL is positively contraindicated under these circumstances is a common misconception. Provided the cadmium is localized in the lungs and has not yet been systemically distributed, nephrotoxic complications are less likely and BAL administration to seriously poisoned individuals becomes worth the risk [385]. It has also shown that BAL can sometimes remove cadmium from the liver without affecting its deposition in the kidneys [386–389].

As a result of many reports on the effectiveness of DMPS against cadmium toxicity, this compound should be tested in man as adjunctive therapy with BAL. The outcome promises well for this unsatisfactory area of chelation therapy [390–393].

Mercury toxicity

Mercury is a notoriously toxic element. However, in contrast to metals such as lead and cadmium, its ill-effects are nowadays largely confined to specific and limited incidents, albeit sometimes of epidemic proportions. The most widely publicised of these outbreaks of mercury poisoning have involved methylmercury. The extremely lipophilic nature of this compound means that is almost completely absorbed from the gastrointestinal tract and that it can readily cross the blood-brain barrier or the placenta. It thus tends to interfere with the nervous system, leading to sensory disturbances, visual constriction, ataxia, dysarthia and, ultimately, involuntary spasms that precede death.

In Minamata, Japan, polluted fish and other seafood, containing as much as 10 ppm of mercury on a wet weight basis, caused over fifty fatalities [394]. There have also been mass poisonings in various countries where mercury-

based fungicides such as Granosan M are used for agricultural purposes. In Iraq, during the winter of 1971/72, there were hundreds of deaths and thousands of hospital cases caused when local farmers consumed bread prepared from wheat which had been treated in this way [87].

Mercury poisoning by inorganic salts or from mercury vapour is much less common, but it may, nevertheless, be very hazardous in certain particular circumstances. Liquid mercury has an appreciable vapour pressure and should consequently be handled in laboratories with precaution. There is some evidence that dentists are a group at special risk. It is interesting that the characteristic neurological lesions of subacute mercury poisoning which were commonly experienced by those working with mercuric nitrate in the felting industry, led to the expression 'as mad as a hatter' [80]. Kidney damage is the other prominent toxic effect. However, a case of massive oral ingestion of elemental mercury without the appearance of any toxic symptoms has been reported [395].

The affinity of mercury for HSAB 'soft' donors is a major factor in its biochemical effects. The reaction with sulphydryl groups in enzymes and proteins in membranes is the major cause of cellular dysfunction. In bile, methylmercury is known to be associated with glutathione [396]. This seems the most probable low-molecular-weight ligand in cytoplasm as well. Although the complex with glutathione is thermodynamically quite stable, it is also labile and is probably involved in the enterohepatic recirculation of methylmercury.

In severe cases of mercury poisoning, simultaneous haemodialysis and infusion of mercury-specific chelating agents is recommended [397]. In less serious accidents, the chelators may be administered in the more conventional manner. BAL was proposed as the drug of choice in 1945 [398], but since then *N*-acetylpenicillamine has also been advanced [399], particularly for treating exposure to mercury vapour. It has been reported from the Soviet Union that DMPS can be successfully used to counter accidental poisonings by various mercury compounds [400]. It has also been suggested that other sulphydryl chelating agents could prove effective [401]. However, BAL still appears to be the best choice for organic compounds, in spite of the danger that it may increase uptake of the metal into the brain. Of the very many other sulphur-containing compounds that have been examined, very few have proved to be of any value. It is noteworthy that neither DMPS nor any of the other possibilities have yet been listed in the British Pharmacopeia.

Plutonium decorporation

Very few people have ever received chelating agents for the purpose of plutonium decorporation. Volf has listed the only 54 cases involving all transuranic elements reported from 1954 to 1976 [200]. In view of the extensive publicity given to the hazards which are generally supposed to be associated with nuclear technology, it is a most striking fact that no significant harmful effects attributable to plutonium have ever been demonstrated in man [402]. This makes it difficult, if not impossible, to assess the real benefits of chelation therapy in humans.

On the other hand, a prolonged and internationally concerted research effort has been directed at investigating radionuclide contamination and its treatment. Most concern has focussed on accident prevention, but the best way to deal with acutely exposed workers has also received much attention. The high cost-to-benefit ratio of the work devoted to radionuclide chelating agents provides an interesting insight into the concerns and priorities of the general public and into the unsurpassed safety record of the nuclear industry.

Recommended treatment procedures for accidental radionuclide exposure depend largely on the way in which the metal has been incorporated. However, in all cases, administration of Ca(II)-DTPA should be commenced as a matter of great urgency. Doses of 1–2 g are administered [403], preferably within minutes of the accident, if possible by intravenous injection [200]. If the radionuclide has been inhaled, aerosols of the chelating agent are appropriate. They have the advantage that they can be administered by unskilled personnel [404]. Alternatively, intramuscular injections may be given for the same reason. In flesh wounds, surgical excision of contaminated tissue is standard practice, but when this is not practicable, irrigation of the injury with chelating agent solutions has been recommended.

Ca(II)-DTPA is the chelating agent of choice in all the above procedures. It is contraindicated only in pregnancy or in patients with severe disorders of the kidney or liver. In such cases, DFOA is probably the best substitute. There are grounds for thinking that a combination of DTPA and DFOA may prove most satisfactory [405].

It is important to continue chelation therapy for a considerable period after the contaminating incident. This is because microdeposits of the radioactive material may take a long time before being solubilized and entering the circulation. As Zn(II)-DTPA is much less toxic than Ca(II)-DTPA [406, 407] and as it is just as efficacious in delayed treatment [408], it has been suggested for prolonged chelating regimens.

It is interesting to note that Ca(II)-DTPA is more effective than Zn(II)-

DTPA so long as the radionuclide has not had time to translocate into tissues [409–412]. This effect has been examined by computer simulation [413] and has been attributed to the fact that, in plasma, DTPA is preferentially bound to Zn(II). Thus, when the chelating agent is administered as the Ca(II) complex, an exchange with Zn(II) tends to occur. At the plasma levels normally administered, there is insufficient exchangeable zinc in the biofluid to satisfy all the DTPA. So, a different equilibrium is set up compared with that reached when the Zn(II)-complex is injected. It is evident, under such circumstances, that the circulating concentration of free (uncomplexed) DTPA is increased and is thus more effective in complexing other metal ions present in plasma. Moreover, it is consistent to find that the difference between the zinc and calcium DTPA complexes diminishes as the target radionuclide is increasingly deposited in tissues and the process of leaching it back into plasma becomes the rate-limiting step in its decorporation.

Another observation which computer simulations help to explain, relates to the increased urinary excretion of plutonium, well above control levels, for many days after a single injection of polyaminocarboxylic acid. This appears to conflict with well established data showing that the chelating agents are almost entirely eliminated within 24 h. However, Schubert and Lindenbaum long ago appreciated that the levels of DTPA and EDTA would, nevertheless, remain very much higher than the levels of radionuclide even after a week [414]. This is a general fact in chelation therapy [65] which is often neglected. For the same reason, oral administration of polyaminocarboxylic acids may be quite adequate for long-term radionuclide decorporation, in spite of their limited absorption (D. Taylor, personal communication).

Nickel intoxication

In the early years of this century, nickel salts appear to have been something of a therapeutic vogue [415]. However, it was recognized quite early that nickel carbonyl ($Ni(CO)_4$) was extremely toxic: the lung, brain and kidneys [416, 417] are the critical organs.

Exposure to nickel usually occurs by ingestion or inhalation. Around 10% or less of soluble nickel salts taken orally will be absorbed across the gut wall. Acidic foodstuffs can also extract nickel from cooking utensils and storage containers. Nickel dermatitis is common especially amongst women in contact with nickel alloys used in the manufacture of clothes fasteners, spectacle frames, watches and coins. It has even been associated with the use of some detergents.

Nickel carbonyl poisoning of workers in the nickel refining industry is a

specific cause for concern [418–421]. However, nickel carbonyl may also be assimilated from cigarette smoke [422]. Rapid pulmonary absorption and facile transfer into tissues are responsible for the lethal nature of this compound. The metal is deposited inside cells, first by decomposition of the carbonyl to Ni(0), after which it is slowly oxidized to Ni(II). Thus, the metal ion and the released carbon monoxide both contribute to the toxicity.

The most effective antidote to nickel carbonyl at present is DDC [417]. Other powerful nickel chelating agents such as EDTA and TRIEN are of no use [423], even though they may be effective when the metal is administered as a Ni(II) salt. The fact that DDC has access to intracellular nickel, whereas the other agents generally do not, is undoubtedly the reason for this. The marginal benefits of BAL, which was used prior to the introduction of DDC [424], strengthen this argument.

From computer simulation studies [425] it seems that DDC *in vivo* is preferentially bound to Cu(II). On the basis that transition metal complexes of this agent are all very lipophilic and widely used for the extraction of these metals into organic phases, it seems probable that the DDC will thus soon become localized in cell membranes as $[Cu(II)(DDC)_2]$. Accordingly, its effectiveness against nickel carbonyl poisoning may be due to an ability to act as an ionophoric shuttle for Ni(II) across the membrane, exchanging the toxic metal ion inside for Cu(II) outside the cell. If this is so, co-administration of TRIEN to collect the Ni(II) as it appears in plasma would seem well worth investigating.

DDC is administered orally, as the sodium salt, in doses of about 1 g per day. In spite of its unique benefits in nickel carbonyl toxicity, it cannot be regarded as a very satisfactory agent for clinical use [426]. This is primarily because of some pronounced psychological side-effects which it possesses, most noticeably including acute depression and loss of sexual libido. Co-administration with drugs affecting the central nervous system is also contra-indicated because it inhibits certain enzymes involved in the metabolism of sedatives and anaesthetics. Furthermore, as it is unstable in acid solutions, it tends to decompose in the stomach before being absorbed. Attempts to overcome this using enteric-coated capsules have not met with much success (L. Morgan, personal communication). In addition, as DDC is a primary metabolite of disulfiram (tetraethylthiuram disulphide, Antabuse), the agent used for treating alcoholics, those receiving DDC for nickel carbonyl poisoning are required to abstain; this strains patient compliance. Interestingly, disulfiram has been administered, with some benefit, to sufferers of nickel dermatitis [417].

Aluminium osteomalacia and encephalopathy

Chelation therapy to counter aluminium intoxication has not been considered necessary until recent years. However, the high incidence of osteomalacia and encephalopathy in patients on renal dialysis has prompted a spate of research activity in this area. The aluminium is derived from the water supply used by the dialysis machines [427]. It tends to lead to the development of hypercalcaemia, which is responsible for bone demineralization [428], and the progressive manifestation of a devastating and ultimately fatal dementia, the detailed pathogenesis of which remains unknown.

Similarities between the symptoms of this dialysis encephalopathy and the senile disorder known as Alzheimer's disease as well as increased concentrations of aluminium in the brains of individuals with Alzheimer's disease, led to the suggestion that the two conditions may be associated [429]. However, opinion is sharply divided about this conclusion [430–435].

Nevertheless, the need for an efficient aluminium chelating agent is clear. The only one to have been tried so far appears to be DFOA [436–439]. Initial reports suggest that it is capable of removing aluminium from patients with dialysis encephalopathy but it is not satisfactorily efficient. It also has the disadvantage of removing iron from subjects, many of whom are already relatively iron deficient. So, there is an urgent need to find a superior antidote.

ORPHAN DRUGS FOR ORPHAN DISEASES

The term 'orphan diseases' was coined by Petersen and Cerami to describe those disorders for which drug research and development is not economically viable [440]. Many of the conditions requiring chelation therapy fall into this category either because those affected are relatively few in number or because they cannot afford treatment. It is a most disturbing matter with profound philosophical, political, ethical and legal overtones. So complicated is it that any decided view probably depends on personal feeling rather than objective criteria. Nevertheless, the problem cannot be ignored, since it is perhaps the single most restricting factor confronting those searching for new chelating drugs.

The development of drugs nowadays is very expensive [441]. Pharmaceutical companies which nurture a prospective agent through the many stages leading to full-scale production and marketing must typically invest sums between £16 million and £40 million [442]. This amount, and any profit, has to be recovered over the relatively short period during which the initial lead over competitors can be maintained. Twenty years of patent protection is not much

when at least half this period is often spent on animal and clinical trials before the agent can be sold commercially. It is thus not surprising that the pharmaceutical industry tends to restrict its research activities to very widespread medical conditions [443].

At the root of these enormous development costs is public demand for totally safe drugs [444]. There is unremitting social pressure to protect everyone from any harm which the practice of medicine in all its variety may ever bring about. Both mistakes and side-effects are regarded as a betrayal of good faith.

This feeling is understandable enough, particularly in view of the publicity given to tragedies such as happened with thalidomide. But there is a price to pay which is sometimes equally unacceptable. It is that many benefits of modern medicine are lost by default. Thus, public opinion needs to be made more aware of the damage which can be done by clamour for drug safety at all costs.

Almost every facet of chelation therapy is beset by this problem. Disorders of mineral metabolism are relatively rare and improvements in industrial hygiene and greater domestic precaution have considerably reduced the frequency of heavy metal poisoning. So, in practice, the development of chelating therapies has fallen to either state-funded institutions or individual physicians dealing with the particular problems of their own patients. In both cases, objective assessment of potentially better agents is seldom a high priority. So, alternative treatments are usually attempted in desperate circumstances only and defects in conventional approaches are endured for the sake of proven benefits. This reflects a *de facto* acceptance by the medical profession that, in all but the most hopeless of cases, avoidance of risk to the individual is paramount even if this means relinquishing opportunities to improve the patient's quality of life. It also, of course, justifies slower clinical progress.

In spite of this manifest conservatism (or, perhaps, because of it) many of the pioneers in chelation therapy have found themselves in a most invidious position. For example, one can cite the criticism [445, 446] of the work by Sternlieb and Scheinberg on patients with asymptomatic Wilson's disease [447]. Also in the context of Wilson's disease, there is the battle Walshe has fought to ensure that sufficiently pure TRIEN will continue to be available to treat those who are allergic to penicillamine [448, 449]. The abrupt withdrawal of DTPA from clinical testing in the USA in 1967, even though its efficacy in plutonium removal was clearly established and no significant toxic effects had occurred in man, is a case where over-zealous safety considerations have subsequently been reversed [450].

It should be emphasized that this conservative approach is probably for the best. It is not always clear when it is ethically correct to administer chelating

agents [360, 445, 451, 452] or, for that matter, any drug. However, it would be less worrisome if the costs of the policy were more universally debated instead of being so studiously ignored [444].

POSSIBLE DEVELOPMENTS IN CHELATION THERAPY

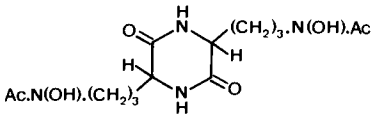
SURVEY OF AGENTS TESTED IN ANIMALS

In this section an account is presented of laboratory investigations of mainly novel chelating agents which may have implications for the future management of metal imbalances.

Iron

The expense of DFOA and the problems presently associated with the therapy of iron overload have provided considerable impetus for research into new iron chelating agents. The need for alternatives to DFOA has prompted work in three directions.

1. The search for new microbial siderophores has identified several chelating agents which might replace DFOA. Foremost amongst these [453] is rhodotorulic acid (RHODA, 16) which was originally isolated by Atkins and Neilands [454]. Unlike DFOA, it can be isolated easily in a very pure form from the spent growth media of the yeast *Rhodotorula pilimanae* in yields as high as 10 g l^{-1}. It would, therefore, be much cheaper than DFOA. The toxicity of the chemical is minimal at parenteral doses of less than 250 mg kg^{-1}, although it does increase the excretion of zinc and causes local inflammatory reactions [455]. The low aqueous solubility of RHODA means that it can be used in a depot form: DFOA injected intrasmuscularly into dogs is eliminated from the body within 2 h, whereas RHODA is still detectable at 12 h. In humans, however, RHODA, administered intravenously was only 16% more efficient than DFOA in enhancing excretion of iron. Moreover, injections of the suspension proved very painful. It is thus unlikely to be used in the clinic unless the inflammatory effects can be suppressed [455, 456].



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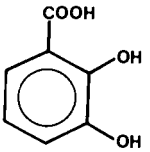
An evaluation of the efficacy of desferrichrome A, hadacidin and aerobactin in the removal of iron from hypertransfused mice showed that they were all ineffective [457]. Also shown to be without effect in this study were sodium thioformin and the desferri forms of schizokinen, fusarinine, triacetylfusarinine C and *N*-(2,3-dihydroxybenzoyl)glycine. Liposomally entrapped lasalocid A, when administered concurrently with DTPA, failed to clear ^{59}Fe from mice [163]. As it is the salicyl moiety of this calcium-selective ionophore which complexes Fe(III), it is possible that the iron became associated with the cell membrane. In contrast, ionophore A23187 administered in liposomes concurrently with Ca-DTPA produced a greater iron excretion than DTPA alone. This is interesting, since the calcium-selective ionophore has very little affinity for Fe(III).

As agrobactin (*N'*, *N''*, *N'''*-tri(2,3-dihydroxybenzoyl)spermidine), a siderophore from *Agrobacterium tumefaciens* [458], is superior to DFOA in suppressing the uptake of ^{59}Fe into cultured rat myocytes [459], it might prove capable of achieving a negative iron balance in the clinic. Related to agrobactin is N^1,N^8-bis(2,3-dihydroxybenzoyl)spermidine, which has been shown to decrease uptake of ^{59}Fe into Chang cells and also inhibit uptake of iron into ferritin [460]. Three properties of the latter – its straightforward laboratory synthesis, an LD_{50} in mice >800 mg/kg and its ready absorption from the intestine – suggest some potential as an alternative iron-chelating agent [461].

2. Synthetic ferric-complexing agents have continued to be examined in spite of the apparent success of the microbial siderophores.

Sixteen synthetic hydroxamic acids, typified by species such as hydroxamic acid derivatives of amino acids and 3,3'3''-nitrilotris(propionohydroxamic acid) hydrochloride, failed to produce any significant change in the distribution of iron in hypertransfused mice [457]. 3-Isopropyltropolone, 3-(1'-methylprop-1'-enyl)- and tropolone-5-sulphonic acid also failed this screening test. In fact, some of these compounds actually increased the uptake of iron into the liver. Furthermore, it was noted that the bone-seeking chemical, ethane-1-hydroxy-1,1-diphosphonic acid (EHDP), elevated the hepatic and spleenic iron levels by 55%, yet enhanced the urinary clearance of iron as much as 150%. This elevation of iron in the liver by a hydrophilic chemical is unexpected; it may arise from the formation of an insoluble complex in plasma. Of the other compounds examined, *N*-(2,3-dihydroxybenzoyl)glycine was the only catechol to enhance urinary excretion of iron. However, it failed to alter hepatic and spleenic iron contents. In comparison, 2,3-dihydroxynaphthalene-6-sulphonic acid lowered the iron burden of the spleen and liver by 39% and 26%, respectively. The tetradentate catechol, tetramethyl-1,3-bis(3'-[2'',3''-diacetoxyphenyl]propyl)disiloxane, produced no change in the hepatic and spleenic iron burdens, but lowered the urinary clearance.

The last compound is an example of some of the innovative work which has recently come from several organic chemistry laboratories. The introduction of the siloxane moiety into molecules facilitates the formation of strain-free ferric complexes. There is the additional advantage that siloxanes are non-toxic and are normally not metabolized. To protect the air-sensitive catechol groups in this molecule, they were acetylated. However, like the above siloxane, the closely related hexadentate catechol derivative, 1,1,3,5,5-pentamethyl-1,3,5-tris[3-(2,3-diacetoxyphenyl)propyl]trisiloxane, also showed no activity.



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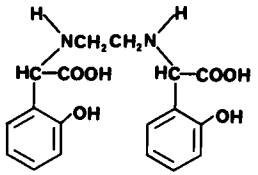
Developments with 2,3-dihydroxybenzoic acid (DHB, 17) have proved disappointing [462] after the initially encouraging reports [463–465]. Administered orally to rats, this agent causes a significant increase in urinary iron excretion. However, in a recent clinical trial, a single oral dose of 25 mg kg^{-1} DHB yielded an average net drug-induced iron excretion of 4.5 mg day^{-1} [466]. Most of this iron appeared in the faeces, in contrast to the iron-overloaded rat in which the mobilized iron appears largely in the urine. It was concluded that DHB was unlikely to restore patients into iron balance. However, the compound might still serve as a useful sink for iron-induced free-radical reactions which are believed to be responsible for the tissue damage caused by iron deposition [467].

Very few of the chelating agents examined have potential as orally administered drugs. An interesting exception is cholyhydroxamic acid, which enhances the excretion of iron from the hypertransfused rat [468]. It is also possible that ethylenediamine-*N*,*N*′-di(*o*-hydroxyphenylacetic acid) (EDDHA, 18) has been overlooked for many years as a potential therapeutic chelating agent [155, 469, 470]. Administered intravenously at a dose of 3 g per patient, EDDHA produced urinary clearances of iron around 18 to 25 mg day^{-1} [469]. It is at least as effective as DFOA in the new, rapid assay developed by Pippard, Johnson and Finch [471]. The absence of nephrotoxicity and the fact that it can be taken orally might make it worth re-examining [457].

Macromolecular forms of chelating agents have been examined occasionally for ability to mobilize metals, and a few will be discussed in other sections of



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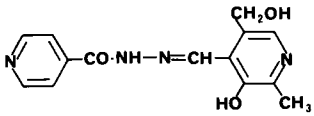
this review. Poly(*N*-methacryl-*β*-alanine hydroxamic acid) administered at 300 mg kg^{-1} decreased hepatic and spleenic iron levels by 52% and 20%, respectively, and raised urinary levels by 380% [457].

Several pyridine derivatives have been examined for their ability to mobilize iron [457, 472–475]. In human studies, 5-hydroxypyridine-2-carboxaldehyde thiosemicarbazone enhanced excretion of iron by 6–100-times the control levels in cancer patients [472]. However, it should be noted that DFOA gives a similar elevation and that the pyridine derivative, at least, is far too toxic to be useful as an iron chelator. Interestingly, the ferric complexes may, themselves, be the active anti-cancer species *in vivo* [475]. As 5-hydroxypyridine-2-carboxaldehyde thiosemicarbazone appears to complex iron in the ferrous form [472], the preparation of Fe(II)-specific complexing agents to complement ferric-specific complexing might be rewarding.



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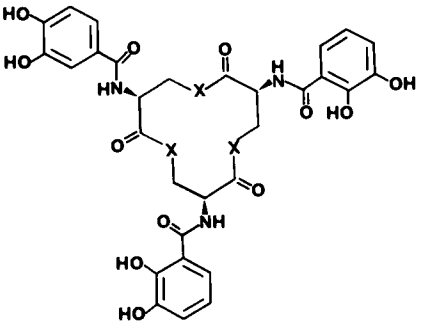
There has been a flurry of activity concerned with pyridoxal isonicotinoyl hydrazone (PIH, 19) [471, 474, 476–480]. Initial signs are very promising, not least because it appears to be effective when given orally [477, 478]. It has been shown to increase biliary excretion of iron derived from the catabolism of haemoglobin [474]. However, this effect is probably due to interference with some metabolic process rather than to chelation *per se*. Administered to rats in two doses of 250 mg kg^{-1}, it elevated the biliary excretion of iron from 1.9 μg per 24 h to 82.7 ±17.5 μg per 24 h. In comparison, DFOA administered under the same conditions enhanced biliary excretion to 75.7 ± 8.6 μg per 24 h. Subsequently, it was shown that iron is depleted from both parenchymal and reticuloendothelial stores. Urinary excretion of iron is also elevated in

animals injected with this hydrazone. Whether comparable effects can be achieved in humans, however, remains to be seen.

Although there was no change in the iron levels in the spleen and liver in the mouse, di(pyridine-2-carboxaldehydo)azine increased clearance of iron into urine by over 300% [457].

3. The biomimetic approach has produced several compounds which appear to merit further investigation at the preclinical stage.

The structure of enterobactin (20, X = O) has served as the platform for a wide variety of biomimetic compounds. A trimeric cyclic ester of 2,3-dihydroxybenzoyl-*N*-serine, it forms an exceptionally stable complex with Fe(III). Unfortunately, it is too readily degraded to be useful in chelation therapy. Unlike the hydroxamates, these catechol-based siderophores have the kinetic



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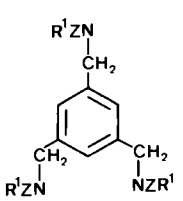

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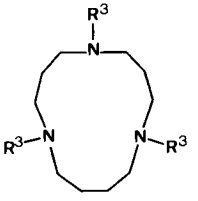

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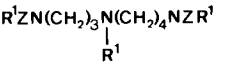

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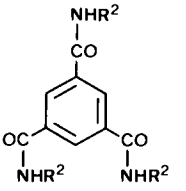

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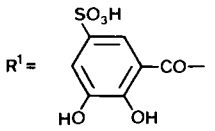
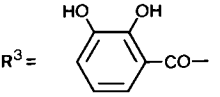
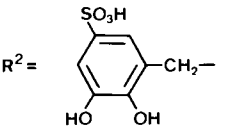






ability, as well as the thermodynamic strength, to remove iron from transferrin [481].

Also, unlike enterobactin itself, several synthetic derivatives, namely, the catechol (20, X = NH), MECAM (21, Z = H, $R^1 = R^3$ = 2,3-dihydroxybenzoyl), MECAMS (21, Z = H, R^1 = 2,3-dihydroxy-5-sulphobenzoyl), CYCAM (22, R^3 = 2,3-dihydroxybenzoyl), LICAMS (23, Z = H, R^1 = 2,3-dihydroxy-5-sulphobenzoyl) and COCAMS (24, R^2 = 2,3-dihydroxy-5-sulphobenzyl) are resistant to hydrolysis over a wide range of pH [482–484]. The introduction of sulphonic acid groups increases the water solubility of the chelating agents and also stabilizes the catechol groups to air oxidation.

Investigations of the kinetics of the reaction of these chelating agents with iron-saturated transferrin show a rapid formation of a ternary complex with transferrin followed by a slow step in which the apotransferrin and the Fe-LICAMS complex is liberated. The formation constants of MECAM and CYCAM with Fe(III) have been estimated to be log β = 46 and log β = 40, respectively [485].

As the proton-dependent formation constants are not a good indicator of the ferric ion sequestering properties of a chelating agent, Weitl, Harris and Raymond have determined the equilibrium concentration of $[Fe(H_2O)_6]^{3+}$ in solutions having 1 μM total iron and 10 μM total ligand at pH 7.4 [484]. The results ($pM = -\log[Fe(H_2O))_6]^{3+}$) permit comparison of the various chelating agents as presented in *Table 5.2*. The larger the value of p*M*, the more effective is the ligand as a ferric-chelating agent. As all the chelating agents had p*M* values greater than the p*M* value of transferrin, they are assumed to be thermodynamically capable of depleting iron from the metalloprotein.

In another study it was shown that LICAMS is capable of sequestering iron from ferritin but that the kinetics of iron labilization, involving reduction of the metal ion, controlled its removal [486]. In conjunction with the fact that

Table 5.2. p*M* VALUES FOR SYNTHETIC CATECHOLAMIDES AND PERTINENT BIOLOGICAL IRON CHELATORS

Chelating agent	*p*M
Enterobactin	35.5
MECAMS	29.4
MECAM	29.1
LICAMS	28.5
DFOA	26.6
COCAMS	25.1
Transferrin	23.6

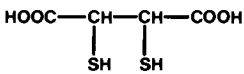
ascorbate enhances the action of agents such as DFOA, this observation suggests that ascorbic acid should routinely be co-administered during the evaluation of new iron-chelating agents.

Copper

Other than TRIEN, several polyamines, both linear and cyclic, have been examined for cupriuretic activity in laboratory animals [175, 487–489]. Borthwick, Benson and Shugar have reported that 3,7-diazanonane-1,9-diamine (2,3,2-tetramine, $NH_2(CH_2)_2NH(CH_2)_3NH(CH_2)_2NH_2$) possesses considerable cupriuretic activity and is effective when administered by gavage [487]. In addition, they considered 2,3,2-tetramine to be superior to TRIEN in the following respects: (i) the Cu(II) complex with 2,3,2-tetramine is more stable than the equivalent TRIEN complex ($\log \beta = 23.9$ as opposed to $\log \beta = 20.2$) and (ii) the purification of 2,3,2-tetramine is straightforward and no potentially toxic isomers are formed. In an evaluation of TRIEN, 2,3,2-tetramine and PEN, the agents were infused into rats and the extent of cupriuresis noted at various times [488]. During the infusion period, 2,3,2-tetramine was the best cupriuretic agent. TRIEN was only 66% and PEN 40% as effective. In the post-infusion period, 1.5 to 3.5 h after infusion, 2,3,2-tetramine remained the most effective. Two studies from West Germany show that simultaneous application of TRIEN and PEN does not produce an additive effect in depleting rats of copper [489, 490]. TRIEN administered orally is inferior to TRIEN administered subcutaneously by a factor of three [489]. TRIEN-induced teratogenesis in rats has been reported [491], which contrasts with Walshe's experience in both animals and humans [241].

One of the problems associated with studying copper-overload conditions is the difficulty in developing a suitable experimental model. Recently, Sternlieb has recommended the Bedlington terrier as a suitable animal model for chelation therapy [492].

It has been reported that DMPS was the most effective agent in a sparing-test conducted on mice injected with a solution of copper sulphate at a dosage rate sufficient to yield a fatality rate of 95% [493]. Whereas the mice that



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received DMPS had a survival rate of 83%, those animals that received 2,3-dimercaptosuccinic acid (DMSA, 25), BAL and TRIEN had survival rates of 20%, 20%, and 33%, respectively. However, it is relevant to note that there are few fatalities following accidental consumption of solutions of copper salts.

Although DDC is an effective chelating agent for depleting the body of nickel, it is of no value in combatting copper-load, for it enhances the uptake of copper into the liver and central nervous system [494].

Lead

Graziano, Leong and Friedheim have compared BAL, EDTA, DMSA and PEN for efficacy in removing lead from minimally lead-poisoned rats [495]. In this study it was reported that PEN was ineffective and that orally administered DMSA was as effective as the recommended counter-measure against lead-poisoning, parenterally injected BAL and EDTA. DMSA is less toxic than BAL.

As lead in soft tissues is not available to hydrophilic chelating agents such as EDTA and DTPA, there have been investigations into the value of more lipophilic forms of chelating agents. The bisbutyl ester of DTPA injected into the intraperitoneum of mice, subjected to intravenous injections of lead citrate, was more effective than DTPA and EDTA in reducing the uptake of lead into liver [496]. In this study the chelating agents were injected 4, 18 and 24 h after injection of lead and the animals were killed after 48 h. Under these conditions, both forms of DTPA appeared equally effective in reducing the uptake of lead into the brain.

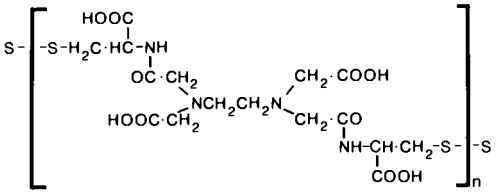
Baudot, Jacque and Robin have demonstrated that the cryptand (10, X = O) administered intravenously, 18 and 24 h after feeding rats with lead acetate, elevated urinary excretion of lead around 4-fold [497]. However, as observed by the authors, this kind of increase has also been found for BAL, EDTA and DTPA. Unfortunately, no investigation of the ability of this cryptand to mobilize lead from intracellular sites was carried out.

Cadmium

As early as 1946, forty-four mercaptans were screened for potential as therapeutic counter-measures to cadmium-poisoning [498], but of all the compounds examined, only one, BAL, really proved to be sufficiently powerful. The following mercaptans have been found to be of little or no value in mobilizing cadmium from the body once metallothionein synthesis has started: PEN [499, 500]; mercaptosuccinic acid and *N*-acetylpenicillamine [500];

DMPS, 1,9-dimercaptononane, cysteine and 3,4-dimercaptotoluene [387]; 1,3-dimercaptopropan-2-ol [388]; dithioerythritol and DMSA [501]; 1,2-dimercaptoethane, 1,2-dimercaptopropane, 1,3-dimercaptopropane [502]. On the other hand, 1,2,3-trimercaptopropane (TMP) is slightly better than BAL at enhancing cadmium clearance into the bile. In control rats, cadmium levels averaged 0.04% of the injected dose, whereas in animals injected after a delay of 24 h with BAL and TMP, the level of cadmium in bile reached 3.1% and 5.5%, respectively. Gel permeation chromatography on Sephadex G-75 of bile from rats treated with either TMP or with BAL suggested that the cadmium was associated with different components of the bile. In the bile of animals injected with TMP, cadmium was bound to high-molecular-weight components, but similar species could not be detected in the bile from animals injected with BAL. It was thus conjectured that TMP, or its metabolites, had formed disulphide bonds with the proteins in bile.

When administered soon after injection of cadmium into animals, DTPA is very effective but it fails to mobilize cadmium once it has been sequestered by metallothionein [503]. Biscysteineamido derivatives of EDTA (26) and of DTPA have been synthesized in NRPB laboratories. The reduced form has been examined for ability to clear cadmium from rats and hamsters, but was found to be of limited value (F.D. Bonner, personal communication). DDC increases brain uptake of cadmium by forming a lipophilic complex which permeates the blood-brain barrier [504].



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L-Amino acid oligopeptides possessing three cysteine residues and analogous to the cadmium-binding site of a variety of metallothioneins have been synthesized [505] and their effectiveness against cadmium-induced fatalities in mice examined. Mice injected subcutaneously with the oligopeptides 15 min prior to injection of cadmium chloride into the intraperitoneal cavity had survival rates ranging from 80 to 100% at 48 h after injection of cadmium. In contrast, the control animals had survival rates of only 20–38%. As linear peptides are readily degraded, cyclic peptides may be more effective.

Lehn has reported that the cryptand (10, X = NMe) displays a high selectivity ($10^6 \approx 10^7$) for cadmium with respect to the zinc and calcium cations [506]. It is unlikely, however, that the cryptand will remove cadmium from the body once it becomes incorporated into metallothionein.

Bakka, Aaseth and Rugstad have developed a screen for cadmium-chelating agents based on cultured epithelial cells [140]. This shows DMPS and DMSA to be superior to BAL and with less toxic effect. The claims advanced for mixed-ligand chelation therapy of cadmium and plutonium [507] were contested [508–511] and have been withdrawn [512].

Mercury

Many chelating agents, principally sulphur-containing ones, have been examined for ability to clear mercury from the body. From studies on rats it would appear that DMPS is superior to DMSA in removing mercury initially injected as mercuric chloride [513–515]. However, from investigations into their efficiency in removing methylmercury from rats the order is DMSA > DMPS > *N*-acetylpenicillamine [516]. This order of efficacy parallels the decrease in lipophilicity of these chelating agents. On the other hand, in acute mercuric chloride poisoning it would appear that *N*-acetylpenicillamine and DMPS are superior to DMSA, BAL and PEN [517]. Planas-Bohne and Olinger have also recently considered the interaction of various chelating agents with methylmercury bound to erythrocytes [518].

In the last decade, several entirely different chelating agents have been examined for ability to counter mercury poisoning. These new mercury-binding chemicals fall into two groups: (i) sulphur-containing macromolecules and (ii) low-molecular-weight sulphur-containing agents.

Several studies have shown that the hepatic recirculation of methylmercury can be suppressed by ingestion of methylmercury-binding macromolecules [87, 519–524]. The macromolecules investigated are either polysaccharides derivatized so that thiol groups are introduced [521], or polymers generated from dialdehydes and oligothiol-containing monomers [522–524].

The most effective thiolated polysaccharide is mercaptostarch, for mercaptodextrans are either degraded or oxidized in the gut [521]. Orally administered mercaptostarch added to food to give a concentration of 2%, together with *N*-acetylpenicillamine therapy (four subcutaneous injections of 1 mmol kg^{-1}) was found to be an effective procedure for mobilizing methylmercury from mice [521]. Therapy commenced 4 days after intravenous injection of the methylmercuric chloride. In the treated group, retention of methylmercury in the liver, kidneys, brain and blood was 46%, 30% 58% and 30%, respectively,

compared with control animals. Of particular note was the effectiveness of low doses of *N*-acetylpenicillamine; much higher doses are normally used.

Unlike mercaptostarch and the polymers generated from dialdehydes and oligothiols, such as pentaerythritol tetrathioglycolate, the polymers produced from dialdehydes and dithiols such as mercaptoethyl sulphide possess no free thiols. However, the effectiveness of these latter compounds can be judged by the reduction in the biological half-life of methylmercury from 10 days in control mice to 4.5 days in mice maintained on a diet containing 1% of a polymer prepared from terephthalaldehyde and mercaptoethyl sulphide [525]. Initial investigations of the toxic properties of the aforementioned polymer indicate an LD_{50} value in excess of 5 g kg^{-1} and no uptake from the gut of the ^{14}C-labelled polymer.

Sulphur-containing steroids have been introduced as mercury-binding agents on the assumption that their highly lipophilic nature would minimize the burden placed upon the kidneys [526, 527]. The sulphur-containing steroids were entrapped in liposomes and administered by injection into the intraperitoneal cavity. The efficacy of the sulphur-containing steroids was then judged by life-prolongation and survival rate. BAL, PEN and the steroids were injected into the intraperitoneal cavity of mice 48 h and 24 h before injection of methylmercuric chloride (16 mg kg^{-1}) and this regimen was continued for a further 24 h. The animals that received no agent to counter methylmercury had a 13% survival rate after 14 days, compared with those animals treated with BAL and PEN which had rates of 24% and 33%, respectively [526]. In contrast, 80% of those mice receiving thiocholesterol and 3β-mercapto-5β-cholan-24-oic acid survived. Under a similar screening procedure, mice injected with 5α-cholestane-2β,3α-dithiol and 5β-cholane-3β,24-dithiol had survival rates of 77% and 60%, respectively [521]. By way of a comparison, all the mice treated with 1-dodecanethiol died.

Yonaga and Morita have evaluated *N*-(2,3-dimercaptopropyl)phthalamidic acid for ability to mobilize mercury from mice. They found it superior to BAL and PEN in increasing urinary, and faecal, excretion [528]. The particular enhancement of biliary clearance serves to minimize the exposure of the kidneys to mercury. The authors also found this agent to be most effective when therapy commenced after a delay of 24 h.

The mercury in bile from mice treated with *N*-(2,3-dimercaptopropyl)-phthalamidic acid was readily absorbed from the small intestine and was, thus, different from the mercury in bile from BAL-treated animals. If the animals were to be maintained on a diet supplemented with mercaptostarch, this new chelating agent might prove even more effective. Other amide derivatives of succinic and malonic acids would be another promising line for further investigation.

Aluminium

An examination of several polyaminocarboxylic acids has shown that EDDHA and *N,N'*-di(2-hydroxybenzyl)ethylenediamine-*N,N'*-diacetic acid form very stable mononuclear chelates with aluminium [529]. These chelates are not susceptible to hydrolytic changes at neutral pH, unlike the EDTA chelate, which hydrolyzes above pH 5.0.

Some evidence that aluminium is normally cleared into bile comes from the observations of Soroka, who obtained enhanced urinary clearance of aluminium in animals with ligated bile ducts [530]. If there is biliary clearance of aluminium, it is possible that partially lipophilic polyaminocarboxylic acids might well be of some value in reducing aluminium retention in renal dialysis patients. Partially lipophilic phosphonic acid analogues of the polyaminocarboxylic acids might also prove useful agents for complexing this HSAB 'hard' acid.

The demonstration that gallium can be cleared from the body by the biomimetic iron-chelating agents [531], leads one to suppose that they might also mobilize aluminium. DHB derivatives, generally, would seem promising.

Chromium

Investigations into therapeutic procedures to counter chromate burdens have been limited to only a few studies. Behari and Tandon injected rats intraperitoneally with the following chelating agents: ethylenediamine-*N,N'*-diacetic acid (EDDA), ethyleneglycolbis(2-aminoethyl)tetraacetic acid (EGATA), hexamethylene-1,6-diaminetetraacetic acid (HDTA), EDDHA, HEDTA and TTHA [532]. All the agents were administered at 0.0385 mmol kg^{-1} some 48 h after the termination of the injection regime, by which time the animals had received potassium chromate (2 mg Cr kg^{-1}) 6 days a week for 45 days. The efficacy of the chelating agents was: HDTA (52% depletion) $\approx$ EDDA > TTHA > EGATA $\approx$ EDDHA (20% depletion) for decorporation of chromium in the liver; in the kidney the order was HEDTA (78% depletion) > EDDHA > EGATA > EDDA (9.6% depletion); in the heart, EDDHA (55.2% depletion) > EDDA > HDTA > EGATA > TTHA (0.4% depletion); in the brain, EDDHA (67.2% depletion) > HDTA > EDDA > EGATA > HEDTA (0.0% depletion). In a similar experiment with mice using other chelating agents, the order of efficacy was: EDTA (61% depletion) > DTPA > NTA > DDTA (38.5% depletion) in the liver; DDTA (66.9% depletion) > DTPA > NTA $\approx$EDTA (27.5% depletion) in the kidney; DTPA (72% depletion) > EDTA > DDTA > NTA (34.6% depletion) in the brain;

EDTA (72% depletion) > NTA ≈ DTPA > DDTA (14% depletion) in the testes [533]. In this study, DDTA was 3,6-dioxooctamethylenedinitrilotetraacetic acid. These differences make it difficult to draw any conclusions about the ideal chelating agent for removing chromium from the body. The authors did not include in the study an additional group of rats which had been injected with HDTA, TTHA and EDDHA.

It is reported that an ointment containing 10% sodium calcium EDTA gives some protection to the skin against development of chromate-induced ulcers [534].

Manganese

Eleven oxidation states (+7 to −3) of manganese and its wide distribution in animals and plants makes manganese chemistry of considerable interest to bioinorganic chemists. Animals deficient in manganese exhibit symptoms such as malformation of neonates, infertility and ataxia. Excessive manganese intake may result in retardation of growth, interference in the uptake of calcium and phosphorus, reduction of haemoglobin formation and the development of neurological disorders. Its distribution in mammalian tissues and fluids tends to remain constant due to controlled excretion, principally in bile, rather than to variable absorption. Injection of ^{54}Mn(II) into the peritoneal cavity of rats demonstrates its immediate accumulation in the brain, where it can remain for up to 30 days [535]. An inability to clear manganese from the brain could be responsible for the neuronal damage caused by chronic exposure to the element. The element exhibits marked elevation in the hypothalamus and in the pineal gland [536]. The symptoms of manganese poisoning are strikingly similar to the psychiatric and neurological symptoms encountered in Parkinson's disease [537]. Manganese intoxication and its attendant neuropsychiatric manifestations have been observed after drinking water containing high levels of manganese [538]. Manganese poisoning is also frequently noted in manganese ore miners [537]. EDTA has been used occasionally, with inconsistent success, in workers exhibiting symptoms of manganese toxicity [539, 540].

Investigations into chelation therapy to counter manganese intoxication have been limited. Most work has been concerned with various polyaminocarboxylic acids [197, 541–544]. In a recent study of PEN and NTA, cyclohexane-1,2-diaminotetraacetic acid (CDTA), DTPA and *p*-aminosalicylic acid (PAS) [545], Tandon administered manganese dioxide by intratracheal incision to rabbits. After a delay of 120 days, faecal and urinary samples were collected for 3 days prior to injection of PAS (80 mg kg^{-1}), CDTA (80 mg kg^{-1}) and a combination of the two. CDTA increased urinary excretion from 10.8 μg l^{-} to 19.4 μg l^{-1}.

Post-treatment response lasted for 3 days. A similar enhancement was noted for the faecal clearance at 49.9 $\mu g\ g^{-1}$, compared with a pretreatment level of 28 $\mu g\ g^{-1}$. The biliary clearance of manganese would indicate that further research into countering manganese poisoning should investigate chelating agents cleared through bile. In this context, it would be interesting to determine the effectiveness of partially hydrophilic phospholipids.

Administration of DTPA to rabbits in a similar experiment produced only a marginal increase in faecal clearance of manganese, whereas in HEDTA-treated animals the faecal clearance was raised to 328 $\mu g\ g^{-1}$ 48 h after injection [546]. In the same study, further evidence of the ineffectiveness of sulphur-containing chelating agents such as DDC and DMSA was obtained. Tandon and Khandelwal have also found that polyaminocarboxylic acids are generally more effective than sulphydryl chelating agents [547]. In view of the HSAB 'hard' characteristics of Mn(II), these observations are only to be expected.

The chelating agents CDTA and DTPA are of limited value in treating long-term manganese poisoning because it is necessary to remove manganese from neuronal sites of deposition. The similarity of manganese-poisoning to Parkinson's disease initiated an evaluation of drugs used in the treatment of Parkinson's disease as therapeutic countermeasures to manganism in man [548]. L-3,4-Dihydroxyphenylalanine (DOPA) and 5-hydroxytryptophan, precursors of dopamine and serotonin, respectively, given in oral doses of 8 g day^{-1} resulted in improvements in five out of eight patients. However, on cessation of treatment all the patients relapsed.

Nickel

Several chelating agents for nickel have been examined for therapeutic properties. An evaluation for ability to prevent death in rats after a single parenteral injection of nickel chloride of six chelating agents administered in equimolar dosages showed that PEN and TRIEN were approximately equal in efficacy and were superior to diglycyl-L-histidine-*N*-methylamide, DDC and EDTA [549]. Other workers have subsequently come to compatible conclusions in respect of nickel acetate [550]. A converse relationship is found with nickel carbonyl.

An early examination of a series of alkyl dithiocarbamates showed that the dimethyl, diethyl, diisopropyl and morpholine-1 derivatives, at parenteral dosages of 50 mg/kg, provided 100% protection against the toxic effects of nickel carbonyl [11]. DDC, with an LD_{50} of 1.5 g in mice, was found to be the least toxic of the dialkyl dithiocarbamate series. It has subsequently been established that DDC and PEN are more effective than TRIEN as antidotes for

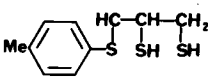
acute nickel carbonyl poisoning in rats [551]. However, PEN was found to have toxic side-effects that outweighed therapeutic benefit [11]. BAL, although partially effective, increased the LD_{50} value of nickel carbonyl in rats by a factor of 2 [417].

Experiments with DDC have shown that it reduced the incidence of rhabdomyosarcomas to 50% in rats which had received nickel subsulphide implants, compared with an incidence of 84% in animals which received no therapy [417]. On the basis of such observations, Sunderman has suggested that patients with nickel prostheses should receive periodic treatment with DDC to guard against the possibility of nickel-induced tumour development [417].

Arsenic, antimony, bismuth, thallium and gold

Pronounced similarities are evident in both the toxicity and chelation therapy of As(III) and Sb(III). As 'soft' HSAB acids, they interfere with sulphydryl-dependent enzyme systems and are most effectively sequestered by dithiols.

BAL has been recommended to counter arsenic poisoning since the late 1940's. Although DMPS and DMSA appear to be just as effective, they are not listed in the British Pharmacopoeia. The studies of Tadlock and Aposhian on mice would indicate that DMPS and DMSA are capable of protecting against the lethal effects of subcutaneously administered sodium arsenite [552]. In these studies, both PEN and *N*-acetyl-DL-penicillamine afforded no protection. This absence of any protection from PEN is in contrast to its reported use on a child who was believed to have ingested arsenic trioxide [553]. However, as the child was initially treated with BAL, it is possible that this prompt administration, rather than the subsequent oral treatment with PEN, saved the child's life. Tadlock and Aposhian also found that DMPS and DMSA were effective if taken orally 15 min before ingestion of the arsenite [552]. Subsequent work showed that DMSA was superior to DMPS and that, by comparison, PEN and *N*-acetylpenicillamine were without benefit [554]. In the Soviet Union it has been proposed that arsine poisoning can be countered by the administration of 3-(4-tolylthio)propane-1,2-dithiol (*p*-tolylthioether-1-(2,3-dimercaptopropane), 27) [555]. A recent study suggests DMPS and DMSA may also be very effective [556].



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In a study similar to that of Tadlock and Aposhian, Basinger and Jones have demonstrated that DMPS and DMSA are the most effective agents for protecting mice against the effects of potassium antimonyl tartrate [557]. The tartrate was injected into the intraperitoneum at doses of 120 mg kg^{-1} and the chelating agents administered in a 10 molar excess, also injected into the intraperitoneum, after a delay of 20 min. In contrast to the arsenite study, PEN was found to be moderately effective, achieving a survival rate of 40%. Of particular interest was the ineffectiveness of BAL, although it should be noted that because of its toxicity is was used only at a molar ratio of 1 : 1. This study also found that DMSA, with a survival rate of 93% was much more efficient than DMPS, for which the corresponding rate was only 63%.

It has been concluded from some related work that BAL can be used to combat bismuth poisoning [558]. Presumably DMPS and DMSA might also be effective.

Bendl has reported the successful treatment of thallium poisoning with diphenylthiocarbazone (dithizone) [559]. However, there is the danger that this chemical might induce diabetes, possibly by complexation of zinc in the pancreatic β-cells [560]. As DDC redistributes thallium to the brain, it is not a suitable chelating agent to use in cases of thallium poisoning [561]. A similar danger exists with BAL [562].

Although gold toxicity is a fairly common complication of the use of gold salts in the treatment of rheumatoid arthritis, little appears to have been published about the relative efficacies of different chelating agents. Most of the sulphydryl-containing compounds have been tried, with PEN and *N*-acetylpenicillamine being two of the most popular.

Lithium

Lithium salts were employed indiscriminately in the early years of the present century to treat gout, epilepsy, insomnia, hypertension and cardiac disease [563]. As a result, their use has been discouraged for many years. However, more careful modern approaches have now restored lithium carbonate to clinical medicine for the control of manic symptoms. The narrow therapeutic index of this agent has led to an investigation of therapeutic counter-measures for lithium overdoses.

Martin and Martin [564] determined the lithium-chelating tendencies of a series of β-diketones. They found formation constants for dipivaloylmethane (2,2,6,6-tetramethyl-3,5-heptadione) and 2,6-dimethyl-3,5-heptadione to be $\log \beta = 5.75$ and 9.23, respectively. The corresponding values for sodium were 4.08 and 4.47, respectively, whereas for potassium they were 3.9 and 3.86. The

larger stability constants of these two β-diketones with lithium compared with the values for sodium and potassium would suggest that they might be used to deplete the body of excess lithium. Dipivaloylmethane is known to enhance the clearance of lithium from bovine red blood cells, but is was concluded that 2,6-dimethyl-3,5-heptadione was unsatisfactory as it tended to haemolyze the cells [565].

Radionuclides

The use of chelating agents to remove radionuclides from experimental animals commenced soon after they began to be used to counter heavy-metal poisoning. Over the last 35 years, research into chelating agents which might remove radiocations from the body has been extensive and several reviews and books have been published on the relevant decorporation procedures [200, 566, 567]. Only work conducted since 1970 will be considered in any detail here.

Inevitably, BAL has been screened for ability to remove a wide variety of radiocations and, as might have been expected, found to be ineffective for nearly all radionuclides other than polonium (^{210}Po) [568]. In some later studies of polonium decorporation, it was found that in DMPS-treated rats there was an accumulation of polonium in the kidneys, whereas in DDC-treated rats polonium was cleared from the kidneys [569]. However, DDC did slightly increase the uptake of polonium into the brain.

Until the partial test ban treaty on the atmospheric explosion of nuclear weapons severely curtailed the release of strontium (^{90}Sr), there had been considerable concern about possible human uptake of this bone-seeking radionuclide. Several countries maintained research programmes aimed at developing chelating agents that would preferentially complex this metal ion. Only two procedures warrant mention:

(i) orally administered alginates have been shown to be effective in reducing the uptake from the gut of ^{84}Sr in children [570];

(ii) strontium-specific cryptands (10, X = O) must be administered immediately after uptake of strontium to be effective [571].

On several occasions, citric acid has been examined for a role in chelation therapy. With the exception of its synergistic effect with DTPA in the irrigation of wounds [572], neither citric acid nor its derivatives appear to have any role in chelation therapy as a counter-measure to plutonium [573, 574].

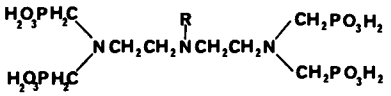
The realization that DTPA and EDTA are not able to enter cells to any appreciable extent because they are too hydrophilic has prompted investigations into the efficacy of esterified forms of these chelating agents. The morpholinoethyl ester of HEDTA (13) and 2,2′-bis[di(carboxymethyl)amino]-

diethyl ether (BADE, 14, $n = 1$, X = O) and later the pentaethyl ester of DTPA were examined for ability to clear radionuclides [160]. Studies by Catsch showed that this derivative of HEDTA and BADE was partially successful in mobilizing cerium (^{144}Ce) and yttrium (^{91}Y) from rats when administered after delay of 24 and 30 h [160]. The faecal clearance of these radionuclides leads to the supposition that they had been excreted into bile and this would, therefore, indicate that the complexes of these radiocations had crossed cell membranes. Although these chemicals appeared to be superior to DTPA, they have not been used in any further chelation therapy. Co-administration of DTPA and the pentaethyl ester of DTPA proved effective in removing plutonium from mice [161], but the toxic properties of the ester stopped it from being investigated further.

Other means of achieving intracellular uptake of plutonium chelating agents have also been considered. The results with liposomally encapsulated and other phagocytizable forms of EDTA and DTPA have been ambivalent [162, 575, 576]. Some chemical modifications to enhance lipophilicity were not successful, either. Pyridine-2,6-dihydroxamic acid, *N*-stearoyl-DFOA and phosphatidylethanolamide-EDTA were not superior to DTPA in mobilizing plutonium from the hamster liver [577]. Also ineffective were RHODA and 2,3-dihydroxybenzoyl-*N*-glycine [577]. Moreover, although 1,2-diheptanoyl-DL-glycerophosphoric acid is an effective agent for enhancing the hepatic accumulation of protactinium and uranium [578, 579], it has proved to be ineffective in mobilizing either plutonium or americium from the liver (R.A. Bulman, unpublished observations).

An evaluation of DFOA has shown it may sometimes be superior to DTPA in clearing plutonium from rats, except that it increases the amount taken up by the kidneys [405]. However, if DTPA is injected along with DFOA, radionuclide incorporation by these organs is suppressed.

Replacement of carboxylate groups by phosphonate groups in EDTA increases the affinity of the chelating agent for lanthanides and actinides [580]. Investigations of the therapeutic potential of such derivatives have shown that *N*,*N*′-ethylenebis[*N*-phosphonomethyl]glycine removes more plutonium from rats than does either EDTA or CDTA [581]. However, in the fully phosphonated EDTA analogue, steric effects could contribute to the observed decrease in therapeutic potential [580].



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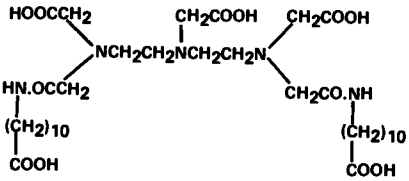
There have been some promising results from investigations of diethylenetriaminepentamethylenephosphonic acid (DTPP, 28, R = $CH_2PO_3H_2$) for ability to clear plutonium from mice [582]. Unfortunately, the chelating agent was administered with a delay of only 1 h. So, although it proved to be quite effective under these conditions, possessing an efficacy similar to DTPA, it is unlikely that sufficient time had elapsed for the plutonium to have been incorporated into cells. Nevertheless, DTPP might well be of value in mobilizing plutonium from wounds. As dioxo cations, such as UO_2^{2+}, form particularly stable complexes with phosphates and phosphonates, DTPP might even prove to be superior to DTPA for removing uranium. Partially lipophilic agents related to DTPP (28, R = C_6H_{11}) have also been examined but were ineffective [583].

Cycloeicosoheptane-1,1,9,9,18,18-hexacarboxylic acid, a highly selective host for UO_2^{2+} [584], has been examined for ability to mobilize uranium from hamsters, but found to be ineffective (R.A. Bulman and I. Tabushi, unpublished observations).

Work conducted in the laboratories of the NRPB in an attempt to find agents which are reasonably lipophilic but not toxic has concentrated on introducing lipophilic moieties with slightly polar functional groups into the chelating agent's structure. The idea has been to reduce the detergent-like properties often exhibited by this kind of molecule and which are believed to have detrimental effects on biological membranes. Of all the compounds examined in this context, PUCHEL (29) has proved the most interesting. It is more effective than DTPA at mobilizing plutonium from the liver of Syrian hamsters [585]. It is also capable of clearing plutonium from the lungs of rats [586]. In both cases, the radionuclide is discharged into the faeces. However, PUCHEL is much less effective in removing plutonium from the liver of rats (R.A. Bulman, unpublished observations) and of Chinese hamsters [587]. These differences can probably be attributed to different rates of clearance of PUCHEL from the livers of the two species. It has been demonstrated using tritiated PUCHEL that clearance of the chelating agent itself is much slower in the Syrian hamster than it is in the rat (F.E.H. Crawley, R.A. Bulman and J.W. Haines, unpublished data).



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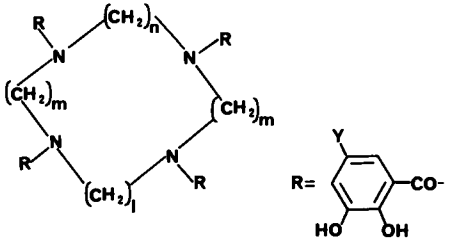
Investigations with PUCHEL in the laboratories of other institutes has shown that: (i) PUCHEL inhaled by rats 90 min after plutoniumtributylphosphate administration reduced the amount of plutonium retained in the lungs at 30 days by 50%. However, when the delay between exposure to plutonium and inhalation of PUCHEL was extended to 1 day, therapy was ineffective [588], and (ii) subcutaneous injections of PUCHEL, administered to rats either 90 s or 4 days after intravenous injection of thorium nitrate, increased the amount of thorium in the liver from 4% in the control animals to over 7% in the treated animals [589]. In contrast to plutonium, this increased retention might be due to incomplete co-ordination of Th(IV) by PUCHEL, as the latter metal ion is more likely to form ternary complexes [590].

Unfortunately, evidence has recently accumulated concerning the toxicity of PUCHEL which has tended to restrict further research with this compound [591]. When injected intravenously into mice as the sodium salt at 200 μmol kg^{-1}, cardiac irregularities are induced. However, these can be suppressed by co-administration of calcium chloride. More significantly, repeated i.p. injection causes irreversible liver damage. This was observed as a compensatory regenerative response, with the cells often being hyperplastic in appearance and possessing large nuclei and abundant cytoplasm [591].

The nature of the histological changes observed in the liver could be an inherent manifestation of lipophilic chelating agents which complex essential metal ions. It might, therefore, be more apposite to develop lipophilic chelating agents which have less affinity for Ca(II), Zn(II), Mn(II), and Cu(II). To this end, a rationale for developing actinide-specific chelating agents has been presented by Raymond and Smith [592]. In essence, their approach requires that the chelating agent hold the tetravalent actinide cation in a cavity with a radius of approximately 24 nm. An examination of molecular models reveals that tetracatechol chelating agents prepared from DHB and 1,4,8,11-tetraazacyclotetradecane or 1,5,9,13-tetraazacyclohexadecane might be particularly well suited to complex plutonium.



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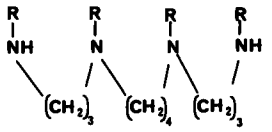

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These chemicals, for which the abbreviated nomenclature system CYCAM has been suggested, exert a variety of effects on plutonium clearance when injected into mice 1 h after plutonium citrate [593]. 3,3,3,3-CYCAM (30, $l = m = n = 3$, Y = H), which is sensitive to low pH, resulted in 41% renal uptake of plutonium compared with approximately 2% in control animals. An examination of the CYCAM-derivatives which are not as acid-sensitive showed that 2,3,2,3-CYCAM (30, $m = 2$, $l = n$, $n = 3$, Y = NO_2) also causes retention of plutonium in soft tissues and, in addition, it is very toxic. However, the sulphonated CYCAM-derivatives (30, Y = SO_3Na) have a much reduced toxicity and are effective in clearing plutonium from the mice. Typically, the liver, skeleton and soft tissue of mice treated with 3,3,3,3-CYCAM-SO_3Na retained 23%, 18% and 3% compared with control animals which retained 27%, 24% and 36% of the intramuscularly injected activity.

A series of acyclic analogues was also examined. Of these, N^1,N^5,N^{10},N^{14}-tetra(2,3-dihydroxy-5-sulphobenzoyl)tetraazatetradecane (31, Y = SO_3Na) proved to be the most effective. Injected intravenously 1 h after plutonium citrate, this chelating agent reduced the plutonium burden in the skeleton, liver and soft tissue to 6.6%, 22% and 1.8%, respectively. The plutonium burden in the equivalent samples from control animals was 30%, 23% and 32%, respectively. So, although these chelating agents cannot remove plutonium from the liver, they do represent a significant advance in achieving total plutonium decorporation from animals. The special significance of this is that they have a low affinity for divalent cations and appear to be effective at doses as low as 2 μmol kg^{-1}. No deaths were observed among mice which received five consecutive daily injections of 20 μmol kg^{-1}.

SYNERGISTIC CHELATION THERAPY

One way of overcoming the restrictions imposed by the mutual exclusiveness of ideal properties discussed earlier is to administer not one but a combination of chelating agents. There are excellent theoretical grounds for thinking that the overall clinical effects of two agents may sometimes be far greater than the sum of their individual contributions. This concept has been termed synergistic chelation therapy [594]. The idea is that each of the critical steps in the sequence required for optimum metal ion decorporation can be accomplished by different ligands with suitable specialized properties. If the agents have complementary roles which can be successfully integrated, the dramatic improvements in chelation therapy which have seemed feasible for so long but which have remained so elusive, could at last be realized.

There is already a certain amount of experimental evidence that synergism

between chelating agents can be accomplished. Volf has shown that a combination of DTPA and DFOA is substantially better treatment of intramuscularly deposited plutonium than either agent alone [200]. It is also known that a variety of low-molecular-weight ligands are able to increase dramatically the rate of iron exchange between transferrin and DFOA [110, 595]. As mentioned previously, this has the potential to revolutionize iron chelation therapy. Pollack and Ruocco have shown that NTA is effective *in vivo* [596] and from the rates of dissociation of the mixed ligand complexes formed by transferrin, it has been calculated that synergistic chelation therapy might be capable of removing tens of grams of iron per month [597]. It is surprising that no one yet seems to have tested DHB for this kind of synergism, even though its evaluation as adjuctive therapy with parenteral DFOA has been suggested [466]. One might also note that current treatment of lead poisoning employs EDTA in combination with either BAL or penicillamine. This has been rationalized on the basis that the sulphydryl compounds are able to penetrate into cells and carry the toxic metal back into plasma where it may be complexed by the polyaminocarboxylic acid and hence excreted in the urine [66]. Similar conclusions have recently been reached from clinical observations [598].

Notwithstanding these examples, the theoretical expectations remain largely unfulfilled. Hundreds of combinations of chelating agents have been tested together, usually with indifferent results. Additive (rather than synergistic) effects are sometimes observed, but this often can be attributed to chelation of the target metal ion from different body pools. Clearly, few pairs of chelating agents capable of interacting synergistically are going to be discovered by chance alone.

A generalization of some importance which has been established by computer simulations concerns the role of mixed ligand complex formation in chelation therapy. Contrary to the common supposition that ternary complexes (with their additional stability) ought to be exploited [599, 600], the models of chelating agents in blood plasma suggest that, if either ligand is naturally occurring, no such benefit is likely to occur. This is because the ternary species can form even when the primary ligand is administered on its own. Many experimental studies support this conclusion.

Synergistic activity requires each chelating agent to have very specific and well-matched properties. Effective combinations of such agents are thus unlikely to be found before much more sophisticated computer simulation models have been developed. These models will need to take account of both equilibrium and kinetic considerations, especially the way in which labile metal binding alters the distribution of metal and ligand in various body compartments.

OTHER ASPECTS IN MEDICINE

In the final part of this review, several other aspects relevant to the use of chelating agents in medicine are presented.

CHELATING AGENTS IN NUCLEAR MEDICINE

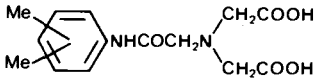
In the last few years, the development of non-invasive diagnostic imaging of the body has provided one of the greatest benefits of the 'nuclear age'. The cations of gallium (^{67}Ga), indium (^{111}In) and technetium (^{99m}Tc) are among the most widely used radionuclides for this purpose. By carefully selecting the chelating agent, it is possible to direct them to predetermined organs or regions of the body. For example, the affinity of tetracyclines for calcium results in their uptake into myocardium and this has been exploited to delineate infarcted areas by the uptake of the ^{99m}Tc-chelate [601]. ^{99m}Tc-tetracycline complexes have also been used to detect tumours in the extremities of man [602]. Furthermore, as a generalization, lipophilic chelates are cleared to the bile and hydrophilic ones directed to the kidney, so chelated radionuclides can be used to measure kidney function and check heptobiliary clearance.

The literature contains many accounts of new radionuclide complexes. However, it is not generally apparent which complexed form of the radiocations is the most satisfactory. One sometimes suspects that many of the complexes are used only in the medical school from which they originated.

Hepatobiliary diagnostic agents

Several groups have considered the iminodiacetates and have investigated the structural features which determine their mechanism of clearance from the body. *N*-(Dimethylphenylcarbamoylmethyl)iminodiacetic acid (HIDA, 32) has been examined in the rabbit for hepatobiliary and renal clearance [603]. The lowest clearance of activity to the bladder (9–10%) was given by the 2,3-, 2,5- and 3,4-dimethyl-HIDA isomers and a slightly higher clearance (14–16%) by the 2,6-, 2,4- and 3,5-dimethyl-HIDA isomers. Even slight increases in molecular mass can result in a significant reduction of the renal clearance rate: excretion values in the baboon for 2,6-dimethyl-HIDA and 2,6-diethyl-HIDA were 20% and 5%, respectively [604]. Chiotellis and Varvarigou have considered the relationship between compounds with more complex substitutions in the aromatic ring of HIDA derivatives [605]. Again, it was found that low-molecular-weight compounds and, particularly those which are *ortho*-substituted, exhibit preferential renal clearance. The clinical value of ^{99m}Tc-

aminodiacetic acid derivatives has been established for diagnosis of a variety of liver dysfunctions in man [606].



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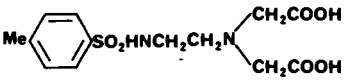

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Closely related to these HIDA structures are the derivatives formed by reacting arylacyl and arylsulphonyl halides with ethylenediamine-*N*,*N*-diacetic acid (33) [607]. The technetium complexes formed from these compounds are soluble in both acidic and neutral aqueous media. Species such as the *p*-toluenesulphonyl derivative are cleared more rapidly from the blood to the small intestine than the HIDA complexes. Hepatobiliary clearance of technetium can also be achieved by ethylenediamine-*N*,*N'*-bis(α-2-hydroxy-5-bromophenyl)acetate [608]. As a moderately 'soft' HSAB acid, Tc(IV) is complexed by DDC and rapidly transferred from blood to the liver. This is then followed by slow hepatic clearance [609]. The poor quality of gall bladder visualization by the complex may arise from the interaction of Tc(IV) with reduced glutathione.

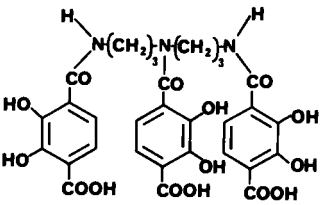
The imaging potential of the radiolabelled Schiff bases formed from pyridoxal and amino acids has been investigated [610, 611]. This work showed that the ^{99m}Tc complexes of pyridoxylidene-L-leucine and pyridoxylidene-L-phenylalanine were the most effective derivatives for imaging the gall bladder of the rabbit. It appears that the complex is stabilized by the interaction of technetium with the Schiff-base nitrogen, the phenolic oxygen of the pyridine ring and the carboxylic group. The side-chain of amino acids exerts little influence upon the *in vivo* kinetics of the various compounds. The reduction of pertechnate by pyridoxal during the autoclaving of the reactants affords a rapid method of synthesis.

Renal imaging agents

In the previous section it was stated that low-molecular-weight HIDA complexes of ^{99m}Tc generally undergo renal clearance. Another example of this is provided by the low-molecular-weight mercaptan complexes which are taken up by the kidneys and excreted into the urine [612]. In an evaluation, using mice, of ^{99m}Tc complexed by a series of dimercaptodicarboxylic acids, the

following order of kidney specificity was demonstrated: DMSA > 2,4-dimercaptoadipic acid > 2,5-dimercaptoglutaric acid. These differences were attributed to the increase in distance between the mercapto groups.

The biomimetic approach used in the development of chelating agents for the binding of iron and plutonium has been extended to nuclear medicine [613]. The introduction of isopropyl moieties onto the amide nitrogens of LICAMS to enhance lipophilicity yielded TiP-LICAMS (23, Z = isopropyl, R^1 = 2,3-dihydroxy-5-sulphobenzoyl). A similar modification of MECAMS gave DiP-MECAMS (21, Z = isopropyl, R^1 = 2,3-dihydroxy-5-sulphobenzoyl). DiP-LICAMS and TiP-MECAMS were found to clear ^{67}Ga(III) and ^{111}In(III) primarily through the kidneys, whereas the even less polar DiP-LICAM (23, Z = isopropyl, R^1 = 2,3-dihydroxybenzoyl) was excreted via the liver. However, this N-substitution of the amide nitrogens did not make the compound so lipophilic that it was able to penetrate the central nervous system. These chelating agents were capable of *in vivo* binding of ^{67}Ga(III) previously injected as the citrate. An extension of this work showed that the introduction of a carboxylic group at C-4 on the catecholamide ring (LICAM-C, 34) gave rise to increased aqueous solubility as well as enhanced resistance to oxidation of the phenol substituents [531]. LICAM-C was shown to be an effective agent for enhancing the clearance of gallium from blood, where the metal is bound to transferrin. It also proved to be of value in enhancing the abscess-to-blood and abscess-to-bone ratios in rats. Administration of LICAM-C could prove to be of value in reducing the radiation burden from ^{67}Ga, which is otherwise unsatisfactorily slow to clear.



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Of a slightly peripheral relevance to this review is the demonstration that an enterochelin specific immunoglobin has been detected in normal human serum [614]. However, an interaction between this antibody and biomimetic chelating agents incorporated into diagnostic imaging kits might have a profound effect upon the distribution of imaging radionuclides in patients. Such a possibility should be borne in mind if anomalous results are ever noted.

In addition to the development of renal imaging kits based on ^{67}Ga and ^{111}In, a renal imaging procedure based upon complexation of cationic forms of ^{99m}Tc by 1,4,8,11-tetraazacyclotetradecane has been reported [615]. In unanaesthetized mice, 74% of the radioisotope appeared in the urine within 20 min, while only 1% remained in the blood.

Fritzberg, Lyster and Dolphin [616] have demonstrated the modification of organ specificity which can be achieved by incorporating into complexing agents moieties with different cation specificity. Unlike other ^{99m}Tc-monophosphonates, ethylthiomethylphosphonate complexes of ^{99m}Tc showed no uptake onto bone. When pertechnate was reduced by stannous reductants, the clearance by the kidneys was slower than when the complex was generated by formamidine sulphinic acid reduction. It is thus possible that the tin became bound to the sulphydryl groups of proteins in the kidneys.

Radiolabelling of cellular blood elements

Chromate (^{51}Cr) was used as one of the earliest techniques for labelling cells in blood, but much more advanced methods are now available by which a variety of cells can be specifically radiolabelled.

8-Hydroxyquinoline (HQ, 12) was used by McAfee and Thakur to radiolabel blood cells with ^{111}In [617]. Later, the technique was extended to label platelets [615, 617, 618] and leukocytes [615]. These labelled cells can be reintroduced into the blood pool to locate thrombi and to detect inflammation and abscesses. The value of radiolabelled cells in exploiting the natural defence processes of the body has been demonstrated by the monitoring of rejection of heterotrophic heart transplants in rats [619]. In this procedure, lymphocytes were radiolabelled with ^{111}In-HQ.

However, as always, some limitations have emerged: the most important of these are that (i) HQ is toxic to cells, (ii) ^{111}In-HQ has a low formation constant ($\log \beta = 11$), and (iii) cytoplasmic components also tend to become radiolabelled. In addition, free HQ is believed to impair the chemotactic mobility and the antimicrobial capacity of neutrophils. So, other lipophilic complexing agents have been examined in the hope of finding an improved cell-labelling procedure.

Although acetylacetone is capable of transporting radiocations through cell membranes, it is also likely to be too toxic. Several groups have shown tropolone to be an effective lipophilic agent for radiolabelling cells [620–622] and, in terms of chemotaxis and phagocytosis, it is not as toxic as HQ or acetylacetone [620]. Fe(III) tropolonate has a higher formation constant than transferrin, so it might be predicted that both the indium and gallium tropolo-

nates will possess a higher specificity in radiolabelling than the equivalent HQ complexes.

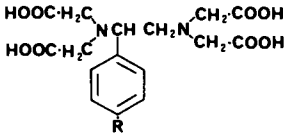
The potential radiotoxicity of ^{111}In to radiolabelled cells, especially lymphocytes, has prompted some investigations into the potential of other radionuclides [623]. The physical characteristics of ^{97}Ru are similar to those of ^{111}In, but the radiation dose from it could be one-third of that from the latter radionuclide. However, studies with ^{103}Ru-HQ have shown that less than 55% of this radionuclide is incorporated into human platelets suspended in plasma. Generally, cell-labelling yields for radionuclides are found to be in the order $^{111}In \gg {}^{67}Ga \gg {}^{57}Co > {}^{99m}Tc > {}^{51}Cr > {}^{54}Fe$ [624], so it would appear that unless yields can be increased by the use of new lipophilic chelating agents, ^{111}In will continue to be used in spite of its radiotoxicity and the danger of possible neoplastic transformation of T-lymphocytes.

The search for new chelating agents which can be used to label the cellular elements of blood will no doubt continue. Lipophilic derivatives of EDTA and DTPA might prove to be suitable for labelling cells through an association of hydrophobic moieties. For instance, monooctadecylamido-DTPA associates strongly with lecithin liposomes [625]. In addition, a micellar association of 1-[*p*-(palmitamido)phenyl]ethylenedinitrilotetraacetic acid (33, R = palmitamido) has been demonstrated [626]. Similarly, an association with cell membranes of phosphatidylamido-EDTA (see Ref. 577) might be expected. Another potential means of radiolabelling cells might be to use 1,2-diheptanoyl-DL-glycerophosphate which is soluble in aqueous media and extracts polyvalent cations into organic solvents [578, 579] and, thus, might be well absorbed by cells. Manipulation of the lipophilicity of the acyl groups could conceivably yield chemicals with varying specificity for the different cells in blood: replacing alkyl groups to give other species such as $CH_3O(CH_2CH_2O)_n$-CH_2CO and $CH_3(CH_2)_mSO(CH_2)_nCO$ is an interesting possibility. Similarly, lipophilic ^{111}In-porphyrin has been used to detect lymphomas [627]. It might also prove possible to radiolabel cells with monoclonal antibodies that have had chelating agents such as DTPA or EDTA covalently bound to them.

Chelating agents bound to macromolecules

Since the initial report in 1974 on the binding of 1-(*p*-aminophenyl)EDTA (35, R = NH_2) to human serum albumin and bovine fibrinogen [628], several similar processes have been described in the literature [575, 629, 630]. DTPA has been bound to the free amino groups of albumins by the mixed anhydride coupling process [629] and by the reaction of DTPA bisanhydride with albumin [575, 630]. Initially, investigations of the reaction of the bisanhydride were

conducted in non-aqueous solvents, but it has now been reported that the reaction also proceeds in an aqueous solution buffered at pH 7.0 [631]. It has also been shown that DTPA can be coupled to cyanogen bromide-activated polysaccharides through a spacer-arm such as 1,6-diaminohexane [575].



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A significant development in the detection of tumours has been made by coupling derivatives of 1-phenyl-EDTA to bleomycin. Bleomycin, a fermentation product of *Streptomyces spp.*, possesses marked antineoplastic properties and is known to be selectively accumulated in some cancer cells [632, 633]. Radiolabelled with ^{57}Co, it has been used to diagnose cancer in over 1000 French patients [634]: ^{57}Co-bleomycin is particularly useful for detecting metastases, especially in lungs. Unfortunately, the long half-life of ^{57}Co (270 days) poses radiotoxicity problems. As chelates of other radionuclides (^{111}In, ^{64}Cu, ^{67}Cu, ^{99m}Tc, ^{62}Zn, ^{67}Ga) do not have the *in vivo* stability of the cobalt chelate, they could not simply be substituted, and more sophisticated procedures for the radiolabelling of bleomycin have had to be developed. A conjugate of bleomycin and 1-phenylEDTA (BLEDTA) when labelled with ^{111}In possessed excellent imaging properties in tumour-bearing experimental animals [635]. In clinical studies with ^{111}In-BLEDTA, positive scans were found in 81% of the cases examined. In 22%, tumour sites were detected where other procedures had failed [636]. Unfortunately, only tumours larger than 1.5–2.0 cm were visualized. An additional limitation which emerged was the result of radiolabelling of polymorphonucleocytes by ^{111}In-BLEDTA. This resulted in false-positive uptake by sites of inflammation. Notwithstanding these defects, ^{111}In-BLEDTA represents a very significant step forward in the development of procedures for directing complexing agents to specific targets in the body.

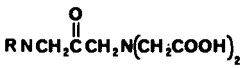
Transport across the blood-brain barrier

Oldendorf has suggested that technetium complexes with an octan-1-ol-water partition coefficient of 0.5 can be expected to cross the blood-brain barrier

[637]. Such radiolabelled species are thus of potential value for measuring regional cerebral perfusion. Two general mechanisms for transport across the blood-brain barrier are known: passive diffusion, which is determined by substrate lipophilicity, and carrier-mediated transport, based on specific interactions between substrates and membrane-bound carriers. Exploitation of the latter will tax the ingenuity of organic chemists. However, as some studies have already shown, the problems associated with passive diffusion are somewhat less daunting. In one case [638], the ^{99m}Tc complexes of HQ and 5,7-diiodo-8-hydroxyquinoline were found to have a high partition coefficient between octan-1-ol and 0.05 M phosphate-buffered saline, and in both cases the percentage of protein binding and the brain-uptake index were also high. Of several chelating agents derived from polyaminocarboxylic acids, three (36, R = $(CH_2)_{10}Me$; 36, R = $(CH_2)_{17}Me$; 37, R = $C_{14}H_{28}COOH$) had partition coefficients in excess of the 0.5 limiting value specified by Oldendorf.

These studies demonstrate that large, highly lipophilic substituents must be attached to polyaminocarboxylates to achieve transport across the blood-brain membrane. It is possible, however, that sufficient lipophilicity might also be introduced by derivatizing with fluorinated aminoalkanes or alcohols of shorter chain length.

In a departure from the conventional use of polyaminocarboxylate-based chelating agents, some aminothiol ligands (38) have been prepared and the partition coefficients of their ^{99m}Tc complexes determined [639]. Several of these are sufficiently lipophilic to be of interest.



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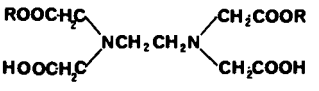

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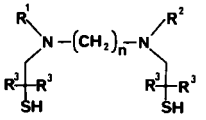

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Uptake by bone

The uptake of radionuclides by bone is a potentially valuable aid in detecting fractures, primary or metastatic neoplasms in the bones and skeletal metabolic disorders such as Paget's disease. Unfortunately, there are no radioisotopes of calcium or strontium which are well suited to such clinical applications. Moreover, other than the 1,1-diphosphonates, such as ethane-1-hydroxy-1,1-diphosphonic acid (EHDP), few organic compounds that complex radiocations are

actually taken up into bone. The most notable exceptions to this are alizarin and related anthroquinone dyes, tetracyclines and *N*-(2-carboxy-4-iodophenyl)-iminodiacetic acid [640]. Shtacher and Anbar have shown that radionuclide uptake with a series of iminodiacetic acids could be correlated with the formation constants of their calcium complexes [641]. They found that after 3 h, the uptake of *N*-(*p*-bromoacetanilido)iminodiacetic acid onto the tibial epiphysis of the rat reached a maximum of 0.18%.

The extensive uptake of EHDP by bone occurs primarily because of the structural similarity between 1,1-diphosphonates (H_2O_3–C–PO_3H_2) and pyrophosphate. Accordingly, research into the chemistry of 1,1-diphosphonates has been extensive, as they are used both in detergents and in the treatment of Paget's disease. A variety of structures have been synthesized but none of them has yet given rapid skeletal imaging. In a recent investigation of ^{99m}Tc-labelled aminomethanediphosphonic acids (AMDP), Unterspann and Fink found the following order of uptake into bone: *N*-methylAMDP > *N*-dimethylAMDP > *N*-trimethylAMDP > AMDP [642].

Other organs

Imaging procedures for the prostate and the pancreas have not advanced as far as those for visualizing other parts of the body. Cationic complexes of radionuclides for this purpose have been particularly lacking. The high pancreas-to-liver uptake ratios reported for the β-D-thioglucose complexes of ^{105}Ag, ^{199}Au and ^{203}Pb [643] were later reported as inconsistent and difficult to repeat [644]. In studies with mice it has been shown that 11% of ^{65}Zn, injected as the complex of β-D-thioglucose, accumulated in the pancreas within 3 h. In the rat, however, only 3% of the activity was found in the pancreas after 2 h [644]. A pancreas-to-liver ratio of 1.44 was achieved when the radiozinc was injected as amino acid chelates. Sufficient specificity of pancreas and prostate uptake occurred to permit a feasible emission computed tomography of ^{62}Zn. ^{99m}Tc-2-mercaptopropionylglycine injected into mice achieved a pancreas-to-liver ratio of 4.5 and pancreas-to-blood ratio of 5.8 [645]. These results are sufficiently promising to suggest that the search for an efficient pancreatic imaging agent might now be drawing to a close.

Imaging procedures for the heart have conventionally exploited the replacement of potassium by thallium (^{201}Th). There have been, however, some investigations into alternatives. As ^{99m}Tc is readily available, it has been proposed that it may be used to replace ^{201}Th in some cases. The imidodiphosphonate of technetium has been reported as giving a good definition of the infarcted myocardium [646]. The uptake of fatty acids into the myocardium

prompted Karesch, Eckelman and Reba [647] to synthesize lipophilic polyaminocarboxylic acids in the hope that they would behave similarly. Although it was concluded from some *in vitro* experiments that these chelating agents could complex cations of ^{57}Co and ^{99m}Tc, less than 1% of the injected activity was found to be localized in the heart. It is possible that the hydroxamic acid derived from alkylmalonic acids might be more effective.

CHELATING AGENTS AS CYTOTOXIC COMPOUNDS

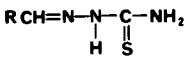
The important role of metal ions in cell physiology makes them a suitable target for agents intended to be cytotoxic. About 12% of all known enzymes require a metal ion for activity [648] and metal ions are also fundamentally involved in the stabilization of various nucleic acid structures. Thus, chelating agents which penetrate into cells and which are sufficiently powerful to interfere with any of several critical biochemical systems can cause metabolic malfunction and, ultimately, cell death.

To exploit this characteristic feature of chelating agents, it is evident that to be beneficial, the compounds must be selective in their toxicity [649]. In particular, it is necessary to find those that, ideally, inhibit enzymes which are specific to neoplastic or virus-producing cells. Failing this, there needs to be some differential in response between healthy and pathological growth. This goal forms a large and expanding area of interest to medicinal chemists.

Antiviral properties

As Perrin and Stünzi have reviewed the application of chelating agents in viral chemotherapy [4], only a limited discussion of this topic is necessary here.

A few examples serve to illustrate the use of chelating agents as viriostatic drugs. Aromatic and heteroaromatic thiosemicarbazones are powerful chelating agents which have been useful in treating complications of smallpox vaccination. They are also effective prophylactics when administered to people in intimate contact with cases of smallpox [650]. Thiosemicarbazones (39) possess some antineoplastic activity in animals, but are of little value in treating neoplasia in man [651]. The common site of action of their antiviral and antineoplastic activity could be the inhibition of DNA synthesis by complexation of Fe(II), a co-factor of ribonucleoside diphosphate reductase [652].



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Antiviral activity has been reported for HQ [653], isonicotinic acid hydrazide [653], DTPA and 1,10-phenanthroline [654]. The hydrazides of partially lipophilic forms of polyaminocarboxylic acids (derivatives which may cross cell membranes) have yet to be examined for cytotoxic properties, but would seem potentially useful.

Phosphonoacetic and phosphonoformic acids are active against the herpesviruses and several other viruses [655, 656]. The antiviral activity of β-diketones, presumably arising from metal complexation through the ketoenol tautomeric forms, has been demonstrated. It might arise from the complexation of iron, copper or zinc [4].

Antiviral activity has also been shown for bleomycin [657] and for rifamycin, the latter having been modified so that additional chelating sites are introduced [658].

Inhibition of the synthesis of DNA in the HeLa cells by DFOA [659] has prompted further investigations into the action of various iron-specific chelating agents [660]. In this study, inhibition of DNA synthesis was measured by inhibition of tritiated-thymidine incorporation into the nucleus of phytohaemagglutinin-stimulated lymphocytes. In general, aliphatic hydroxamic acids were the most active agents examined, although salicylhydroxamic acid was also effective. Cory, Lasater and Sato [661] have thus suggested that the therapeutic efficacy of hydroxyurea, guanazole and pyrazoloimidazole might be enhanced by the incorporation of DFOA into the treatment schedule. Of particular note was the poor inhibitory action of DHB and DTPA.

Antimicrobial properties

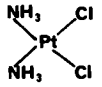
The antimicrobial action of chelating agents has also been reviewed [6]. Foremost among the chelating agents used for this purpose is HQ. From an examination of the oil-water partition coefficient of HQ and some metal-binding structural analogues, it is presumed that the site of action is inside the cell or at least within the cytoplasmic membrane. The bacterial action of HQ with iron is particularly rapid and can be attributed to the introduction of toxic amounts of Fe(III) into the cell. As this action can be inhibited by cobaltous sulphate at levels as low as 4×10^{-5} M, it seems likely that it is due to the catalytic degradation of hydrogen peroxide.

The chelating action of the tetracyclines has also been recognized for many years [662]. Their selective uptake by bacteria (which is in contrast to mammalian cells) has been attributed to the presence of magnesium in the bacterial plasma membrane [663].

In more recent years, phenanthrolines appear to have replaced hy-

droxyquinolines as the most intensely investigated lipophilic chelating agents possessing antimicrobial action [664–666]. The mode of action of the 2,9-dimethyl-1,10-phenanthroline against *Paracoccus dentrificans* and *Mycoplasma gallisepticum* has been attributed to the ionophoretic transport of Cu(I) to receptor ligands buried inside cell membranes [656, 666]. From an investigation of 4,7-phenanthroline-5,6-quinone (phanquone) against Gram-positive and Gram-negative organisms, it would appear that at least two modes of action are possible [664]. Against *Staphlococcus aureus*, and possibly other Gram-positive organisms, phanquone may be killing the cells by transporting metal ions into the cells. However, with *E. coli* and, possibly, other Gram-negative organisms, phanquone appears to be chelating intracellular metal ions, and thus inactivating some essential metal-containing system. 3,4,7,8-Tetramethyl-1-10-phenanthrolinate nickel(II) has been used as a disinfectant in the cleansing of new-born babies [667].

Several chelating agents are known to possess anti-amoebic activity. Currently, 5,7-diiodo-8-hydroxyquinoline is most commonly used. As it exhibits ionophoretic activity for Zn(II), it may well be that this is responsible for its beneficial action in treating acrodermatitis enteropathica [668].



(40)

Roles for chelating agents in more exotic areas of medicinal chemistry have appeared. Recently, the molluscidal action of 5,2′-dichloro-4′-nitrosalicylanilide has been attributed to its chelation of Fe(III) [669]. In addition to its antineoplastic action, cisplatin (40) shows some promise as a trypanocide when administered in conjunction with Antabuse, which is known to be metabolized to DDC [670]. Other uses of chelating agents in tropical medicine are readily envisaged: lipophilic chelating agents developed to penetrate the cell membrane of the human malaria parasite *Plasmodium fulciparum*, and thus inactivate metalloprotein oxidase enzymes, might lead to a whole new generation of antimalarial drugs [671, 672].

Anticarcinogenic properties

The intimate relationship which exists between metals, chelating agents and cancer has been steadily elucidated ever since Furst's original speculations on

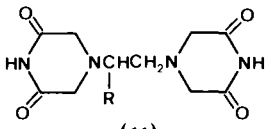
this subject in 1960 [673]. By 1980, it had been estimated that over 11,000 complexes of fifty-five metals had been examined for antitumour activity [674]. The subject has been extensively reviewed on many occasions and from a variety of standpoints.

Schwartz considered the role of trace elements as substances which may both cause and inhibit the disease [675]. Seelig has pointed to magnesium and other trace substances in cancer pathogenesis [67]. Furst and Radding have dealt with a variety of unusual metals as carcinogens [677] and Weinberg has reviewed the relationship between iron and neoplasia [678]. In 1980, an international workshop under the chairmanship of Friberg and Nelson was held to evaluate the state of knowledge concerning metal carcinogenicity, particularly in respect of the underlying mechanisms of action [679]. A multicontributor volume, edited by Sigel, has been devoted to metal complexes as anti-cancer agents, focussing attention on a wide variety of metal ions and, in particular, on the role of complexes as alkylating agents [680]. Mildvan and Loeb have covered the way in which metal ions participate in nucleic acid polymers and have outlined some approaches to malignant diseases which this perspective suggests [681]. Heck and Costa have shown that the activity of metal compounds in mammalian cell transformation assays correlates well with the known carcinogenic activity of the compounds *in vivo* [682].

In spite of the progress covered by these reviews, few clear, fundamental principles about the interrelationship between cancer and metals, chelating agents or their complexes have emerged. There is considerable ambivalence about using either chelating agents or metals in cancer chemotherapy because sometimes they may be used to prevent malignant growth, but on other occasions they may promote it. For instance, it has been suggested that certain tumours can be selectively inhibited by acute zinc deficiency [683] yet this might damage the immune response to allogeneic cells [684]. Similarly, it has been shown that 1,10-phenanthroline is capable of reversibly inhibiting lymphoblast cell cycles but, on the other hand, depletion of intracellular metal ion levels may produce nucleic acid conformational changes which may ultimately initiate neoplasia [649]. The induction of urinary tract transitional cell carcinoma by nitrilotriacetic acid may well arise from chelation of intracellular calcium [685]. It is interesting to note that L-alanosine lowers serum copper levels but lacks cupriuretic activity [686].

It is clear that present knowledge of intracellular metal binding will have to be considerably improved in order to realize the full potential of chelating agents in cancer chemotherapy. However, several promising areas of investigation have already been established. In particular, the thiosemicarbazones are one class of compound which have proved interesting in a variety of contexts

[687–690]. The free radical-producing drugs are another which may well function by forming metal complexes. The naturally occurring antibiotic, bleomycin, is an example of this [691–695]. It is noteworthy that manganese and, more likely, copper-zinc superoxide dismutase may selectively protect normal tissue against the effects of such drugs [696]. Similarly, it has been suggested that the Fe(III) complex of adriamycin, known as quelamycin [697], may be less toxic because free-radical formation is inhibited [698].



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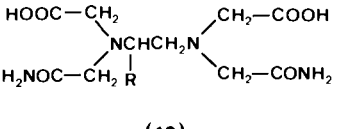

(42)

The bisdioxopiperazines, a class of antitumour agents developed at the Imperial Cancer Research Fund laboratories [699], are derivatives of EDTA and its analogues. It was speculated that the reduced polarity of these derivatives might facilitate penetration of cell membranes, to be followed by degradation to give chelating species which were cytotoxic [700]. A study of the structure-activity relationships, particularly the modification of the central chain, identified ICRF 159 (41, R = Me) as an antineoplastic agent worthy of further investigation. This is now marketed as Razoxane; it is used for the suppression of soft tissue metastases and, increasingly, in the treatment of psoriasis.

However, the mode of action of this class of drug at the molecular level remains obscure and the role of chelation uncertain. In contrast to ICRF 159, the homologue ICRF 192 (41, R = Et) is inactive. An investigation of the interaction of Ca(II), Mg(II), Zn(II), Cu(II), Fe(II), and Mn(II) with the diacid diamide hydrolysis products of ICRF 159 (42, R = Me) and ICRF 192 (42, R = Et) revealed similar metal-binding affinities in all cases except one [698]. The exception occurred with zinc, which was complexed much less avidly by the derivative of ICRF 159 than by the corresponding compound from ICRF 192. It is possible that this effect could be responsible for the difference in the cytotoxic properties of ICRF 159 and 192. The mechanism through which this might occur is, however, obscure. It is unlikely that zinc can be the target in the action of ICRF 159, as the hydrolysis product of ICRF 192 binds zinc the most avidly [698].

Cytotoxicity arising from the depletion of cells of essential cations is not the only manner in which complexing agents can act on cells. The complexation

of a wide range of metal ions in stereochemical forms which confer antineoplastic activity upon the metal ion has considerably extended the spectrum of cancer chemotherapeuticals. In this context, the initial demonstration that cisplatin promoted filamentous growth of *E. coli* and the way in which its antineoplastic potential was developed has been reviewed by Rosenberg [701].

Investigations into the antineoplastic activity of the complexes of the noble metals and other transition elements have since been pursued with much vigour.

The low therapeutic index and toxic effects of cisplatin have encouraged the search for safer and more effective analogues. For optimum cytotoxic activity, the leaving groups must be easily displaced [702]. If the leaving groups are not easily displaced, the *cis*-platinum(II) complex is inactive, whereas leaving groups which are readily displaced give extremely toxic compounds. The replacement of the ammine groups by amines can modify the activity of the complexes by altering the aqueous solubility and by increasing lipid solubility. In addition to cisplatin and *cis*-dichlorodiaminoethaneplatinum(II), other complexes have been shown to possess antineoplastic properties which merit their addition to the list of potential cancer chemotherapeuticals. Foremost amongst these are [sulphato-1,2-diaminocyclohexaneplatinum(II)], [malonato-1,2-diaminocyclohexaneplatinum(II)] and [cyclobutane-1,1-dicarboxylatodiammineplatinum(II)] [702]. A wealth of evidence discussed by Roberts and Thompson [703] indicates that the principal target site for neutral platinum complexes is the impairment of DNA replication of Pt(II) by DNA bases.

It has been proposed, on the basis of laboratory studies with the rat, that DDC may be used to suppress nephrotoxicity of cisplatin [704]. PEN has also been suggested for this purpose, but it appears to suppress the anticancer action of the platinum complex.

COPPER COMPLEXES AS ANTI-ARTHRITIC DRUGS

As two reviews on the role of copper complexes in arthritic conditions have appeared in recent volumes of *Progress in Medicinal Chemistry* [1, 705], and the subject has also been discussed by May and Williams [322], coverage here will be very brief.

The role of copper can be summarized thus: (i) there is a marked increase in total serum copper in rheumatoid arthritis (RA) patients; (ii) there is an increased rate of synthesis and accelerated turnover of caeruloplasmin and this can be related to the activity of, and natural response to, the disease; (iii) increased levels of copper and of caeruloplasmin are found in the synovial fluid of patients during prolonged bouts of RA; (iv) the distribution of copper

between exchangeable and non-exchangeable forms in biofluids such as blood plasma is altered by the disease; (v) intravenous administration of copper has a marked anti-inflammatory effect; and (vi) drugs such as PEN which interact with copper *in vivo* may facilitate remission of RA by promoting tissue utilization of copper. It thus seems likely that rheumatoid diseases require copper (in a complexed form) to counteract such pathogenic processes associated with the diseased state. A model for the role of copper in inflammatory processes has been proposed [706].

To date, the copper complexes most effective in treating RA have been those formed by the salicylates and structurally related drugs. Cu(II) salicylate and other reasonably lipophilic complexes administered in non-aqueous solution to the skin of the rat are capable of suppressing experimental arthritis [707–710]. These preparations are effective because the complex is able to penetrate the dermis [711] and, presumably, enter the circulation via the lymph system. West has shown that other less lipophilic species such as Cu(II)-histidine are particularly effective only when given systemically [712]. Histidine is the most important ligand for naturally occurring low-molecular-weight Cu(II) complexes in blood plasma [22], so it is interesting that in RA there is a specific lowering of its concentration [713]. Moreover, it has been found that a significant increase in serum histidine levels occurs in patients with RA treated with PEN [714].

The anti-ulcer activity of various copper complexes has also attracted considerable attention [715], particularly because most of the current drugs used to treat arthritis are prone to be ulcerogenic.

MISCELLANEOUS ASPECTS

Chelating agents have been used for an incredible variety of medicinal purposes. In the late 1950's and early 1960's EDTA almost achieved the status of a panacea. At one time it was considered to have a role in controlling arteriosclerosis [716] but this has now been dismissed [717]. Nevertheless, EDTA has been used in very diverse roles: as the cobalt salt, it is an effective therapy for cyanide poisoning [718] and it has also been used to dissolve kidney stones [719]. Computer simulation models are playing a fascinating role in the selection of drugs for urolithiasis therapy [720]. This is an important development, since about one person in every thousand suffers from urinary calculi [720].

The chelation of Cu(II) or Zn(II) by L-3,4-dihydroxyphenylalanine (DOPA), the drug used to replenish dopaminergic pools in patients suffering from Parkinson's disease, suppresses the pyridoxal-dependent decarboxylation

of the agent in the precerebral areas [721]. Studies with radiolabelled DOPA have shown an increased uptake into the brains of rats following intraperitoneal injection of the copper and zinc chelates.

The prophylactic effects of chelating agents have also been examined in several less well-known forms of copper dysfunction. These are Menkes's disease, a condition first described in 1962 which is characterized by progressive degeneration of the brain and spinal cord in infants [722], Indian childhood cirrhosis and primary biliary cirrhosis (PBC) [488]. Indian childhood cirrhosis differs from Wilson's disease by the early onset of the disease and the normal ceruloplasmin concentrations of its victims [488]. It is not yet clear whether excess hepatic copper is an expression of a primary inherited metabolic defect, or is a secondary event. In Wilson's disease the excess copper is associated with the lysosomes [723], whereas in PBC the copper is in the supernatant fraction [724]. Additionally, there are also differences in the distribution of copper in the proteins in the periportal hepatocytes [725].

Clinical studies on Menkes's disease patients indicates that the transport of copper across the gut wall is impaired [726]. Copper supplementation is thus potentially capable of preventing the development of the neuropathological lesions. Administration of Cu(II)-EDTA to a new-born baby, the sibling of a baby who had died from Menkes's syndrome, has demonstrated that it can, indeed, be beneficial [726]. From experiments with pregnant mutant mice – bred to possess a disorder similar to Menkes's syndrome – it would appear that Cu(II)-NTA might similarly meet fœtal or neonatal requirements for copper [727, 728].

Trials with PEN as a treatment for PBC have yielded conflicting results. One showed that PEN (900 $\mu g\ day^{-1}$) over 1 year reduced mean liver concentrations of copper in biopsy from $310 \pm 128\ \mu g\ g^{-1}$ dry weight to $84 \pm 20\ \mu g\ g^{-1}$ [225]. Patients in a placebo group exhibited no significant reduction. However, a more recent study (4×250 mg per day) concluded that PEN was not an effective treatment [226]. TRIEN has been used as an alternative to PEN in some patients, but its application is limited by the occurrence of side-effects [729].

CONCLUSION

After a comprehensive review on the clinical uses of metal-binding drugs published in 1968, Chenoweth [730] concluded that the status of chelating agents in medicine could be summarized as follows: "Arsenic and mercury poisonings call for immediate and systematic treatment with dimercaprol; symptoms of

chronic lead poisoning respond well to systemic calcium disodium edetate; Wilson's disease is ameliorated by oral D-penicillamine and all other situations discussed are still experimental and *sub judice*".

Progress since then has largely been a fulfilment of earlier discoveries. DFOA has become established as the agent for transfusional siderosis, DTPA should be used for radionuclide decorporation and TRIEN is now an accepted alternative to PEN for Wilson's disease. Current treatment of acute iron and lead poisoning is usually gratifyingly successful and a few new agents have been found which appear to have considerable potential. Yet, this is not much reward for nearly two decades of intensive research.

On the other hand, these efforts have accumulated a great deal of data concerning the behaviour of chelating agents in biological systems. The need now is to collate and understand it. As Albert has maintained [731], this requires greater emphasis to be placed on the comparative biochemistry, distribution and cytology of the agents in question. In particular, the interrelationship between these three properties will be the factor of paramount importance.

Special attention must be devoted to this interrelationship if metal complexation in areas of medicine other than chelation therapy is to be effectively exploited. As Mahler observed over 20 years ago [732], there is not a single enzyme-catalysed reaction in which the enzyme itself, the substrate or the product is not directly and specifically influenced by the nature and concentration of the inorganic ions which surround it. The therapeutic potential of chelating agents as a class of compounds is thus, probably, unparalleled. However, all depends on the future design of agents with sufficient chemical and physical selectivity to achieve very specific biochemical objectives.

REFERENCES

1. J.R.J. Sorenson, Prog. Med. Chem., 15 (1978) 211.
2. J.R.J. Sorenson (ed.), Inflammatory Diseases and Copper (Humana Press, New Jersey, 1980).
3. R.L. Gross and P.M. Newberne, Physiol. Rev., 60 (1980) 188.
4. D.D. Perrin and H. Stunzi, Pharmacol. Ther., 12 (1981) 255.
5. J.L. Sullivan, Lancet, i (1981) 1293.
6. A. Albert, Selective Toxicity (Chapman and Hall, London) 5th edn. (1973) p. 367.
7. S. Kirschner and S.H. Kravitz, Adv. Exp. Med. Biol., 91 (1978) 151.
8. R.A. Peters, L.A. Stocken and R.H.S. Thompson, Nature (London), 156 (1945) 616.
9. E.L. Belknap, Ind. Med. Surg., 21 (1952) 305.
10. J.M. Walshe, Am. J. Med., 21 (1956) 487.

11. B. West and F.W. Sunderman, Am. J. Med. Sci., 236 (1958) 15.
12. H. Bickel, E. Gaumann, W. Keller-Schierlein, V. Prelog, E. Vischer, A. Wettstein and H. Zahner, Experientia, 16 (1960) 129.
13. J.F. Fried, E.H. Graul, J. Schubert and W.M. Westfall, Atompraxis, 5 (1959) 1.
14. J.M. Walshe, Lancet, ii (1969) 1401.
15. S.C. Wang, K.S. Ting and C.C. Wu, Chin. Med. J., 84 (1965) 437.
16. W.F. Anderson, in: Proceedings of the Symposium on Development of Iron Chelators for Clinical Use, eds. W.F. Anderson and M.C. Hiller (D.H.E.W. Publication No. NIH 76-994, 1976) pp. 1–4.
17. A.E. Martell, W.F. Anderson and D.G. Badman, in: Proceedings of the 2nd Symposium on Development of Iron Chelators for Clinical Use, eds. A.E. Martell, W.F. Anderson and D.G. Badman (Elsevier, New York, 1981) pp. x–xi.
18. G.B. Kauffman, Alfred Werner – Founder of Coordination Chemistry (Springer-Verlag, Berlin, 1966).
19. C.F. Bell, Metal Chelation Principles and Applications (Oxford University Press, London, 1977) p. 3.
20. G. Beech, Qt. Rev., 23 (1969) 410.
21. M.R. Rosenthal, J. Chem. Educ., 50 (1973) 331.
22. P.M. May, P.W. Linder and D.R. Williams, J. Chem. Soc., Dalton Trans., (1977) 588.
23. G.R.H. Jones and R. Harrop, J. Inorg. Nucl. Chem., 35 (1973) 173.
24. F.A. Cotton and G. Wilkinson, Advanced Inorganic Chemistry (Wiley, New York) 4th Edn. (1980).
25. A. Vacca, A. Sabatini and M.A. Gristina, Coord. Chem. Rev., 8 (1972) 44.
26. F. Gaizer, Coord. Chem. Rev., 27 (1979) 195.
27. A.E. Martell and R.M. Smith, Critical Stability Constants (Plenum Press, New York) Vols. I–V (1974, 1975, 1977, 1976, 1982).
28. D.D. Perrin, Stability Constants of Metal-ion Complexes. Part B. Organic Ligands (Pergamon Press, Oxford, 1979).
29. A.M. Fiabane and D.R. Williams, The Principles of Bio-inorganic Chemistry (Chemical Society, London) Monographs for Teachers No. 31 (1977) p. 82.
30. D.D. Perrin, Masking and Demasking of Chemical Reactions (Wiley, New York, 1970) p. 183.
31. S. Ahrland, J. Chatt and N.R. Davies, Chem. Soc. Rev., 12 (1958) 265.
32. R.J.P. Williams and J.D. Hale, Struct. Bonding (Berling), 1 (1966) 249.
33. R.G. Pearson, J. Am. Chem. Soc., 85 (1963) 3533.
34. R.G. Pearson, Hard and Soft Acids and Bases (Dowden, Hutchinson and Ross, Pennsylvania, 1970).
35. R.G. Pearson, J. Chem. Educ., 45 (1968) 643.
36. H.M. Irving and R.J.P. Williams, Nature (London), 162 (1948) 746.
37. J.J. Christensen, J.O. Hill and R.M. Izatt, Science, 174 (1971) 459.
38. J.-M. Lehn, Struct. Bonding (Berlin), 16 (1973) 1.
39. W. Simon, W.E. Morf and P.C. Meiier, Struct. Bonding (Berlin), 16 (1973) 113.
40. Y.A. Ovchinnikov, V.T. Ivanov and A.M. Slikrob, Membrane-Active Complexones (Elsevier, Amsterdam. 1974).
41. F. Vogtle and E. Weber, Angew. Chem., Int. Edn. Engl., 18 (1979) 753.
42. B.D. Gomperts, S. Cockroft, J.P. Bennett and C.M.S. Feurtrell, J. Physiol. (London), 76 (1980) 383.

43. C.M. Deber and P.D. Adauradkar, Biopolymers, 18 (1979) 2375.
44. B. Sarkar, in: Metal-Ligand Interactions in Organic Chemistry and Biochemistry, ed. B. Pullman and N. Goldblum (Reidel, Dordrecht) Part I (1977) pp. 193–228.
45. K.S. Iyer, J.P. Laussac, S. Lau and B. Sarkar, Int. J. Peptide Protein Res., 17 (1981) 549.
46. D.D. Perrin, Top. Curr. Chem., 64 (1976) 183.
47. D.D. Perrin, Nature (London), 206 (1965) 170.
48. D.D. Perrin, Suomen Kemi., 42 (1969) 205.
49. P.S. Hallman, D.D. Perrin and A.E. Watt, Biochem. J., 121 (1971) 549.
50. B. Branegard and R. Osterberg, Clin. Chim. Acta, 54 (1974) 55.
51. P.Z. Neumann and A. Sass-Kortsak, J. Clin. Invest., 46 (1967) 646.
52. B. Sarkar and T.P.A. Kruck, Can. J. Biochem., 45 (1967) 2046.
53. A.S. Prasad and D. Oberleas, J. Lab. Clin. Med., 76 (1970) 416.
54. E.L. Giroux and R.I. Henkin, Biochim. Biophys. Acta, 273 (1972) 64.
55. B. Sarkar, Can. J. Biochem., 48 (1970) 1339.
56. A.S. Prasad and D. Oberleas, Proc. Soc. Exp. Biol. Med., 138 (1971) 932.
57. N. Asato, M. Van Soestbergen and F.W. Sunderman, Clin. Chem., 21 (1975) 521.
58. M. Lucassen and B. Sarkar, J. Toxicol. Environ. Health, 5 (1979) 897.
59. B. Lonnerdal, A.G. Stanislowski and L.S. Hurley, J. Inorg. Biochem., 12 (1980) 71.
60. G.W. Evans and P.E. Johnson, Pediatr. Res., 14 (1980) 876.
61. P.M. May, G.L. Smith and D.R. Williams, J. Nutr., 112 (1982) 1990.
62. D.D. Perrin and R.P. Agarwal, in: Metal Ions in Biological Systems, ed. H. Sigel (Marcel Dekker, New York) Vol. II (1973) pp. 167–206.
63. R.P. Agarwal and D.D. Perrin, Agents Actions, 6 (1976) 667.
64. E.W. Moore, J. Clin. Invest., 49 (1970) 318.
65. P.M. May, P.W. Linder and D.R. Williams, Experientia, 32 (1976) 1492.
66. P.M. May and D.R. Williams, FEBS Lett., 78 (1977) 134.
67. M. Micheloni, P.M. May and D.R. Williams. J. Inorg. Nucl. Chem., 40 (1978) 1209.
68. P.M. May, J. Whittaker and D.R. Williams, Inorg. Chim. Acta Bioinorg. Lett., in press.
69. P.M. May, Agents Actions, 11 (1981) 294.
70. E.B. Dowdle, D. Schachter and H. Schenker, Am. J. Physiol., 198 (1960) 609.
71. P. Jacobs, T.H. Bothwell and R.W. Charlton, Am. J. Physiol., 210 (1966) 694.
72. P.M. May, D.R. Williams and P.W. Linder, in: Ref. 62, Vol. VII (1978) pp. 29–76.
73. R.G. Sheehan, Am. J. Physiol., 231 (1976) 1438.
74. J.J.M. Marx and P. Aisen, Biochim. Biophys. Acta, 649 (1981) 297.
75. R. Albert, M. Berlin, J. Finklea, L. Friberg, R.A. Goyer, R. Henderson, S. Hernberg, G. Kazantzis, R.A. Kehoe, A.C. Kolbye, L. Magos, J.K. Miettinen, G.F. Nordberg, T. Norseth, E.A. Pfitzer, M. Piscator, S.I. Shibko, A. Singerman, K. Tsuchiya and J. Vostal, Environ. Physiol. Biochem., 3 (1973) 65.
76. L.W. Chang, P.R. Wade, J.G. Pounds and K.R. Reuhl, Adv. Pharmacol. Chemother., 17 (1980) 195.
77. F.H. Nielsen, D.R. Myron and E.O. Uthus, in: Trace Element Metabolism in Man and Animals, ed. M. Kirchgessner (A.T.W., Freising-Weihenstephan) Vol. III (1978) pp. 244–247.
78. G. Bertrand, Proc. Int. Congr. Appl. Chem., 28 (1912) 30.
79. A.I. Venchikov, in: Trace Element Metabolism in Animals, ed. W.G. Hoekstra, J.W. Suttie, H.E. Ganther and W. Mertz (University Park Press, Baltimore) Vol. II (1974) pp. 295–310.

80. E.J. Underwood, Trace Elements in Human and Animal Nutrition (Academic Press, New York. 1977).
81. K.E. Mason, J. Nutr., 109 (1979) 1979.
82. A.V. Hoffbrand, in: Iron in Biochemistry and Medicine, eds. A. Jacobs and M. Worwood (Academic Press, London) Vol. II (1980) pp. 499–527.
83. P. Saltman, J.C. Hegenauer and J.P. Christopher, Ann. Clin. Lab. Sci., 6 (1976) 167.
84. G.C. Cotzias, Physiol. Rev., 38 (1958) 503.
85. J.J. Chisolm and D. Barltrop, Arch. Dis. Child., 54 (1979) 249.
86. P.A. Lucas, A.G. Jariwalla, J.H. Jones, J. Gough and P.T. Vale, Lancet, ii (1980) 205.
87. F. Bakir, S.F. Damluji, L. Amin-Zaki, M. Murtadha, A. Khalidi, N.Y. Al-Rawi, S. Tikriti, H.I. Dhahir, T.W. Clarkson, J.C. Smith and R.A. Doherty, Science, 181 (1973) 230.
88. R.P. Wedeen, D.K. Mallik and V. Batuman, Arch. Intern. Med., 139 (1979) 53.
89. E.L. Baker, P.J. Landrigan, A.G. Barbour, D.H. Cox, D.S. Folland, R.N. Ligo and J. Throckmorton, Br. J. Ind. Med., 36 (1979) 314.
90. S. Hernberg and S. Tola, Scand. J. Work Environ. Health, 5 (1979) 336.
91. D.F. Flick, H.F. Kraybill and J.M. Dimitroff, Environ. Res., 4 (1971) 71.
92. E. Mastromatteo, J. Occup. Med., 9 (1967) 127.
93. M. Margoshes and B.L. Vallee, J. Am. Chem. Soc., 79 (1957) 4813.
94. S.B. Gross, E.A. Pfitzer, D.W. Yeager and R.A. Kehoe, Toxicol. Appl. Pharmacol., 32 (1975) 638.
95. E.R. Humphreys and V.A. Stones, Int. J. Radiat. Biol., 33 (1978) 571.
96. J.L. Walker and H.M. Brown, Physiol. Rev., 57 (1977) 729.
97. C.O. Lee, Am. J. Physiol., 241 (1981) H459.
98. A.B. Borle and K.W. Snowdowne, Science, 217 (1982) 252.
99. S. Pors Nielsen, Scand. J. Clin. Lab. Invest., 23 (1969) 219.
100. J.K. Chesters and M. Will, Br. J. Nutr., 46 (1981) 111.
101. R.J.P. Williams, FEBS Lett., 140 (1982) 3.
102. R.M. Leach, Fed. Proc., 30 (1971) 991.
103. C.C. Widnell and J.R. Tata, Biochim. Biophys. Acta, 123 (1966) 478.
104. J.K. Chesters, Biochim. Biophys. Acta, 114 (1966) 385.
105. Y. Nagamine, D. Mizuno and S. Natori, Biochim. Biophys. Acta, 519 (1978) 440.
106. P.M. May and D.R. Williams, in: Ref. 82, pp. 1–28.
107. E. Giroux and J. Schoun, J. Inorg. Biochem., 14 (1981) 359.
108. S.H. Laurie and B. Sarkar, J. Chem. Soc., Dalton Trans., (1977) 1822.
109. A.M. Fiabane, M.L.D. Touche and D.R. Williams, J. Inorg. Nucl. Chem., 40 (1978) 1201.
110. S. Pollack, P. Aisen, F.D. Lasky and G. Vanderhoff, Br. J. Haematol., 34 (1976) 231.
111. P. Aisen and I. Listowsky, Annu. Rev. Biochem., 49 (1980) 357.
112. R.C. Najarian, D.C. Harris and P. Aisen, J. Biol. Chem., 253 (1978) 38.
113. P. Aisen, A. Leibman and J. Zweier, J. Biol. Chem., 253 (1978) 1930.
114. H. Huebers, B. Josephson, E. Huebers, E. Csiba and C. Finch, Proc. Natl. Acad. Sci. U.S.A., 78 (1981) 2572.
115. N.D. Chasteen and J. Williams, Biochem. J., 193 (1981) 717.

116. C. van der Heul, M.J. Kroos, W.L. van Noort and H.G. van Eijk, Clin. Sci., 60 (1981) 185.
117. H. Huebers, W. Bauer, E. Huebers, E. Csiba and C. Finch, Blood, 57 (1981) 218.
118. J. Williams, N.D. Chasteen and K. Moreton, Biochem. J., 201 (1982) 527.
119. E.F. Workman and G.W. Bates, J. Inorg. Biochem., 10 (1979) 41.
120. R.G. Batey, K. Williams and J.P. Milsom, Am. J. Physiol., 238 (1980) G30.
121. K. Konopka and I. Romslo, Eur. J. Biochem., 117 (1981) 239.
122. H.M. Schulman, A. Wilczynska and P. Ponka, Biochem. Biophys. Res. Commun., 100 (1981) 1523.
123. J. Zahringer, A.M. Konijn, B.S. Baliga and H.N. Munro, Biochem. Biophys. Res. Commun., 65 (1975) 583.
124. C.C. McCormick, M.P. Menard and R.J. Cousins, Am. J. Physiol, 240 (1981) E414.
125. M.R. Swerdel and R.J. Cousins, J. Nutr., 112 (1982) 801.
126. J.F.B. Mercer, I. Lazdins, T. Stevenson, J. Camakaris and D.M. Danks, Biosci. Rep., 1 (1981) 793.
127. H.N. Munro and M.C. Linder, Physiol. Rev., 58 (1978) 317.
128. I. Bremner, in: Trace Element Metabolism in Man and Animals, ed. J. McC. Howell, J.M. Gawthorne, and C.L. White (Australian Academy of Science, Canberra) Vol. IV (1981) pp. 637–642.
129. V.F. Fairbanks and G.G. Klee, Prog. Clin. Pathol., 8 (1981) 175.
130. R.W. Chen, D.J. Eakin and P.D. Whanger, Nutr. Rep. Int., 10 (1974) 195.
131. M.G. Cherian and R.A. Goyer, Life Sci., 23 (1978) 1.
132. J.K. Piotrowski, B. Trojanowska and A. Sapota, Arch. Toxicol., 32 (1974) 351.
133. E. Sabbioni and E. Marafante, Environ. Physiol. Biochem., 5 (1975) 132.
134. J.K. Piotrowski and J.A. Szymanska, J. Toxicol. Environ. Health, 1 (1976) 991.
135. M. Webb, L. Magos and E.H. Schaffer, Chem. Biol. Interactions, 32 (1980) 137.
136. J.H.R. Kagi and B.L. Vallee, J. Biol. Chem., 235 (1960) 3460.
137. D.Y. Mason and C.R. Taylor, J. Clin. Pathol., 31 (1978) 316.
138. K. Lerch, in: Ref. 62, Vol. XIII (1981) pp. 299–318.
139. R.R. Crichton, F. Roman and F. Roland, J. Inorg. Biochem., 13 (1980) 305.
140. A. Bakka, J. Aaseth and H.E. Rugstad, Acta Pharmacol. Toxicol., 49 (1981) 432.
141. R.E. Burch, H.J.K. Hahn and J.F. Sullivan, Clin. Chem., 21 (1975) 501.
142. R.L. Aamodt, W.F. Rumble, G.S. Johnston, D. Foster and R.I. Henkin, Am. J. Clin. Nutr., 32 (1979) 559.
143. R.M. Leach and M.S. Lilburn, World Rev. Nutr. Diet., 32 (1978) 123.
144. C.A. Finch, K. Deubelbeiss, J.D. Cook, J.W. Eschbach, L.A. Harker, D.D. Funk, G. Marsaglia, R.S. Hillman, S. Slichter, J.W. Adamson, A.M. Ganzoni and E.R. Giblett, Medicine, 49 (1970) 17.
145. P.B. Hammond, Annu. Rev. Pharmacol. Toxicol., 17 (1977) 197.
146. Z.A. Shaikh and O.J. Lucis, Arch. Environ. Health, 24 (1972) 410.
147. L. Golberg and L.E. Martin, Life Sci., 3 (1964) 1465.
148. A. Oskarsson and H. Tjalve, Br. J. Ind. Med., 36 (1979) 326.
149. C.D. Klaassen, Fundam. Appl. Toxicol., 1 (1981) 353.
150. J.A. Millar, G.G. Thompson, A. Goldberg, P.S.I. Barry and E.M. Lowe, Br. J. Ind. Med., 29 (1972) 317.
151. L.S. Schanker, D.J. Tocco, B.B. Brodie and C.A.M. Hogben, J. Pharmacol. Exp. Ther., 123 (1958) 81.

152. A. Albert, M.I. Gibson and S.D. Rubbo, Br. J. Exp. Pathol., 34 (1953) 119.
153. W.-W. Tso and W.-P. Fung, Inorg. Chim. Acta, 55 (1981) 129.
154. J.V. Princiotto, M. Rubin, G.C. Shashaty and E.J. Zapolski, J. Clin. Invest., 43 (1964) 825.
155. E.P. Haddock, E.J. Zapolski, M. Rubin and J.V. Princiotto, Proc. Soc. Exp. Biol. Med., 120 (1965) 663.
156. M. Rubin, E. Pachtman, M. Aldridge, E.J. Zapolski, D.H. Bagley and J.V. Princiotto, Biochem. Med., 3 (1970) 271.
157. D.H. Bagley, E.J. Zapolski, M. Rubin and J.V. Princiotto, Clin. Chim. Acta, 35 (1971) 311.
158. R.M. McClain and J.J. Siekierka, Toxicol. Appl. Pharmacol., 31 (1975) 443.
159. A. Catsch and A.E. Harmuth-Hoene, Pharmacol. Ther., 1 (1976) 1.
160. A. Catsch, Int. J. Appl. Radiat. Isot., 11 (1961) 131.
161. J.F. Markley, Int. J. Radiat. Biol., 7 (1963) 405.
162. Y. Rahman, M.W. Rosenthal and E.A. Cerny, Science, 180 (1973) 300.
163. S.P. Young, E. Baker and E.R. Huehns, Br. J. Haematol., 41 (1979) 357.
164. R. Green, J. Miller and W.H. Crosby, Clin. Res., 26 (1978) 503A.
165. R. Green, J. Miller and W. Crosby, Blood, 57 (1981) 866.
166. R. Green, J.L. Lamon and D. Curran, Lancet, ii (1980) 327.
167. H.S. Waxman and E.B. Brown, Prog. Hematol., 6 (1969) 338.
168. A.L. Crumbliss, R.A. Palmer, K.A. Sprinkle and D.R. Whitcomb, in: Ref. 16, pp. 175–197.
169. Y.E. Rahman, in: Liposomes in Biological Systems, ed. G. Gregoriadis and A.C. Allison (Wiley, New York, 1980) pp. 265–298.
170. A. Jacobs, in: Ref. 17, pp. 39–46.
171. E.B. Brown, in: Ref. 17, pp. 47–59.
172. M.M. Jones and M.A. Basinger, in: Inorganic Chemistry in Biology and Medicine, Am. Chem. Soc. Symposium Series 140, ed. A.E. Martell (A.C.S., Washington, 1980) pp. 335–348.
173. H. Foreman and T.T. Trujillo, J. Lab. Clin. Med., 43 (1954) 566.
174. F. Planas-Bohne, A.E. Harmuth-Hoene, K. Kuerzinger and F. Havlicek, Strahlentherapie, 136 (1968) 609.
175. T.R. Borthwick, G.D. Benson and H.J. Schugar, J. Lab. Clin. Med., 95 (1980) 575.
176. T.N. Pullman, A.R. Lavender and M. Forland, Annu. Rev. Med., 14 (1963) 175.
177. J.J. Chisolm, J. Pediatr., 73 (1968) 1.
178. J.M. Walshe, Proc. R. Soc. Med., 70: Suppl. 3 (1977) 1.
179. M.R. Summers, A. Jacobs, D. Tudway, P. Perera and C. Ricketts, Br. J. Haematol., 42 (1979) 547.
180. E.C. Vigliani and N. Zurlo, Br. J. Ind. Med., 8 (1951) 218.
181. H.C. Hodge, L.J. Leach, F.A. Smith, W.H. Strain and D.R. Taves, in: Drill's Pharmacology in Medicine, ed. J.R. DiPalma (McGraw-Hill, New York) 4th Edn. (1971) pp. 1120–1142.
182. M. Berlin and R. Rylander, J. Pharmacol. Exp. Ther., 146 (1964) 236.
183. M. Berlin, L.-G. Jerksell and G. Nordberg, Acta Pharmacol. Toxicol., 23 (1965) 312.
184. A. Catsch and A.E. Harmuth-Hoene, Biochem. Pharmacol., 24 (1975) 1557.
185. H. Foreman, C. Finnegan and C.C. Lushbaugh, J. Am. Med. Assoc., 160 (1956) 1042.

186. P.D. Doolan, S.L. Schwartz, J.R. Hayes, J.C. Mullen and N.B. Cummings, Toxicol. Appl. Pharmacol., 10 (1967) 481.
187. H. Foreman, J. Chron. Dis., 16 (1963) 319.
188. M.J. Seven, in: Metal-Binding in Medicine, ed. M.J. Seven (Lippincott, Philadelphia, 1960) pp. 95–103.
189. L. Marsh and F.C. Fraser, Lancet, 2 (1973) 846.
190. H. Swenerton and L.S. Hurley, Science, 173 (1971) 62.
191. C.A. Kimmel and C.S. Sloan, Teratology, 12 (1975) 330.
192. C.W. Mays, G.N. Taylor and D.R. Fisher, Health Phys., 30 (1976) 247.
193. A. Catsch, Arzneim.-Forsch., 17 (1967) 493.
194. C. Lucke-Huhle, Health Phys., 31 (1976) 349.
195. M.J. Millar, M.I. Fischer, C.A. Mawson and P.V. Elcoate, Nature (London), 174 (1954) 881.
196. P. Dvorak, Strahlentherapie, 139 (1970) 611.
197. W. Nadolny, Strahlentherapie, 141 (1971) 100.
198. F. Planas-Bohne and H. Olinger, Health Phys., 31 (1976) 165.
199. L.R. Cantilena and C.D. Klaassen, Toxicol. Appl. Pharmacol., 63 (1982) 344.
200. V. Volf, in: Treatment of Incorporated Transuranium Elements, Tech. Rep. Ser. 184 (I.A.E.A., Vienna, 1978).
201. N.L. Spoor, National Radiological Protection Board Report NRPB-R59 (H.M.S.O., London, 1977).
202. G. Berthon, C. Matuchansky and P.M. May, J. Inorg. Biochem., 13 (1980) 63.
203. J.R. Duffield, P.M. May and D.R. Williams, in: Ref. 128, pp. 152–154.
204. M.J. Seven, H. Gottlieb, H.L. Israel, J.G. Reinhold and M. Rubin, Am. J. Med. Sci., 228 (1954) 646.
205. A. Catsch, Fed. Proc., Suppl. 10, 20 (1961) 206.
206. J.E. Poldoski and T.J. Bydalek, J. Inorg. Nucl. Chem., 41 (1979) 205.
207. E.P. Abraham, E. Chain, W.H. Baker and R. Robinson, Nature (London), 151 (1943) 107.
208. J.M. Walshe, Q. J. Med., 22 (1953) 483.
209. J.M. Walshe, Lancet, i (1956) 25.
210. J.F. Strauss, R.M. Barrett and E.F. Rosenberg, Ann. Int. Med., 37 (1952) 323.
211. J.C. Crawhall, E.F. Scowen and R.W.E. Watts, Br. Med. J., 1 (1963) 588.
212. M.P. Purkiss and R.W.E. Watts, in: Ref. 178, 27.
213. J.R. Golding, J.V. Wilson and A.T. Day, Postgrad, Med. J., 46 (1970) 599.
214. I.A. Jaffe, Arth. Rheum., 13 (1970) 436.
215. Multicentre Trial Group, Lancet, i (1973) 275.
216. A.T. Day, J.R. Golding, P.N. Lee and A.D. Butterworth, Br. Med. J., 1 (1974) 180.
217. A. Dixon, J. Davies and T.L. Dormandy, Ann. Rheum. Dis., 34 (1975) 416.
218. J.E. Dippy, Br. J. Clin. Pract., 31 (1977) 5.
219. M.I.V. Jayson, C. Lovell, C.M. Black and R.S.E. Wilson, in: Ref. 178, 82.
220. T.B. Deering, E.R. Dickson and C.R. Fleming, Gastroenterology, 72 (1977) 1208.
221. O. Epstein, D. De Villiers, S. Jain, B.J. Potter, H.C. Thomas and S. Sherlock, N. Engl. J. Med., 300 (1979) 274.
222. O. Epstein, S. Jain and R. Lee, Lancet, i (1981) 1275.
223. E.J. Moynahan, in: Ref. 178, p. 73.
224. B.J. Mayou, Br. J. Dermatol., 105 (1981) 87.

225. S. Jain, P.J. Scheuer, S. Samourian, J.O'D. McGee and S. Sherlock, Lancet, 1 (1977) 831.
226. D.S. Matloff, E. Alpert, R.H. Resnick and M.M. Kaplan, N. Engl. J. Med., 306 (1982) 319.
227. F. Planas-Bohne, Arzneim.-Forsch., 22 (1972) 1426.
228. E. Polig and F. Planas-Bohne, Biophysik, 10 (1973) 321.
229. F. Planas-Bohne, Z. Naturforsch., 28c (1973) 774.
230. A.S. Weiss, J.A. Markenson, M.S. Weiss and W.H. Kammerer, Am. J. Med., 64 (1978) 114.
231. P.B. Halverson, F. Kozin, G.C. Bernhard and A.L. Goldman, J. Am. Med. Assoc., 240 (1978) 1870.
232. J. Thorvaldsen, Dermatologica, 159 (1979) 167.
233. H.B. Stein, A.C. Patterson, R.C. Offer, C.J. Atkins, A. Teufel and H.S. Robinson, Ann. Intern. Med., 92 (1980) 24.
234. W.F. Kean, I.L. Dwosh, T.P. Anastassiades, P.M. Ford and H.G. Kelly, Arth. Rheum., 23 (1980) 158.
235. K. Wysocka, F. Fabian and M. Listewnik, Z. Rheumatol., 40 (1981) 135.
236. J.M. Walshe, Ann. Intern. Med., 53 (1960) 1090.
237. K.D. Pool, H. Feit and J. Kirkpatrick, Ann. Intern. Med., 95 (1981) 457.
238. W.A.C. McAllister and J.A. Vale, Lancet, ii (1976) 631.
239. P.E. Lipsky and M. Ziff, J. Immunol., 120 (1978) 1006.
240. E.K. Wong and I.H. Leopold, Metab. Pediat. Opthal., 3 (1979) 1.
241. J.M. Walshe, Lancet, i (1982) 643.
242. S. Moeschlin, H. Bickel, A. Blumberg, F. Frey, L. Heilmeyer, H. Keberle, H.M. Keller, C. Maier, M. Mann, H.R. Marti, J.B. Nielsen, H. Schnack, U. Schnider, J. Tripod and F. Wohler, Schweiz. Med. Woch., 92 (1962) 1295.
243. G. Cartei, T. Barbui, M. Cazzavillan, T. Chisesi and E. Dini, Blut, 31 (1975) 11.
244. N. Cantore, M. Tortarolo, E. Volpe, F. Gonnella, A. Valente and G. Buonanno, Haematologica, 66 (1981) 196.
245. J.B. Neilands, in: Ref. 17, pp. 13-31.
246. C.B. Modell and J. Beck, Ann. N.Y. Acad. Sci., 232 (1974) 201.
247. D.G.D. Barr and D.K.B. Fraser, Br. Med. J., 1 (1968) 737.
248. R.M. Bannerman. S.T. Callender and D.L. Williams, Br. Med. J., 2 (1962) 1573.
249. R.S. Smith, Br. Med. J., 2 (1962) 1577.
250. F. Wohler, Med. Klin., 32 (1962) 1370.
251. F. Wohler, Acta Haematol., 30 (1963) 65.
252. S. Moeschlin and U. Schnider, N. Engl. J. Med., 269 (1963) 57.
253. Y.-F. Hwang and E.B. Brown, Arch. Intern. Med., 114 (1964) 741.
254. J.H. Graziano, Curr. Topics Hematol., 1 (1978) 127.
255. M. Barry, D.M. Flynn, E.A. Letsky and R.A. Risdon, Br. Med. J., 2 (1974) 16.
256. R. Seshadri, J.H. Colebatch, P. Gordon and H. Ekert, Arch. Dis. Child., 49 (1974) 621.
257. R.D. Propper, S.B. Shurin and D.G. Nathan, N. Engl. J. Med., 294 (1976) 1421.
258. A. Cohen and E. Schwartz, Ann. N.Y. Acad. Sci., 344 (1980) 405.
259. R.D. Propper, B. Cooper, R.R. Rufo, A.W. Nienhuis, W.F. Anderson, H.F. Bunn, A. Rosenthal and D.G. Nathan, N. Engl. J. Med., 297 (1977) 418.
260. F. Schettini, A. Mautone, L. Cavallo, M. Altomare, O. Montagna and L. Dell'Edera, Acta Haematol., 66 (1981) 96.

261. G.E. Janka, P. Mohring, M. Helmig, R.J. Haas and K. Betke, Eur. J. Pediatr., 137 (1981) 285.
262. R.W. Grady and A. Jacobs, in: Ref. 17, pp. 133–164.
263. G. Peters, H. Keberle, K. Schmid and H. Brunner, Biochem. Pharmacol., 15 (1966) 93.
264. H.G. Meyer-Brunot and H. Keberle, Biochem. Pharmacol., 16 (1967) 527.
265. R.A. McCance and E.M. Widdowson, Lancet, i (1937) 680.
266. A. Jacobs, Seminars Haematol., 14 (1977) 89.
267. W.H. Crosby, Br. J. Haematol., 4 (1958) 82.
268. B. Modell, Prog. Hematol., 11 (1979) 267.
269. H. Wishinsky, T. Weinberg, E.M. Prevost, B. Burgin and M.J. Miller, J. Lab. Clin. Med., 42 (1953) 550.
270. W.G. Figueroa, in: Ref. 188, pp. 146–153.
271. J.L. Fahey, C.E. Rath, J.V. Princiotto, I.B. Brick and M. Rubin, J. Lab. Clin. Med., 57 (1961) 436.
272. J.B. Neilands, J. Am. Chem. Soc., 74 (1952) 4846.
273. J.B. Neilands, Bacteriol. Rev., 21 (1957) 101.
274. T.F. Emery, Adv. Enzymol., 35 (1971) 135.
275. W-C. Liu, S.M. Fisher, J.S. Wells, C.S. Ricca, P.A. Principe, W.H. Trejo, D.P. Bonner, J.Z. Gougoutos, B.K. Toeplitz and R.B. Sykes, J. Antibiot., 34 (1981) 791.
276. J.B. Neilands, Annu. Rev. Biochem., 50 (1981) 715.
277. K.N. Raymond and C.J. Carrano, Acc. Chem. Res., 12 (1979) 183.
278. C. Hershko and E.A. Rachmilewitz, Isr. J. Med. Sci., 14 (1978) 1111.
279. C. Hershko, R.W. Grady and A. Cerami, J. Lab. Clin. Med., 92 (1978) 144.
280. C. Hershko and E.A. Rachmilewitz, Br. J. Haematol., 42 (1979) 125.
281. C. Hershko, G. Link and L. Eilon, Adv. Red Cell Biol., 1 (1982) 121.
282. J. Fielding, J. Clin. Pathol., 18 (1965) 88.
283. S.P. Balcerzak, W.N. Jensen and S. Pollack, Scand. J. Haematol., 3 (1966) 205.
284. C.D. Karabus and J. Fielding, Br. J. Haematol., 13 (1967) 924.
285. E.B. Brown, Y.-F. Hwang and J.W. Allgood, J. Lab. Clin. Med., 69 (1967) 382.
286. L.A. Harker, D.D. Funk and C.A. Finch, Am. J. Med., 45 (1968) 105.
287. D.A. Lipschitz, J. Dugard, M.O. Simon, T.H. Bothwell and R.W. Charlton, Br. J. Haematol., 20 (1971) 395.
288. I. Cavill, M. Worwood and A. Jacobs, Nature (London), 256 (1975) 328.
289. R. Bailey-Wood, G.P. White and A. Jacobs, Br. J. Exp. Pathol., 56 (1975) 358.
290. G.P. White, A. Jacobs, R.W. Grady and A. Cerami, Blood, 48 (1976) 923.
291. G.P. White, R. Bailey-Wood and A. Jacobs, Clin. Sci. Mol. Med., 50 (1976) 145.
292. G.P. White and A. Jacobs, Biochim. Biophys. Acta, 543 (1978) 217.
293. E. Baker, F.R. Vicary and E.R. Huehns, Br. J. Haematol., 47 (1981) 493.
294. R. Rama, J.-N. Octave, Y.-J. Schneider, J.-C. Sibille, J.N. Limet, J.-C. Mareschal, A. Trouet and R.R. Chrichton, FEBS Lett., 127 (1981) 204.
295. A. Jacobs, Blood, 50 (1977) 433.
296. L. Hallberg and L. Hedenberg, Scand. J. Haematol., 2 (1965) 67.
297. R.W. Charlton, D.A. Lipschitz, J. Dugard, M.O. Simon and T.H. Bothwell, S. Afr. Med. J., 45 (1971) 178.
298. L.R. Weintraub, M.E. Conrad and W.H. Crosby, Med. Clin. North. Am., 50 (1966) 1579.

299. Y. Sidi, M. Shaklai, E. Liban and J. Pinkhas, Isr. J. Med. Sci., 17 (1981) 348.
300. A.A. Wapnick, S.R. Lynch, R.W. Charlton, H.C. Seftel and T.H. Bothwell, Br. J. Haematol., 17 (1969) 563.
301. J.E. Ploem, J. de Wael, M.C. Verloop and K. Punt, Br. J. Haematol., 12 (1966) 396.
302. J.K. Hampton, Am. J. Physiol., 176 (1954) 20.
303. T.G. Hoy, P.M. Harrison, M. Sabir and I.G. Macara, Biochem. J., 137 (1974) 67.
304. H.G. Meyer-Brunot and H. Keberle, Am. J. Physiol., 214 (1968) 1193.
305. M.J. Pippard, S.T. Callender and C.A. Finch, Blood, 60 (1982) 288.
306. C. Hershko, Blood, 51 (1978) 415.
307. M. Barry, G. Cartei and S. Sherlock, Gut, 11 (1970) 891.
308. C. Hershko, J. Lab. Clin. Med., 85 (1975) 913.
309. S.P. Young and P. Aisen, Biochim. Biophys. Acta, 633 (1980) 145.
310. S.A.K. Wilson, Brain, 34 (1912) 295.
311. A.J. Glazebrook, Edinburgh Med. J., 52 (1945) 83.
312. I.H. Scheinberg and I. Sternlieb, Pharmacol. Rev., 12 (1960) 355.
313. B.M. Mandelbrote, M.W. Stanier, R.H.S. Thompson and M.N. Thruston, Brain, 71 (1948) 212.
314. J.N. Cumings, Brain, 71 (1948) 410.
315. D. Denny-Brown and H. Porter, N. Engl. J. Med., 245 (1951) 917.
316. J.N. Cumings, Brain, 74 (1951) 10.
317. I. Sternlieb, C.J.A. Van Den Hamer, A.G. Morell, S. Alpert, G. Gregoriadis and I.H. Scheinberg, Gastroenterology, 64 (1973) 99.
318. J.M. Walshe, Q. J. Med., 42 (1973) 441.
319. T.U. Hoogenraad, R. Koevoet and E.G.W.M. de Ruyter Korver, Eur. Neurol., 18 (1979) 205.
320. D.M. Danks, Lancet, ii (1982) 435.
321. J.M. Walshe, in: Ref. 188, pp. 265–268.
322. P.M. May and D.R. Williams, in: Ref. 62, Vol. XII (1981) pp. 283–317.
323. J.R. Wright and E. Frieden, Bioinorg. Chem., 4 (1975) 163.
324. T. Shalouhi, P.T. Evans and J.R. Wright, Physiol. Chem. Physics, 8 (1976) 337.
325. M. Kekki, P. Koskelo and E.A. Nikkila, Metabolism, 15 (1966) 1029.
326. G.E. Jackson, P.M. May and D.R. Williams, FEBS Lett., 90 (1978) 173.
327. H.K. Sachs, L.A. Blanksma, E.F. Murray and M.J. O'Connell, Pediatrics, 46 (1970) 389.
328. W.R. Lee, J. R. Coll. Physicians Lond., 15 (1981) 48.
329. D.D. Perrin, in: An Introduction to Bio-inorganic Chemistry, ed. D.R. Williams (Thomas, Springfield, Il., 1976) pp. 361–381.
330. S. Nagy and R.L. Rouseff, J. Agric. Food Chem., 29 (1981) 889.
331. A. Cavalleri, A. Baruffini, C. Minoia and L. Bianco, Environ. Res., 25 (1981) 415.
332. J.M. Guerit, M. Meulders, G. Amand, H.A. Roels, J.P. Buchet, R. Lauwerys, P. Bruaux, F. Claeys-Thoreau, G. Ducoffre and A. Lafontaine, Clin. Toxicol., 18 (1981) 1257.
333. J.A.G. Graham, D.G. Maxton and C.H.C. Twort, Lancet, ii (1981) 1159.
334. J.S. Lin-Fu, in: Low Level Lead Exposure, ed. H.L. Needleman (Raven Press, New York, 1980) pp. 5–16.
335. V.N. Houk, in: Ref. 334, pp. 231–237.
336. United States Environmental Protection Agency, Air Quality Criteria for Lead, Publ. No. EPA-600/8-77-017 (1977).

337. Lead and Health: The Report of a DHSS Working Party on Lead in the Environment (HMSO, London, 1980).
338. Anon., New. Sci., 96 (1982) 281.
339. M. Rutter, Dev. Med. Child Neurol., 22: Suppl. 42 (1980).
340. J.M. Hunter, Soc. Sci. Med., 11 (1977) 691.
341. G.B. Gerber, A. Leonard and P. Jacquet, Mutat. Res., 76 (1980) 115.
342. D.M. Berwick and A.L. Komaroff, N. Engl. J. Med., 306 (1982) 1392.
343. D. Goldberg and B. Davidow, N. Engl. J. Med., 307 (1982) 1268.
344. R.K. Byers and E.E. Lord, Am. J. Dis. Child., 66 (1943) 471.
345. K.M. Jason and C.K. Kellogg, in: Lead Toxicity, eds. R.L. Singhal and J.A. Thomas (Urban and Schwarzenberg, Baltimore, MD, 1980) pp. 241–271.
346. H.L. Needleman, C. Gunnoe, A. Leviton, R. Reed, H. Peresie, C. Maher and P. Barrett, N. Engl. J. Med., 300 (1979) 689.
347. M.R. Moore, Nature (London), 283 (1980) 334.
348. H.L. Needleman and D. Bellinger, J. Am. Acad. Child Psychiatr., 20 (1981) 496.
349. H.L. Needleman, A. Leviton and D. Bellinger, N. Engl. J. Med., 306 (1982) 367.
350. J.S. Lin-Fu, New Engl. J. Med., 307 (1982) 615.
351. O. David, E. Grad, B. Mcgann and A. Koltun, Am. J. Psychiatr., 139 (1982) 806.
352. D.C. Rice, S.G. Gilbert and R.F. Willes, Toxicol., Appl. Pharmacol., 51 (1979) 503.
353. P.J. Bushnell and R.E. Bowman, Neurobehav. Toxicol., 1 (1979) 207.
354. V. Albergoni, N. Favero and G.P. Rocco, Bioinorg. Chem., 9 (1978) 431.
355. D.P. Alfano and T.L. Petit, Behav. Neural Biol., 32 (1981) 319.
356. E. Gross-Selbeck and M. Gross-Selbeck, Clin. Toxicol., 18 (1981) 1247.
357. D.H. Minsker, N. Moskalski, P.P. Chennekatu, R.T. Robertson and D.L. Bokelman, Biol. Neonate, 41 (1982) 193.
358. D. Bryce-Smith and H.A. Waldron, Chem. Br., 10 (1974) 205.
359. Anon., Lancet, i (1982) 1337.
360. FDA Drug Bull., 6 (1976) 26.
361. Anon., J. Pediatr., 93 (1978) 709.
362. S.M. Marcus, Vet. Human Toxicol., 24 (1982) 18.
363. P.B. Hammond and A.L. Aronson, Ann. N.Y. Acad. Sci., 88 (1960) 498.
364. C.E.C. Harris, Can. Med. Assoc. J., 79 (1958) 664.
365. W.T.L. Ohlsson, Br. Med. J., 2 (1962) 1454.
366. A. Goldberg, J.A. Smith and A.C. Lochhead, Br. Med. J., 1 (1963) 1270.
367. C.S. Bartsocas, J.A. Grunt, G.W. Boylen and I.K. Brandt, Acta Paediat. Scand., 60 (1971) 553.
368. L.F. Vitale, A.D. Rosalinas-Bailon, Folland, J.F. Brennan and B. McCormick, J. Pediatr., 83 (1973) 1041.
369. F.H. Nielsen, in: Ref. 172, pp. 23–42.
370. H.M. Perry, G.S. Thind and E.F. Perry, Med. Clin. North Am., 60 (1976) 759.
371. M. Piscator, Environ. Health Perspect., 40 (1981) 107.
372. S.J. Kopp, T. Glonek, H.M. Perry, M. Erlanger and E.F. Perry, Science, 217 (1982) 837.
373. G. Huel, C. Boudene and M.A. Ibrahim, Arch. Environ. Health, 36 (1981) 221.
374. A.W. Voors, M.S. Shuman and W.D. Johnson, Arch. Environ. Health, 37 (1982) 98.
375. World Health Organisation, Tech. Rep. Ser., 505 (1972).

376. H. Inskip, V. Beral and M. McDowall, Lancet, i (1982) 896.
377. L. Friberg, M. Piscator and G. Nordberg, in: Cadmium in the Environment (CRC, Philadelphia) Vol. I (1971) pp. 48–54.
378. J.M. Tobias, C.C. Lushbaugh, H.M. Patt, S. Postel, M.N. Swift and R.W. Gerard, J. Pharmacol. Exp. Ther., 87 (1946) 102.
379. T. Dalhamn and L. Friberg, Acta Pharmacol. Toxicol., 11 (1955) 68.
380. L. Friberg, Arch. Ind. Health, 13 (1956) 18.
381. R.J. Wyatt, Arch. Environ. Health, 1 (1960) 487.
382. V. Eybl, J. Sykora and F. Mertl, Acta Biol. Med. Germ., 17 (1966) 178.
383. G. Lehnert, R. Eschstruth, D. Szadkowski and K.-H. Schaller, Med. Welt., 9 (1970) 346.
384. W.H. Lyle, J.N. Green, V. Gore and J. Vidler, Postgrad. Med. J., (1968) 18.
385. H.N. MacFarland, in: Cadmium Toxicity, ed. J.H. Mennear (Marcel Dekker, New York, 1979) pp. 113–132.
386. M.G. Cherian, Nature (London), 287 (1980) 871.
387. R. Von Burg and J.C. Smith, J. Toxicol. Environ. Health, 6 (1980) 75.
388. M.G. Cherian, J. Toxicol. Environ. Health, 6 (1980) 393.
389. M.G. Cherian and K. Rodgers, J. Pharmacol. Exp. Ther., 222 (1982) 699.
390. B. Gabard, Arch. Toxicol., 39 (1978) 289.
391. B. Gabard, F. Planas-Bohne and G. Regula, Toxicology, 12 (1979) 281.
392. B. Gabard and R. Walser, J. Toxicol. Environ. Health, 5 (1979) 759.
393. F. Planas-Bohne, B. Gabard and E.H. Schaffer, Arzneim.-Forsch., 30 (1980) 1291.
394. F. Berglund, M. Berlin, G. Birke, R. Cederlof, U. Von Euler, L. Friberg, B. Holmstedt, E. Jonsson, K.C. Luning, C. Ramel, S. Skerfving, A. Swensson and S. Tejning, Nord. Hyg. Tydskr., Suppl. 4 (1971).
395. N. Wright, W.B. Yeoman and G.F. Carter, Lancet, i (1980) 206.
396. T. Refsvik and T. Norseth, Acta Pharmacol. Toxicol., 36 (1975) 67.
397. F.W. Sunderman, Ann. Clin. Lab. Sci., 8 (1978) 259.
398. L.L. Waters and C.C. Stock, Science, 102 (1945) 601.
399. H.V. Aposhian and M.M. Aposhian, J. Pharmacol. Exp. Ther., 126 (1959) 131.
400. G. Belonozhko, V.I. Uitte-Drozdobsky, V.I. Kefeli and B.M. Shchepotin, Urach. Delv., No. 1 (1957).
401. J.E. Okoshnikova, Prom. Toksikol. Klin. Prof. Zabolevanii Khim. Etiol. Sbornik, 205 (1962); Chem. Abstr. 62:12357h.
402. G.L. Voelz, L.S. Hempelmann, J.N.P. Lawrence and W.D. Moss, Health Phys., 37 (1979) 445.
403. C.W. Mays, G.N. Taylor, R.D. Lloyd and C.W. Jones, Radiat. Res., 87 (1981) 387.
404. V.H. Smith, G.E. Dagle, R.A. Gelman and H.A. Ragan, Toxicol. Lett., 7 (1980) 9.
405. V. Volf, A. Seidel and K. Takada, Health Phys., 32 (1977) 155.
406. A. Seidel and V. Volf, Health Phys., 22 (1972) 779.
407. F. Planas-Bohne and H. Ebel, Health Phys., 29 (1975) 103.
408. L. Ohlenschlager, Health Phys., 30 (1976) 249.
409. V. Volf, Health Phys., 31 (1976) 290.
410. R.D. Lloyd, C.W. Mays, S.S. McFarland, G.N. Taylor and D.R. Atherton, Health Phys., 31 (1976) 281.
411. K. Takada and V. Volf, Radiat. Res., 70 (1977) 164.
412. L. Ohlenschlager, H. Schieferdecker and W. Schmidt-Martin, Health Phys., 35 (1978) 694.

413. P.M. May and D.R. Williams, in: Ref. 77, pp. 179–181.
414. J. Schubert and A. Lindenbaum, in: Ref. 188, pp. 68–74.
415. L. Kolsipinski, Monthly Cyclop. Med. Bull., 4 (1911) 348.
416. E. Horak and F.W. Sunderman, Ann. Clin. Lab. Sci., 10 (1980) 425.
417. F.W. Sunderman, Ann. Clin. Lab. Sci., 11 (1981) 1.
418. L.G. Morgan, J. Occup. Med., 29 (1979) 33.
419. E.J. Bernacki, E. Zygowicz and Sunderman F.W., Ann. Clin. Lab. Sci., 10 (1980) 33.
420. A.P. Polednak, Arch. Environ. Health, 36 (1981) 235.
421. J.E. Cox, R. Doll, W.A. Scott and S. Smith, Br. J. Ind. Med., 38 (1981) 235.
422. E.E. Menden, V.J. Elia, L.W. Michael and H.G. Petering, Environ. Sci. Technol., 6 (1972) 830.
423. F.W. Sunderman, Arch. Ind. Health, 18 (1958) 480.
424. F.W. Sunderman and J.F. Kincaid, J. Am. Med. Assoc., 155 (1954) 889.
425. D.C. Jones, P.M. May and D.R. Williams, in: Nickel Toxicology eds. S.S. Brown and F.W. Sunderman (Academic Press, New York, 1980) pp. 71–76.
426. F.W. Sunderman, O.E. Paynter and R.B. George, Am. J. Med. Sci., 254 (1967) 46.
427. K.C. Hodge, J.P. Day, M. O'Hara, P. Ackrill and A.J. Ralston, Lancet, ii (1981) 802.
428. B.F. Boyce, G.S. Fell, H.Y. Elder, B.J. Junor, H.L. Elliot, G. Beastall. I. Fogelman and I.T. Boyle, Lancet, ii (1982) 1009.
429. D.R. Crapper McLachlan and U. De Boni, Neurotoxicology, 1 (1980) 3.
430. D.P. Perl and A.R. Brody, Science, 208 (1980) 297.
431. H.M. Wisniewski, K. Iqbal and J.R. McDermott, Neurotoxicology, 1 (1980) 121.
432. W.O. Caster and M. Wang, Sci. Total Environ., 17 (1981) 31.
433. J.A. Bjorksten, Sci. Total Environ., 25 (1982) 81.
434. W.O. Caster and M. Wang, Sci. Total Environ., 25 (1982) 85.
435. E.I. Hamilton, Sci. Total Environ., 25 (1982) 87.
436. P. Ackrill, A.J. Ralston, J.P. Day and K.C. Hodge, Lancet, ii (1980) 692.
437. R.S. Arze, I.S. Parkinson, N.E.F. Cartlidge, P. Britton and M.K. Ward, Lancet, ii (1981) 1116.
438. H. Pogglitsch, W. Petek, O. Wawschinek and W. Holzer, Lancet, ii (1981) 1344.
439. D.J. Brown, J.K. Dawborn, K. Hum and J.M. Xipell, Lancet, ii (1982) 343.
440. C.M. Peterson and A. Cerami, N. Engl. J. Med., 292 (1975) 162.
441. F. Gross, Br. Med. J., 285 (1982) 1444.
442. Anon., Lancet, i (1981) 1297.
443. M.H. Van Woert, N. Engl. J. Med., 298 (1978) 903.
444. M. Weatherall, Nature (London), 296 (1982) 387.
445. A.J. Bard and M.D. King, J. Chem. Educ., 42 (1965) 127.
446. R. Arnek, L.G. Sillen and O. Wahlberg, Arkiv Kemi, 31 (1969) 353.
447. I. Sternlieb and I.H. Scheinberg, N. Engl. J. Med., 278 (1968) 352.
448. H.B.F. Dixon, K. Gibbs and J.M. Walshe, Lancet, i (1972) 853.
449. J.M. Walshe, Br. Med. J., 2 (1975) 701.
450. C.W. Mays, G.N. Taylor, R.D. Lloyd and M.E. Wrenn, in: Actinides in Man and Animals, ed. M.E. Wrenn (RD Press, New York, 1980) pp. 351–368.
451. J.T. McEnery, J. Pediatr., 72 (1968) 147.
452. H.L. Hardy, Fed. Proc., Suppl. 10, 20 (1961) 199.

453. R.W. Grady, J.H. Graziano, H.A. Akers and A. Cerami, Blood, 44 (1974) 911.
454. C.L. Atkins and J.B. Neilands, Biochemistry, 7 (1968) 3734.
455. R.W. Grady, C.M. Peterson, R.L. Jones, J.H. Graziano, K.K. Bhargave, V.A. Berdoukas, G. Kokkin, D. Loukopoulos and A. Cerami, J. Pharmacol. Exp. Ther., 209 (1979) 342.
456. A. Jacobs, Br. J. Haematol., 43 (1979) 1.
457. C.G. Pitt, G. Gupta, W.E. Estes, H. Rosenkrantz, J.J. Metterville, A.L. Crumbliss, R.A. Palmer, K.W. Nordquest, K.A. Sprinkle Hardy, D.R. Whitcomb, B.R. Byers, J.E.L. Arceneaux, C.G. Gaines and C.V. Sciortino, J. Pharmacol. Exp. Ther., 208 (1979) 12.
458. S.A. Ong, T. Peterson and J.B. Neilands, J. Biol. Chem., 254 (1979) 1860.
459. C.V. Sciortino, B.R. Byers and P. Cox, J. Lab. Clin. Med., 96 (1980) 1081.
460. A. Jacobs, G.P. White and G.P. Tait, Biochem. Biophys. Res. Commun., 74 (1977) 1626.
461. R.J. Bergeron, P.S. Burton, K.A. McGovern and E.J. St. Onge, J. Med. Chem., 23 (1980) 1130.
462. C.M. Peterson, R.W. Grady, R.L. Jones, A. Cerami, J.H. Graziano, A.L. Merkenson, V. Lavi, V.C. Canale, G.F. Gray and D.R. Miller, N. Engl. J. Med., 297 (1977) 1404.
463. J.H. Graziano, R.W. Grady and A. Cerami, J. Pharmacol. Exp. Ther., 190 (1974) 570.
464. R.W. Grady, J.H. Graziano, H.A. Akers and A. Cerami, J. Pharmacol. Exp. Ther., 196 (1976) 478.
465. M. Peterson, J.H. Graziano, R.W. Grady, R.L. Jones, H.V. Vlassara, V.C. Canale, D.R. Miller and A. Cerami, Br. J. Haematol., 33 (1976) 477.
466. C.M. Peterson, J.H. Graziano, R.W. Grady, R.L. Jones, A. Markenson, V. Lavi, V.C. Canale, G.F. Gray, A. Cerami and D.R. Miller, Exp. Hematol., 7 (1979) 74.
467. J.H. Graziano, D.R. Miller, R.W. Grady and A. Cerami, Br. J. Haematol., 32 (1976) 351.
468. R.W. Grady, J.H. Graziano, G.P. White, A. Jacobs and A. Cerami, J. Pharmacol. Exp. Ther., 205 (1978) 757.
469. F. Cleton, A. Turnbull and C.A. Finch, J. Clin. Invest., 42 (1963) 327.
470. R. Gunther, Naunyn-Sch. Arch. Pharmakol. Exp. Pathol., 262 (1969) 405.
471. M.J. Pippard, D.K. Johnson and C.A. Finch, Blood, 58 (1981) 685.
472. I.H. Krakoff, E. Etcubanas, C. Tan, K. Mayer, V. Bethune and J.H. Burchenal, Cancer Chemother. Rep., 58 (1974) 207.
473. E. Ankel and D.H. Petering, Biochem. Pharmacol., 29 (1980) 1833.
474. M. Cikrt, P. Ponka, J. Neuwirt and E. Necas, Br. J. Haematol., 45 (1980) 275.
475. L.A. Saryan, E. Ankel, C. Krishnamurti, D.H. Petering and H. Elford, J. Med. Chem., 22 (1979) 1218.
476. P. Ponka, J. Borova, J. Neuwirt and O. Fuchs, FEBS Lett., 97 (1979) 317.
477. P. Ponka, J. Borava, J. Neuwirt, O. Fuchs and E. Necas, Biochim. Biophys. Acta, 586 (1979) 278.
478. T. Hoy, J. Humphrys, A. Jacobs, A. Williams and P. Ponka, Br. J. Haematol., 43 (1979) 443.
479. C. Hershko, S. Avramovici-Grisaru, G. Link, L. Gelfand and S. Sarel, J. Lab. Clin. Med., 98 (1981) 99.

480. D.K. Johnson, M.J. Pippard, T.B. Murphy and N.J. Rose, J. Pharmacol. Exp. Ther., 221 (1982) 399.
481. W.R. Harris, C.J. Carrano and K.N. Raymond, J. Am. Chem. Soc., 101 (1979) 2722.
482. M.C. Venuti, W.H. Rastetter and J.B. Neilands, J. Med. Chem., 22 (1979) 123.
483. F.L. Weitl, W.R. Harris and K.N. Raymond, J. Med. Chem., 22 (1979) 1281.
484. W.H. Rastetter, T.J. Erickson and M.C. Venuti, J. Org. Chem., 46 (1981) 3579.
485. K.N. Raymond, V.L. Pecoraro and F.L. Weitl, in: Ref. 17, pp. 165–187.
486. T.P. Tufano, V.L. Pecoraro and K.N. Raymond, Biochim. Biophys. Acta, 668 (1981) 420.
487. T.R. Borthwick, G.D. Benson and H.J. Schugar, Proc. Soc. Exp. Biol. Med., 162 (1979) 227.
488. M.S. Tanner, B. Portmann, A.P. Mowat, R. Williams, A.N. Pandit, C.F. Mills and I. Bremner, Lancet, i (1979) 1203.
489. H. Harders, B. Armah, E. Cohnen, J. Fuhr, N. Heinz and H.F. Schroder,, Arzneim.-Forsch., 30 (1980) 254.
490. F. Planas-Bohne, Toxicol. Appl. Pharmacol., 50 (1979) 337.
491. C.L. Keen, N.L. Cohen, B. Lonnerdal and L.S. Hurley, Lancet, i (1982) 1127.
492. I. Sternlieb, J. Rheumatol., Suppl. 7 (1981) 94.
493. M.M. Jones, M.A. Basinger and M.P. Tarka, Res. Commun. Chem. Pathol. Pharmacol., 27 (1980) 571.
494. H. Iwata, K. Watanabe, H. Miichi and Y. Matsui, Pharmacol. Res. Commun., 2 (1970) 213.
495. J.H. Graziano, J.K. Leong and E. Friedheim, J. Pharmacol. Exp. Ther., 206 (1978) 696.
496. E.M.B. Sorensen, E.S. Moretti and A. Lindenbaum, Bull. Environ. Contam. Toxicol., 22 (1979) 617.
497. P. Baudot, M. Jacque and M. Robin, Toxicol. Appl. Pharmacol., 41 (1977) 113.
498. A. Gilman, F.S. Philips, R.P. Allen and E.S. Koelle, J. Pharmacol. Exp. Ther., 87 (1946) 85.
499. W.H. Lyle, J. Rheumatol., Suppl. 7 (1981) 96.
500. A. Bakka and J. Aaseth, Arh. Hig. Rada Toksikol., 30 (1979) 183.
501. M.G. Cherian, J. Toxicol. Environ. Health, 6 (1980) 379.
502. M.G. Cherian, S. Onosaka, G.K. Carson and P.A.W. Dean, J. Toxicol. Environ. Health, 9 (1982) 389.
503. L.R. Cantilena and C.D. Klaassen, Toxicol. Appl. Pharmacol., 58 (1981) 452.
504. G. Irwin, L.R. Cantilena, C.D. Klaassen and S. Preskorn, Toxicol. Appl. Pharmacol., 63 (1982) 338.
505. A. Yoshida, B.E. Kaplan and M. Kimura, Proc. Natl. Acad. Sci. U.S.A., 76 (1979) 486.
506. J.-M. Lehn, Acc. Chem. Res., 11 (1978) 49.
507. J. Schubert and S.K. Derr, Nature (London), 275 (1978) 311.
508. M.M. Jones and M.A. Basinger, Res. Commun. Chem. Pathol. Pharmacol., 24 (1979) 525.
509. F. Planas-Bohne, Experientia, 36 (1980) 1001.
510. E.R. Humphreys and V.A. Stones, Health Phys., 39 (1980) 103.
511. C.W. Jones, R.D. Lloyd and C.W. Mays, Radiat. Res., 84 (1980) 149.

512. J. Schubert, Nature (London), 281 (1979) 406.
513. M.M. Jones, A.D. Weaver and W.L. Weller, Res. Commun. Chem. Pathol. Pharmacol., 22 (1978) 581.
514. A. Wannag and J. Aaseth, Acta Pharmacol. Toxicol., 46 (1980) 81.
515. F. Planas-Bohne, Toxicology, 19 (1981) 275.
516. F. Planas-Bohne, J. Pharmacol. Exp. Ther., 217 (1981) 500.
517. M.M. Jones, M.A. Basinger, A.D. Weaver, C.M. Davies and W.K. Vaughn, Res. Commun. Chem. Pathol. Pharmacol., 27 (1980) 363.
518. F. Planas-Bohne and H. Olinger, Biochem. Pharmacol., 30 (1981) 667.
519. T. Yonaga and K. Morita, Toxicol. Appl. Pharmacol., 57 (1981) 197.
520. H. Takahashi and K. Hirayama, Nature (London), 232 (1971) 201.
521. J.J. Benes, J. Stanberg, J. Peska, M. Tich and M. Cikrt, Angew. Makromol. Chem., 44 (1975) 67.
522. J. Aaseth and T. Norseth, in: Clinical Chemistry and Chemical Toxicology of Metals, ed. S. Brown (Elsevier, Amsterdam, 1977) pp. 225–228.
523. G.A. Nyssen, M.M. Jones, J.D. Jernigan, R.D. Harbison and J.S. MacDonald, J. Inorg. Nucl. Chem., 39 (1977) 1889.
524. M.M. Jones and G.A. Nyssen, J. Inorg. Nucl. Chem., 40 (1978) 1235.
525. S. Margel, J. Med. Chem., 24 (1981) 1263.
526. R.D. Harbison, M.M. Jones, J.S. MacDonald, T.H. Pratt and R.L. Coates, Toxicol. Appl. Pharmacol., 42 (1977) 445.
527. L.K. Steinrauf, B. Cox, E. Foster, A. Sattar and R.T. Blickenstaff, J. Pharm. Sci., 67 (1978) 1739.
528. R.T. Blickenstaff, B. Cox, E. Foster, L. Roberts and L.K. Steinrauf, J. Pharm. Sci., 69 (1980) 556.
529. K.S. Rajan, S. Mainer, N.L. Rajan and J.M. Davis, J. Inorg. Biochem., 14 (1981) 339.
530. V.R. Soroka, Arkh. Patol., 27 (1966) 58.
531. S.M. Moerlin, M.J. Welch and K.N. Raymond, J. Nucl. Med., 23 (1982) 501.
532. J.R. Behari and S.K. Tandon, Clin. Toxicol., 16 (1980) 33.
533. S.K. Tandon and J.S. Gaur, Clin. Toxicol., 11 (1977) 257.
534. S. Langard and T. Norseth, in: Handbook on the Toxicology of Metals, ed. L. Friberg, G.F. Nordberg and V.B. Vouk (Elsevier, Amsterdam, 1979) p. 395.
535. D.K. Dastur, D.K. Manghani, K.V. Raghavedran and K.N. Jeejeebhoy, Q.J. Exp. Physiol., 54 (1969) 322.
536. A. Barbeau, N. Inoue and T. Cloutier, Adv. Neurol., 14 (1976) 339.
537. I. Mena, O. Marin, S. Fuenzalida and G.C. Cotzias, Neurology, 17 (1967) 128.
538. R. Kawamura, H. Ikuta and T. Fukuzami, Jap. J. Bacteriol., 537 (1940) 687.
539. A.M. Emara, S.H. El-Ghawabi, O.I. Madkour and G.H. El-Samra, Br. J. Ind. Med., 28 (1971) 78.
540. D.G. Cook, S.F. Fahn and K.A. Brait, Arch. Neurol., 30 (1954) 59.
541. J.F. Fried, A. Lindenbaum and J. Schubert, Proc. Soc. Exp. Biol. Med., 100 (1959) 570.
542. A. Kuhn, Strahlentherapie, 137 (1969) 101.
543. S.K. Tandon and J. Singh, Toxicology, 5 (1975) 237.
544. S.K. Tandon, S.V. Chandra, J. Singh, R. Husain and P.K. Seth, Environ. Res., 9 (1975) 18.

545. S.K. Tandon, Toxicology, 9 (1978) 379.
546. S. Khandelwal, D.N. Kachru and S.K. Tandon, Toxicol. Lett., 6 (1980) 131.
547. S.K. Tandon and S. Khandelwal, Arch. Toxicol., 50 (1982) 19.
548. I. Mena, J. Court, S. Fuenzalida, P.S. Papavasilou and G.C. Cotzias, N. Engl. J. Med., 282 (1970) 5.
549. E. Horak, F.W. Sunderman and B. Sarkar, Res. Commun. Chem. Pathol. Pharmacol., 14 (1976) 153.
550. M.A. Basinger, M.M. Jones and M.P. Tarka, Res. Commun. Chem. Pathol. Pharmacol., 30 (1980) 133.
551. R.C. Baselt, F.W. Sunderman, J.R. Mitchell and E. Horak, Res. Commun. Chem. Pathol. Pharmacol., 18 (1977) 677.
552. C.H. Tadlock and H.V. Aposhian, Biochem. Biophys. Res. Commun., 94 (1980) 501.
553. W.A. Watson, J.C. Veltri and T.J. Metcalf, Vet. Human Toxicol., 23 (1981) 164.
554. H.V. Aposhian, C.H. Tadlock and T.E. Moon, Toxicol. Appl. Pharmacol., 61 (1981) 385.
555. I.G. Mizyukova, V.E. Petrunkin and N.M. Lysenko, Farmakol. Tosikol. (Moscow) 34 (1971) 70; Chem. Abstr., 74 (1971) 97223a.
556. H.V. Aposhian, M.M. Mershon, F.B. Brinkley, C. Hsu and B.E. Hackley, Life Sci., 31 (1982) 2149.
557. M.A. Basinger and M.M. Jones, Res. Commun. Chem. Pathol. Pharmacol., 32 (1981) 355.
558. J.M. Arena, Poisoning (C.C. Thomas, Springfield, IL) 3rd Edn. (1974) pp. 81–82.
559. B.J. Bendl, Arch. Dermatol., 100 (1969) 443.
560. Y.A. Lazaris and G.G. Meiramov, Probl. Endokrinol., 20 (1974) 90.
561. H.H. Kamerbeek, A.G. Racours, M. Ten Ham and A.N.P. Van Heijst, Acta Med. Scand., 189 (1971) 149.
562. O. Grunfeld, N. Engl. J. Med., 269 (1963) 1138.
563. T.M. Davis and W.E. Fann, Annu. Rev. Pharmacol., 11 (1971) 285.
564. B.B. Martin and D.F. Martin, J. Inorg. Nucl. Chem., 37 (1975) 1079.
565. D.F. Martin and B.B. Martin, Inorg. Chem., 1 (1962) 404.
566. A. Catsch, Radioactive Metal Mobilization in Medicine (C.C. Thomas, Springfield, IL, 1964).
567. Diagnosis and Treatment of Incorporated Radionuclides (IAEA, Vienna, 1976).
568. J.B. Hursh, Proc. Soc. Exp. Biol. Med., 79 (1952) 210.
569. V. Volf, Experientia, 29 (1973) 307.
570. A. Sutton, G.E. Harrison, T.E.F. Carr and D. Barltrop, Int. J. Rad. Biol., 19 (1971) 79.
571. W.H. Muller, Radiation Protection. A Systematic Approach to Safety (Pergamon Press, Oxford, 1980) pp. 1056–1059.
572. V. Volf, Health Phys., 39 (1980) 364.
573. H. Smith, G.N. Stradling, R.A. Bulman and G.J. Ham, Health Phys., 30 (1976) 318.
574. R.A. Bulman and R.J. Griffin, Health Phys., 40 (1981) 104.
575. R.A. Bulman and R.J. Griffin, Health Phys., 40 (1981) 228.
576. M.L. Blank, E.A. Cress, B.L. Byrd, L.C. Washburn and F. Snyder, Health Phys., 39 (1980) 913.
577. R.A. Bulman, R.J. Griffin and A.T. Russell, Health Phys., 37 (1979) 729.

578. R.A. Bulman and R.J. Griffin, J. Inorg. Biochem., 12 (1980) 89.
579. R.A. Bulman, J. Nucl. Med. Biol., 7 (1980) 295.
580. V.S. Balabukha, L.I. Tikhonava, L.M. Razbitnaya, D.D. Smolin, N.O. Razumovskii and O.L. Torchinskaya, in: Raspred. Biol. Deistive Radioakt. Izot, ed. Y.I. Moskalev (Moscow, 1966) pp. 462–470; USAEC Rep. AEC-TR-6944 (Engl. Transl., 1966) pp. 581–591.
581. Y.A. Belyaev, in: Raspred. Biol. Deistive Radioakt. Izot (Rabot, 1964) pp. 338–342; Chem. Abstr. 62 (1965) 16608a.
582. Z. Szot, R. Zablotna, J. Geisler and A. Poczynajlo, Int. J. Radiat. Biol, 34 (1978) 187.
583. Z. Szot, EULEP Newsl., 30 (1982) 46.
584. I. Tabushi, Y. Kobuke, K. Ando, M. Kishimoto and E. Ohara, J. Am. Chem. Soc., 102 (1980) 5947.
585. R.A. Bulman and R.J. Griffin, Naturwissenschaften, 67 (1981) 483.
586. R.A. Bulman, J.W. Stather, J.C. Strong, P. Rodwell, R.J. Griffin and A. Hodgson, NRPB R & D Rep. (1979) p. 128.
587. E. Peter, V. Volf, F. Planas-Bohne and D.M. Taylor, EULEP Newsl., 30 (1982) 39.
588. H. Metivier, T. Rateau, N. Legendre, R. Masse and J. Lafuma, EULEP Newsl., 30 (1982) 18.
589. E. Peters and V. Volf, Health Phys., 40 (1981) 753.
590. O.P. Pachauri and J.P. Tandon, J. Inorg. Nucl. Chem., 37 (1975) 2321.
591. J.W. Stather, G.N. Stradling, H. Smith, S. Payne, A.C. James, J.C. Strong, S.E. Ham, S.A. Sumner, R.A. Bulman, A. Hodgson, C. Towndrow and M. Ellender, Health Phys., 42 (1982) 520.
592. K.N. Raymond and W.L. Smith, Struct. Bonding (Berlin), 43 (1981) 159.
593. P.W. Durbin, E.S. Jones, K.N. Raymond and F.L. Weitl, Radiat. Res., 81 (1980) 170.
594. P.M. May and D.R. Williams, Nature (London) 278 (1979) 581.
595. S. Pollack, G. Vanderhoff and F. Lasky, Biochim. Biophys. Acta, 497 (1977) 481.
596. S. Pollack and S. Ruocco, Blood, 57 (1981) 1117.
597. R.E. Cowart, N. Kojima and G.W. Bates, J. Biol. Chem., 257 (1982) 7560.
598. H.L. Haust, Ali. Hashmi, D.S. Milton Haines and C.J. Forret, Int. J. Biochem., 12 (1980) 897.
599. J. Schubert, Trends Pharmacol. Sci., 1 (1981) 6.
600. J. Schubert, Trends Pharmacol. Sci., 1 (1981) 50.
601. B.L. Holman, M.K. Dewanjee, J. Idoine, C.P. Fleigel, M.A. Davies, S. Treves and P. Eldh, J. Nucl. Med., 14 (1973) 595.
602. E. Riihimaki, H. Suoranta and K. Tallroth, Eur. J. Nucl. Med., 1 (1976) 123.
603. A.J. van Wyk, P.J. Fourie, W.H. van Zyl, M.G. Lotter and P.C. Minnaar, Eur. J. Nucl. Med., 4 (1979) 445.
604. B.W. Winstow, G. Subramanian, R.L. Heertum, R.W. Henderson, G.M. Gayne, R.C. Hall and J.G. McAfee, J. Nucl. Med., 18 (1977) 455.
605. E. Chiotellis and A. Varvarigou, Int. J. Nucl. Med. Biol., 7 (1980) 1.
606. S.N. Joshi, E.A. George and R.P. Perrillo, Gastroenterology, 81 (1981) 1045.
607. Y. Karube, A. Kono, T. Maeda, M. Ohmya and Y. Mutsushima, J. Nucl. Med., 22 (1981) 619.
608. M.C. Theodorakis, W.C. Groutas, A.J. Bermudez, D. Magnin and S.V.S. Stefanakou, J. Pharm. Sci., 69 (1980) 581.

609. P.M. Pojer and J. Bladae, J. Nucl. Med. Biol., 8 (1981) 112.
610. E. Chiotellis, G. Subramanian and J.G. McAfee, Int. J. Nucl. Med. Biol., 4 (1977) 21.
611. E. Chiotellis, G. Subramanian and J.G. McAfee, Int. J. Nucl. Med. Biol., 4 (1977) 29.
612. D.D. Kubiatowicz, T.F. Bolles, J.C. Nora and D.S. Ithakissios, J. Pharm. Sci., 68 (1979) 621.
613. S.M. Moerlin, M.J. Welch, K.M. Raymond and F.L. Weitl, J. Nucl. Med., 22 (1981) 710.
614. D.G. Moore, R.J. Yancey, G.E. Lankford and C.F. Earhart, Infect. Immun., 27 (1980) 443.
615. M.L. Thakur, R.E. Coleman, C.G. Mayhall and M.J. Welch, Radiology, 119 (1976) 731.
616. A.R. Fritzberg, D.M. Lyster and D.H. Dolphin, Int. J. Nucl. Med. Biol., 4 (1977) 113.
617. J.G. McAfee and M.L. Thakur, J. Nucl. Med., 17 (1976) 480.
618. M.J. Welch, M.L. Thakur, R.E. Coleman, M. Patel, B.A. Siegel, M.M. Ter-Pogossian, J. Nucl. Med., 18 (1977) 558.
619. R.A. Lerch, S.R. Bergmann, E.M. Carlson, J.E. Saffitz and B.E. Sobel, J. Nucl. Med., 23 (1982) 496.
620. J.E.T. Burke, S. Roath, D. Ackery and P. Wyeth, Eur. J. Nucl. Med., 7 (1982) 73.
621. M.K. Dewanjee, S.A. Rao and P. Didisheim, J. Nucl. Med., 22 (1981) 981.
622. L. Hendershott, R. Gentilcore, F. Ordway, J. Fletcher and R. Donati, Eur. J. Nucl. Med., 7 (1982) 234.
623. M.L. Thakur, J. Nucl. Med., 22 (1981) 1011.
624. M.K. Dewanjee and S.H. Rao, J. Labelled Compd. Radiopharm., 18 (1981) 278.
625. D.J. Hnatowich, B. Friedman, B. Clancy and M. Novak, J. Nucl. Med., 22 (1981) 810.
626. S.M. Yeh and C.F. Meares, Experientia, 35 (1979) 715.
627. R. Vaun, N.D. Heidel, H.D. Burns, A.J. Emrich and N. Foster, J. Pharm. Sci., 71 (1982) 1223.
628. M.W. Sundberg, C.F. Meares, D.A. Goodwin and C.I. Diamanti, Nature (London), 250 (1974) 587.
629. G.E. Krejcarek and K.L. Tucker, Biochem. Biophys. Res. Commun., 77 (1977) 581.
630. D.J. Hnatowich and P. Schlegel, J. Nucl. Med., 22 (1981) 623.
631. D.J. Hnatowich, W.W. Layne and R.L. Childs, Int. J. Appl. Radiat. Isot., 33 (1982) 327.
632. H. Umezawa, Pure Appl. Chem., 28 (1970) 665.
633. A. Fujii, T. Takita, K. Maeda and H. Umezawa, J. Antibiotics, 26 (1973) 398.
634. J.P. Nouel, Cancer Res., 19 (1976) 301.
635. L.H. DeRiemer, C.F. Meares, D.A. Goodwin and C.I. Diamanti, J. Labelled Compd. Radiopharm., 18 (1981) 1517.
636. D.A. Goodwin, C.F. Meares, C.I. Diamanti, R.L. Goode, J.E. Baumert, D.J. Sartoris, R.L. Lantieri and H.D. Fawcett, J. Nucl. Med., 22 (1981) 787.
637. W.H. Oldendorf, J. Nucl. Med., 19 (1978) 1182.
638. M.D. Loberg, E.H. Corder, A.T. Fields and P.S. Callery, J. Nucl. Med., 20 (1979) 1181.
639. H.D. Burns, R.F. Dannalls, T.E. Dannalls, A.V. Kramer and L.G. Marzilli, J. Labelled Compd. Radiopharm., 18 (1981) 54.

640. M. Anbar, J. Aviad, R. Rein and S. Schorr, Experientia, 16 (1960) 443.
641. G. Shtacher and M. Anbar, J. Pharmacol. Exp. Ther., 152 (1966) 157.
642. S. Unterspann and W. Fink, Dtsch. Gesundheitswes., 36 (1981) 2205.
643. H.L. Atkins, R.M. Lambrecht, A.P. Wolf, A.N. Ansari and M. Guillaume, Radiology, 115 (1975) 723.
644. Y. Yano and T.F. Burdinger, J. Nucl. Med., 18 (1977) 815.
645. E. Ogawa, Igaku Seibutsugaku, 102 (1980) 13; Chem. Abstr., 95 (1981) 2675j.
646. S.P. Joseph, P.J. Ell, P. Ross, R. Donaldson, A.T. Elliot, N.T.G. Brown and E.S. Williams, Br. Heart J., 40 (1978) 234.
647. S.M. Karesch, W.C. Eckelman and R.C. Reba, J. Pharm. Sci., 66 (1977) 225.
648. Commission on Enzymes. Enzyme Nomenclature (Elsevier, Amsterdam, 1972).
649. I. Sissoeff, J. Grisvard and E. Guille, Prog. Biophys. Mol. Biol., 31 (1976) 165.
650. D. Bauer, C. St. Vincent, C. Kempe and A. Dounie, Lancet, ii (1963) 494.
651. E.C. Moore, M.S. Zedeck, K.C. Agrawal and A.C. Sartorelli, Biochemistry, 9 (1970) 4492.
652. D.S. Auld, H. Kawaguchi, D.M. Livingstone and B.L. Vallee, Proc. Natl. Acad. Sci. U.S.A., 71 (1974) 2091.
653. W. Levinson, W. Rohde, P. Mikelens, J. Jackson, A. Antony and T. Ramakrishnan, Ann. N.Y. Acad. Sci., 284 (1977) 525.
654. J.S. Oxford and D.D. Perrin, J. Gen. Virol., 23 (1974) 59.
655. L.R. Overby, S.E. Robishaw, J.B. Schleicher, A. Reuter, N.L. Shipkowitz and J.C.H. Mao, Antimicrob. Agents Chemother., 6 (1974) 360.
656. J.M. Reno, L.F. Lee and J.A. Boez, Antimicrob. Agents Chemother., 13 (1978) 188.
657. M. Takeshita, S.B. Horovitz and A.P. Grollman, Virology, 60 (1974) 455.
658. C. Gurgo, R.K. Ray, L. Thirg and M. Green, Nature New Biol., 229 (1971) 111.
659. E. Robbins and T. Peterson, Proc. Natl. Acad. Sci. U.S.A., 66 (1970) 1244.
660. K. Ganeshaguru, A.V. Hoffbrand, R.W. Grady and A. Cerami, Biochem. Pharmacol., 29 (1980) 1275.
661. J.G. Cory, L. Lasater and A. Sato, Biochem. Pharmacol., 30 (1981) 1979.
662. A. Albert and C.W. Rees, Nature (London), 177 (1956) 433.
663. T. Franklin, Biochem. J., 123 (1971) 267.
664. R. Husseini and R.J. Stretton, Microbios, 29 (1980) 109.
665. H. Smit, H. Van der Goot, W.T. Nauta, P.P. Pijper, S. Balt, M.W.G. De Boltser, A.H. Stouthamer, H. Verheul and R.D. Vis, Antimicrob. Agents Chemother., 18 (1980) 249.
666. H. Smit, H. Van Der Goot, W.T. Nauta, H. Timmerman, M.W.G. De Bolster, A.H. Stouthamer and R.D. Vis, Antimicrob. Agents Chemother., 21 (1982) 881.
667. Anon., Lancet, i (1971) 72.
668. P.J. Aggett, H.T. Delves, J.T. Harries and A.D. Bangham, Biochem. Biophys. Res. Commun., 87 (1979) 513.
669. J. Nabiln, M.T. El-Wassini, M.M. Karnel and J. Metrii, Egypt. J. Bilh., 2 (1975) 177.
670. M.S. Wysor, L.A. Zwelling, J.E. Sanders and M.M. Grenan, Science, 217 (1982) 454.
671. L.W. Scheibel and A. Adler, Mol. Pharmacol., 18 (1980) 320.
672. L.W. Scheibel and A. Adler, Mol. Pharmacol., 20 (1981) 218.

673. A. Furst, in: Ref. 188, pp. 336–349.
674. P.J. Leonard, J. Persaud and R. Motwani, Clin. Chim. Acta, 35 (1971) 409.
675. M.K. Schwartz, Cancer Res., 35 (1975) 3481.
676. M.S. Seelig, Biol. Trace Element Res., 1 (1979) 273.
677. A. Furst and S.B. Radding, Biol. Trace Element Res., 1 (1979) 169.
678. E.D. Weinberg, Biol. Trace Element Res., 3 (1981) 55.
679. Proc. Workshop/Conference on Role of Metals in Carcinogenesis, Environ. Health Perspect., 40 (1981) pp. 1–252.
680. H. Sigel (ed.), Metal Ions in Biological Systems (Marcel Dekker, New York) Vol. XI (1980).
681. A.S. Mildvan and L.A. Loeb, Crit. Rev. Biochem., 6 (1979) 219.
682. J.D. Heck and M. Costa, Biol. Trace Element Res., 4 (1982) 71.
683. B.J. Mills, W.L. Broghamer, P.J. Higgins and R.D. Linderman, Am. J. Clin. Nutr., 34 (1981) 1661.
684. P. Frost, P. Rabbani, J. Smith and A. Prasad, Proc. Soc. Exp. Biol. Med., 167 (1981) 333.
685. R.L. Anderson, C.L. Alden and J.A. Merski, Food Chem. Toxicol., 20 (1982) 105.
686. G. Powis and J.S. Kokvach, Biochem. Pharmacol., 30 (1981) 771.
687. D. Kessel and R.S. McElhinney, Mol. Pharmacol., 11 (1977) 298.
688. P. Mikelens, B. Woodson and W. Levinson, Bioinorg. Chem., 9 (1978) 469.
689. W. Rohde, R. Shafer, J. Idriss and W. Levinson, J. Inorg. Biochem., 10 (1979) 183.
690. L.A. Saryan, K. Mailer, C. Krishnamurti, W. Antholine and D.H. Petering, Biochem. Pharmacol., 30 (1981) 1595.
691. L.W. Oberley and G.R. Buettner, FEBS Lett., 97 (1979) 47.
692. W.J. Caspary, C. Niziak, D.A. Lanzo, R. Friedman and N.R. Bachur, Mol. Pharmacol., 16 (1979) 256.
693. H. Umezawa and T. Takita, Struct. Bonding (Berlin), 40 (1980) 73.
694. J.M.C. Gutteridge and F. Xiao-Chang, Biochem. Biophys. Res. Commun., 99 (1981) 1354.
695. J.M.C. Gutteridge and X. Fu, FEBS Lett., 123 (1981) 71.
696. H. Rosen and S.J. Klebanoff, Arch. Biochem. Biophys., 208 (1981) 512.
697. M. Gosalvez, M.F. Blanco, C. Vivero and F. Valles, Eur. J. Cancer, 14 (1978) 1185.
698. Z-X. Huang, P.M. May, K.M. Quinlan, D.R. Williams and A.M. Creighton, Agents Actions, 12 (1982) 536.
699. A.M. Creighton, K. Hellmann and S. Whitecross, Nature (London), 222 (1969) 384.
700. B.W. Fox and A.M. Creighton, Adv. Med. Oncol Res. Educ., 5 (1979) 83.
701. B. Rosenberg, in Nucleic Acid and Metal Ion Interactions, ed. T.G. Spiro (Wiley, New York, 1980) p. 1.
702. M.J. Cleare, P.C. Hydes, B.W. Malerbi and D.M. Watkins, Biochimie, 60 (1978) 835.
703. J.J. Roberts and A.J. Thompson, Prog. Nucleic Acid Res. Mol. Biol., 22 (1979) 71.
704. R.F. Borch, J.C. Katz, P.H. Lieder and M.F. Pleasants, Proc. Natl. Acad. Sci. U.S.A., 77 (1980) 5441.
705. I.L. Bonta, M.J. Parnham, J.E. Vincent and P.C. Bragt, Prog. Med. Chem., 17 (1980) 185.
706. R. Milanino, A. Conforti, M.E. Fracasso, L. Franco, R. Leone, E. Passarella, G. Tarter and G.P. Velo, Agents Actions, 9 (1979) 581.

707. M.W. Whitehouse and W.R. Walker, Agents Actions, 8 (1978) 85.
708. W.R. Walker, S.J. Beveridge and M.W. Whitehouse, Agents Actions, 10 (1980) 38.
709. S.J. Beveridge, W.R. Walker and M.W. Whitehouse, J. Pharm. Pharmacol., 32 (1980) 425.
710. S.J. Beveridge, M.W. Whitehouse and W.R. Walker, Agents Actions, 12 (1982) 225.
711. W.R. Walker, R.R. Reeves, M. Brosnan and G.D. Coleman, Bioinorg. Chem., 7 (1977) 271.
712. G.B. West, Int. Arch. Allergy Appl. Immun., 66 (1981) 110.
713. D.A. Gerber and M.G. Gerber, J. Chron. Dis., 30 (1977) 115.
714. M.E. Pickup, J.S. Dixon, J.R. Lowe and V. Wright, J. Rheumatol., 7 (1980) 71.
715. J.R.J. Sorenson, K. Ramakrishna and T.M. Rolniak, Agents Actions, 12 (1982) 408.
716. P.C. Craven and H.F. Morrelli, West. J. Med., 122 (1975) 277.
717. A. Soffer, J. Am. Med. Assoc., 233 (1975) 1206.
718. Anon., Lancet, ii (1977) 1167.
719. F. Ziolkowski and D.D. Perrin, Invest. Urol., 15 (1977) 208.
720. M. Rubin, R. Gohil, A.E. Martell, R.J. Motekaitis, J.C. Penhos and P. Weiss, in: Ref. 172, pp. 381–406.
721. K.S. Rajan, A.A. Manian, J.M. Davis and H. Dekirmenjian, Brain Res., 107 (1976) 317.
722. J.H. Menkes, M. Alter, G.K. Steigleder, D.R. Weakley and J.H. Sung, Pediatrics, 29 (1962) 764.
723. I. Sternlieb, Prog. Liver Dis., 4 (1972) 511.
724. C.A. Owen, E.R. Dickson and N.P. Goldstein, Mayo Clin. Proc., 52 (1977) 73.
725. M. Salaspuro and P. Sipponen, Gut, 17 (1976) 787.
726. H.T. Delves, Essays Med. Biochem., 2 (1976) 37.
727. C.L. Keen, P. Saltman and L.S. Hurley, Am. J. Clin. Nutr., 33 (1980) 1789.
728. H. Nagara, K. Yajima and K. Suzuki, J. Neuropathol. Exp. Neurol., 40 (1981) 428.
729. O. Epstein and S. Sherlock, Gastroenterology, 78 (1980) 1442.
730. M.B. Chenoweth, Clin. Pharmacol. Ther., 9 (1968) 365.
731. A. Albert, J. Med. Chem., 25 (1982) 1.
732. H.R. Mahler, in: Mineral Metabolism, eds. C.L. Comar and F. Bronner (Academic Press, New York) Vol. IB (1961) pp. 743–879.
733. R.H. Dreisbach, Handbook of Poisoning (Lange Medical Publications, Los Altos, CA) 7th Edn. (1971) pp. 192–218.

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6 Ranitidine and Other H_2-Receptor Antagonists: Recent Developments

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INTRODUCTION

Histamine is one of the few naturally occurring substances to have been synthesized and tested biologically before it was found in the body. In addition, despite its simple chemical structure, it has stimulated a great deal of chemical and biological research. Indeed, histamine has many actions in the body and has proved to be a very potent stimulus for the production of research publications (see Ref. 1 for references).

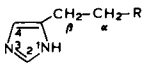
For many years a physiological role for histamine was sought by biologists. This proved to be a difficult task and it was not until the synthesis of the first antihistamines [2] that some of the actions of histamine could be classified. It soon became clear that not all the actions of histamine could be antagonized by the large range of antihistamines available, and Folkow, Haeger and Kahlson [3] postulated, as a result of their studies with diphenhydramine and histamine on the cardiovascular system, that there was more than one histamine receptor. However, the paper of Ash and Schild [4] defined the receptors sensitive to mepyramine as H_1-receptors and left open the question as to how many other types of histamine receptor existed. The tissue defined as containing H_1-receptors was guinea-pig isolated ileum and the tissues containing other histamine receptors were rat isolated uterus and the rat stomach. The question of further classification remained in abeyance until Black and his colleagues published their notable paper [5].

The first H_2-receptor antagonists developed were based on a chemical similarity to histamine [6]. The initial breakthrough which supported the postulation of the two receptor types came with the synthesis and testing of two antagonists, namely 2- and 4-methylhistamine. 4-Methylhistamine was found to have 40% of the activity of histamine *in vivo* for stimulation of gastric acid secretion in the rat and *in vitro* for stimulation of spontaneous atrial frequency in the guinea-pig right atrium. However, 4-methylhistamine had only 0.2% of the activity of histamine for stimulation of contractions of guinea-pig isolated ileum. In contrast, 2-methylhistamine had only 2% of the activity of histamine in stimulating rat gastric acid secretion but 17% of the activity of histamine in stimulating contraction of the guinea-pig isolated ileum. These results and others are summarized in *Table 6.1*. Such results helped to establish the existence of two classes of histamine receptor.

The development of an antagonist, burimamide, for the first time allowed the definition of a second class of receptor, the H_2-receptors. Since that time there has been research which suggests that the various actions of histamine can be explained on the basis of two receptors either alone or in combination.

The clinical implications of histamine antagonists have been very great. The

Table 6.1. AGONIST ACTIVITIES OF CLOSE ANALOGUES OF HISTAMINE [5]



Position of Me in molecule							*Equipotent concn. (histamine = 1)*	
1	2	3	4	α	β	R	*Atria*	*Ileum*
						NHMe	1.3	1.4
						NMe_2	2.0	2.2
					Me	NH_2	167	167
				Me		NH_2	125	125
Me						NH_2		inactive
	Me					NH_2	22.7	6.1
		Me				NH_2		inactive
			Me			NH_2	2.5	500

first antihistamines or H_1-antagonists have had an established place in the symptomatic treatment of various allergic diseases, and the central effects of certain H_1-blockers are of value in suppressing motion sickness. The main clinical indication for H_2-antagonists has been in the control of gastric acid secretion and the treatment of acid aggravated diseases of the upper gastrointestinal tract such as duodenal ulcer, gastric ulcer and reflux oesophagitis.

The imidazole-based H_2-receptor antagonists, burimamide, metiamide and cimetidine have not been without their problems, and the need for an improved H_2-receptor antagonist led us to take a fresh look at the structural requirements for an action at H_2-receptor sites. This work, which led to the development of ranitidine, is the basis of this review.

BIOLOGICAL TEST SYSTEMS FOR MEASURING ACTIVITY AT H_2-RECEPTORS

This section will briefly consider the biological test systems suitable for assessing activity at H_2-receptor sites. Such activity could be produced by an agonist, e.g., histamine or betazole. Alternatively, the activity measured may be that of an antagonist, e.g., burimamide. We have been primarily concerned with antagonists, but appropriate test systems for assessing new structures need to be able to respond to both agonists and antagonists.

There are three main reasons for measuring activity at H_2-receptor sites. (1) To assess new chemical structures both qualitatively and quantitatively.

(2) To investigate the effect of an H_2-agonist or -antagonist in a physiological or pharmacological test system in order to elucidate physiological control mechanisms. (3) To correlate effects in man with those in other test systems and species. We have been primarily concerned with the first objective in testing and developing the novel compounds described in this review.

IN VITRO TESTS

In vitro test systems have the advantage of not being influenced by changes in blood pressure and many of the homoeostatic mechanisms which endeavour to compensate for any response induced *in vivo*. Consequently, *in vitro* tests are very valuable for receptor characterization, but even here precautions may be necessary against compensatory mechanisms. Measurement of a pharmacological response can be carried out using guinea-pig isolated right atrium, rat isolated uterine horn and the rat or mouse isolated stomach or mucosa.

The guinea-pig right atrium is a reliable and reproducible preparation from which cumulative dose-response curves to histamine can be obtained for an increase in contraction frequency. This effect has been characterized as H_2-receptor mediated [5]. In the presence of H_2-receptor antagonists, the histamine concentration-response curve is displaced to the right in a competitive manner (*Figure 6.1*). The competitive nature of the antagonist can be readily demonstrated by means of the Arunlakshana and Schild [7] plot (*Figure 6.2*) and calculation of the pA_2 and slope of the regression of log (DR–1) on log concentration. In contrast, potencies of agonists can be assayed relative to histamine and the competitive nature of H_2-agonists can be assayed by measurement of the maximum response relative to histamine [8].

The rat isolated uterine horn, when contracted, relaxes in response to histamine, and this has been characterized [4] as a non-H_1-receptor-mediated response and it was used [5] to demonstrate the specificity of the H_2-receptor. The experiments which can be carried out are essentially similar to those described above for the atrium, except that cumulative relaxation curves are obtained in response to histamine.

Other *in vitro* tissues such as the mouse [9] or rat [10] stomach can be used to measure the stimulant effect of histamine on gastric acid secretion and inhibition by H_2-receptor antagonists. However, the test model is a complex one due to uncertain interactions between secretagogues. The rat stomach model appears suitable for the study of H_2-blockers, since it has given pA_2 values for metiamide close to those obtained in other tissues such as guinea-pig atrium and rat uterus. But there have been discrepancies with the mouse stomach, where the pA_2 value for metiamide is one log unit lower than the

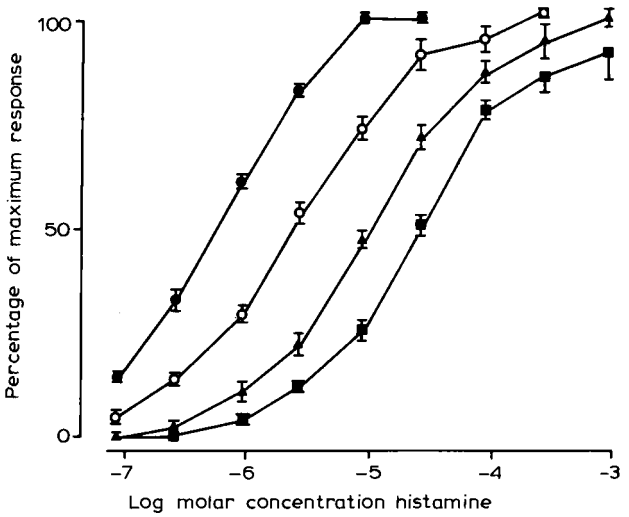


Figure 6.1. The effect of ranitidine on the histamine-induced increase in contraction frequency of guinea-pig isolated right atrium. ●, *control; ranitidine (M):* ○, 3.2×10^{-7}; ▲, 9.5×10^{-7}; ■, 3.2×10^{-6}.

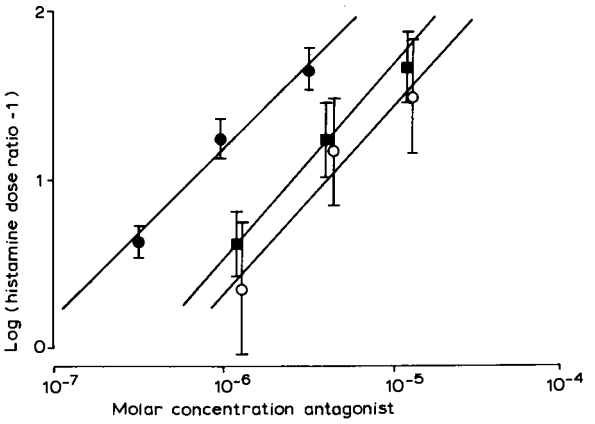


Figure 6.2. Effects of ranitidine, cimetidine and metiamide on histamine-induced increases in the rate of beating of isolated guinea-pig right atrium. ●, *ranitidine;* ■, *cimetidine;* ○, *metiamide.*

reference values for guinea-pig atrium or rat uterus. This discrepancy has been attributed to a failure to achieve a steady-state equilibrium for the antagonist under the conditions occurring in this model [11], but the answer may be more complex.

Isolated mucosal preparations have also been used with tissues from kitten [12], guinea-pig [13], rat [14], rabbit [15] and pig [16]. These preparations provide useful research data on gastric physiology, but they are not suitable for rapid comparative testing of a series of chemical structures.

The use of an enriched parietal cell preparation is a technique intended to allow a much more specific evalution than that which is possible with the whole stomach or gastric mucosa. Methods [17, 19] based on that of Croft and Ingelfinger [18] for the preparation of suspensions of parietal cells have been developed. Basically, these methods require separation of the cells by controlled proteolytic digestion and concentration by centrifugation or elutriation. Cell viability is verified by the ability to exclude dyes such as Erythrosin B or Alcian blue.

Having obtained a viable cell preparation, the problem is how to measure parietal cell function. The ideal measurement would be hydrogen ion secretion. In the normal mucosa, when a hydrogen ion is secreted from the parietal cell surface into the lumen, a bicarbonate ion is secreted from the serosal surface. However, this cell polarity is lost in a cellular suspension and it is not possible to measure acid secretion directly.

Consequently, indirect measurements of parietal cell function must be used. Cyclic AMP is elevated in parietal cells exposed to histamine and to 4-methylhistamine, but not 2-methylhistamine, which is only a weak agonist. These effects can be competitively antagonised by H_2-receptor antagonists [17, 20]. Oxygen consumption is another indicator of cell function. The parietal cell is rich in mitochondria and has high energy requirements for the secretion of acid. Thus, measurements of oxygen consumption by means of an electrode give an indication of cellular activity [19], but this need not totally reflect acid secretion.

Another method is the measurement of amino[^{14}C]pyrine uptake or accumulation. Aminopyrine is a weak base which diffuses across membranes in its non-ionized form, but when ionized in an acidic environment it cannot cross membranes. It is this mechanism which is assumed to trap aminopyrine in the tubulovesicles and secretory canaliculi. Both aminopyrine accumulation and oxygen consumption show similar patterns for the actions and interactions of the secretagogues histamine and gastrin.

A fourth, less quantifiable, measurement involves monitoring the morphological transformation known to occur when the parietal cell changes from

the resting to the active state or *vice versa* [21]. Another step closer to the receptor can be taken by using cell membranes for the study of radioligand binding to receptors. The use of a direct receptor-binding technique in purified membrane fragments is free from some of the potential limitations of pharmacological experiments in intact tissues. Differences in tissue distribution of drugs possessing different physicochemical properties and different rates of equilibrium of drugs in the bulk and receptor phases should not be a problem in receptor-binding studies. However, there are certain disadvantages. It cannot be guaranteed that all the 'specific binding' is in fact binding specifically to the receptor under investigation. Uptake processes and enzymes often show as much specificity as do the receptor sites. Also, binding to the receptor or a high-affinity site does not produce an appropriate pharmacological response to indicate the nature or quality of the binding which is occurring. Thus, receptor binding studies, although of value, need to be interpreted with caution. The need for caution has been well illustrated with regard to H_2-receptors. Initially the binding of [^{3}H]cimetidine to rat and guinea-pig membranes was attributed to an interaction with the histamine-H_2-receptor [22, 23]. However, later detailed studies with both imidazole and non-imidazole structures have shown that cimetidine binds to a high-affinity site in addition to the H_2-receptor [20] and this view has since been confirmed by others [24, 25]

IN VIVO TESTS

In vivo studies of agents affecting H_2-receptors have been largely concerned with gastric acid secretion and this is the aspect which will be considered in some detail.

The choice of species is one consideration. The most commonly used animals are rat and dog. The rat is readily available and easily handled but has distinct gastric anatomical differences to man. However, the basic results obtained with H_2-antagonists in the rat have correlated with those obtained in man. The dog is co-operative and well suited to gastric secretion studies with surgically prepared pouches. A very important factor is the consistency with which results obtained in the dog have proved accurate in predicting the action of novel drugs on gastric secretion in man. The guinea-pig is another widely used laboratory species and it will secrete gastric acid in response to histamine but it is very susceptible to bronchospasm, and this limits its use unless H_1-blockers are administered concurrently.

Gastric juice can be evaluated relatively simply. The volume and pH can be measured, an aliquot can be titrated to determine hydrogen ion concentration and thus total acid secretion can be calculated by reference to the volume.

Pepsinogen secretion can also be measured. The various techniques employed to study gastric acid secretion are essentially different ways of obtaining the gastric juice for analysis.

The perfused stomach preparation of the anaesthetized rat has been very extensively used either in the way described by Ghosh and Schild or in a number of modified forms. The stomach is perfused at a constant rate and the pH or hydrogen ion concentration of the effluent is monitored continuously. This technique measures acid secretion induced by histamine, pentagastrin and muscarinic agonists. It is very useful for the evaluation of both H_2-receptor agonists and antagonists. *Figure 6.3* shows the inhibitory effect of ranitidine and cimetidine. Other species can be used, but this is not common.

The gastric fistula (*Figure 6.4*), either acute or chronic, is a means of gaining access to the stomach. Gastric juice usually drains by gravity into a collecting vessel and can be analysed. It is a simple technique but the juice collected can be contaminated and diluted by saliva and duodenal reflux. Errors can also occur due to loss of gastric juice through the pylorus. The technique has had extensive use in the rat [26, 27] and the dog [28].

Surgically prepared pouches (*Figure 6.4*) have a very long history, as indicated by their names. The Pavlov pouch is a fully innervated gastric pouch which produces gastric juice free from the contamination occurring with the gastric fistula. It is a good physiological model, but the Heidenhain pouch is more convenient and durable. The Heidenhain pouch is a separate and vagally

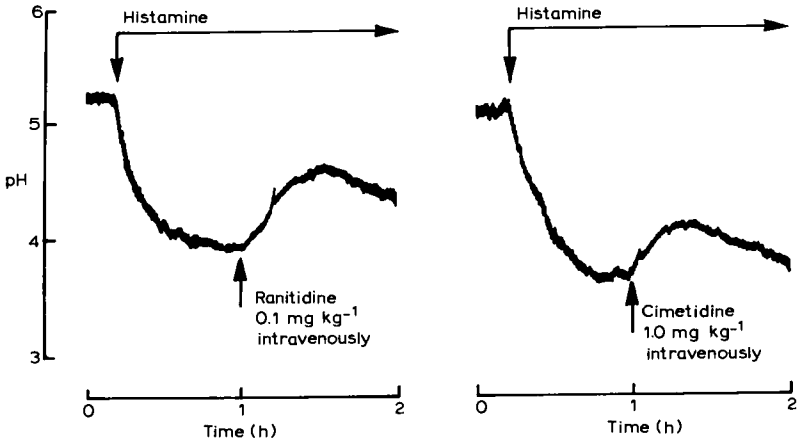


Figure 6.3. Inhibitory effect of ranitidine and cimetidine on histamine-induced gastric acid secretion in the perfused stomach preparation of the anaesthetized rat.

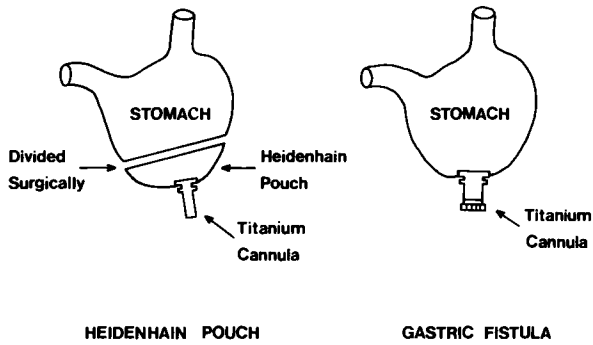


Figure 6.4. Diagram of Heidenhain pouch and gastric fistula preparations.

denervated pouch. These pouches are generally prepared in the dog [28], but the rat has also been used [26, 27, 29].

The duration of antisecretory activity is another facet of activity at H_2-receptor sites which can be measured *in vivo*. The time to 50% or 100% recovery from secretory inhibition can be measured in experiments with the perfused stomach preparation of the anaesthetized rat (*Figure 6.3*). The same experimental design can also be used with the Heidenhain or Pavlov pouch. In such experiments, the duration of activity is related to the intensity of the secretory inhibition. The greater the peak inhibition, the greater is the duration of action (*Figure 6.5*). Therefore it is important when comparing the duration of action of two compounds to use equi-potent antisecretory doses.

The majority of experiments for measuring duration of activity last 4–6 h. When the duration of action is 8 h or more, the use of a continuous secretory stimulus can cause problems for the subject, whether human or animal. Therefore, an alternative technique must be used. When a submaximal dose of secretagogue such as histamine is infused intravenously, the acid output normally reaches a constant level between 1 and 2 h. This plateau of secretion is reproducible for a particular subject. Accordingly, we have measured the secretory output in response to a standard 2 h infusion of histamine alone and at different time intervals after administration of the test compound to conscious dogs with a Heidenhain pouch. Results obtained with this technique are presented in *Table 6.2*.

The various *in vivo* preparations can be used to measure stimulation of secretion by H_2-agonists or inhibition of histamine-induced secretion by H_2-receptor antagonists. However, acid secretion can also be induced by gastrin, muscarinic agonists and food. The stimulation of acid secretion by all these secretagogues can be antagonized by H_2-receptor antagonists. This does not

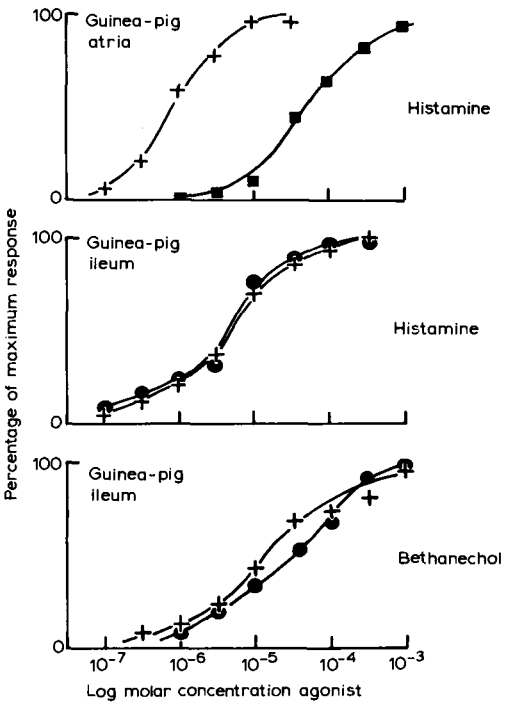


Figure 6.5 Effects of ranitidine on histamine- and bethanechol-induced responses in isolated tissue: + − +, control; ranitidine (M): ■, 3.2 × 10^{-6}; ●, 3.2 × 10^{-4}.

Table 6.2. INHIBITION OF HISTAMINE-INDUCED GASTRIC ACID SECRETION IN THE HEIDENHAIN POUCH DOG BY RANITIDINE AND AH 22216*

Drug	*Oral dose (mg/kg)*	*Mean ± S.E. % inhibition of secretion at:*				
		2 h	*4 h*	*8 h*	*18 h*	*24 h*
Ranitidine	1.0	95 ± 2	62 ± 8	23 ± 9	–	–
AH 22216	0.03	4 ± 8	67 ± 9	55 ± 11	33 ± 10	16 ± 20
	0.10	67 ± 9	95 ± 3	89 ± 2	54 ± 8	32 ± 13

* The formula of this compound (24) is given on p. 364.

reflect a lack of specificity by H_2-antagonists, but rather the complex interactions between secretagogues and nerves in the stomach. These fascinating physiological problems are not within the scope of this review and will not be discussed further. Nevertheless, this situation does mean that inhibition of histamine-induced acid secretion alone is not sufficient evidence to characterize a compound as an H_2-receptor antagonist. Dose-response curves must be displaced and the nature of the antagonism examined [30].

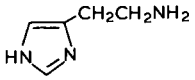
From this brief review of some of the biological test systems available for the evaluation of potential H_2-receptor antagonists, it is clear that a choice of method has to be made if the structure-activity relationship of new chemical structures is to be established. Isolated stomach, isolated mucosa and isolated parietal cell preparations are useful research tools for studying the physiology of gastric secretion, but they do not provide definitive data with regard to specific activity at H_2-receptor sites. This comment also applies to *in vivo* gastric secretion studies, even when histamine is used as the secretagogue. Radioligand binding studies can provide useful information on drug-receptor interactions but for the reasons discussed above, the results should not be viewed in isolation.

In our opinion, the guinea-pig isolated atrium preparation is the most effective test system for characterizing activity at H_2-receptors and measuring relative activity. This technique can be used for qualitative and quantitative assessment of any antagonism. It is not always necessary to prepare a family of curves for screening purposes and the displacement of the histamine response curve in the presence of a single preselected concentration of test compound can provide a useful working guide for structure-activity purposes. However, determination of precise activity and the nature of any antagonism requires experiments with several concentrations (*Figures 6.1* and *6.2*). Results from the rat isolated uterus are of comparable value, but the tissue can be more difficult to work with.

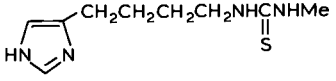
As the prime therapeutic objective for H_2-blockers has been the control of gastric acid secretion, it has been our practice to assess gastric antisecretory activity in parallel with measurements of H_2-blocking activity on the guinea-pig atrium. The perfused stomach preparation of the anaesthetized rat has been our preferred model. It is easy to perform and it produces results quickly; the results correlate well with H_2-receptor blocking activity assessed on the atrium. The other tests described are all valuable for the assessment of a limited number of active compounds but they are not suitable for the rapid evaluation of a series of novel chemical structures.

IMIDAZOLES AS H_2-ANTAGONISTS

The first competitive antagonist at histamine H_2-receptors was synthesized within the research laboratories of Smith, Kline and French. The classical piece of medicinal chemistry which resulted in its synthesis has been described with great clarity by the scientists concerned [6, 31].



(1)

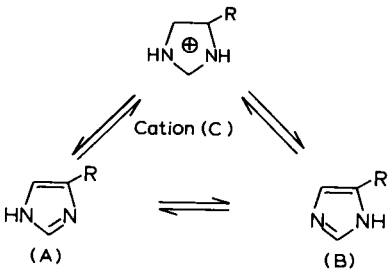


(2)

They succeeded in designing an antagonist by modifying the structure of the agonist histamine (1). Burimamide (2) is an imidazole derivative like histamine; however, the substituent is different in two important respects. The polymethylene chain has been extended to four carbon atoms and the basic nitrogen of histamine has been replaced by the neutral thiourea end-group. Pharmacological investigation of burimamide revealed that it antagonized the action of histamine in stimulating acid secretion in the rat and dog. When the compound was investigated in man, it was shown to inhibit acid secretion stimulated by histamine when given intravenously, but was not potent enough to be given conveniently by the oral route. Thus, it was not developed as a commercial entity.

The same workers considered that the secret of increasing the potency in these compounds lay in a consideration of the relative populations of the three possible species present.

An elegant argument led them to the conclusion that in order to maximize the amount of the tautomeric form (A) which is the one favoured by histamine (*Scheme 6.1*) it was necessary to design an antagonist with two modifications.

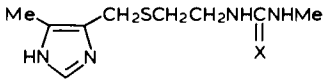


Scheme 6.1.

Firstly, it should have an electronegative atom in the side-chain so that it would function as an electron-withdrawing group. Secondly, an electron releasing substituent should be placed in the vacant 4-position of the ring. The substituent which was chosen was a methyl group, since this should not interfere with the receptor interaction, 4-methylhistamine being a selective H_2-agonist. This led directly to the synthesis of metiamide (3).

This compound was approximately 10-times as active as burimamide in animal experiments as an inhibitor of stimulated acid secretion with good oral activity in man. It produced a significant increase in the healing rate of duodenal ulcers and relief of symptoms and showed great promise. Unfortunately, there were a few instances of granulocytopenia amongst the 700 patients who received metiamide. This precluded further development.

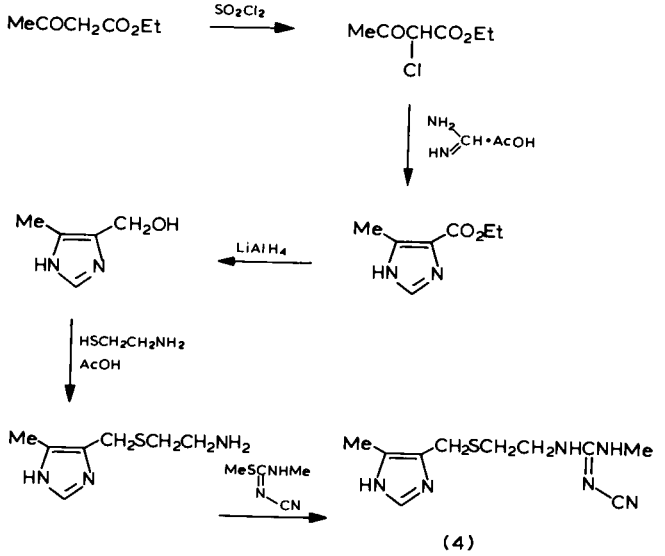
In order to find a molecule which might prove to be as active as an H_2-antagonist without the unwanted side-effects, they investigated the replacement of the thiourea moiety by other groups which might serve for it. The end-group which they chose was the cyanoguanidine, because its partition coefficient and acidity were similar to that of the thiourea. This compound, cimetidine (4), was shown to be just as effective, without producing any haematological changes. One route which has been used to synthesize cimetidine is shown in *Scheme 6.2*. Cimetidine has been shown to be effective in inhibiting histamine on pentagastrin-stimulated acid secretion in the rat and dog (*Table 6.3*).



(3) X = S
(4) X = NCN

Cimetidine is as active as metiamide and has been shown to relieve symptoms and promote healing of lesions in patients with peptic ulcer disease. Since 1976, cimetidine has been marked in the United Kingdom and has proved to be a valuable drug for conditions where control of gastric acid is important.

There is no doubt that this work is a very important piece of drug research and that cimetidine is proving to be valuable in controlling gastric acid secretion. Cimetidine is a compound with a relatively short duration of action, which necessitates a frequent dosing schedule in man. Cimetidine has been shown to have anti-androgenic activity which can lead in extreme cases to gynaecomastia in man. It is also an inhibitor of the cytochrome *P*-450 mixed-function oxygenase metabolizing enzyme system in the liver [32]; thus, if other



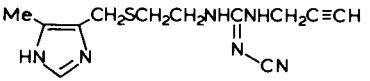
(4)

Scheme 6.2.

Table 6.3. INHIBITION OF GASTRIC ACID SECRETION *IN VIVO* BY CIMETIDINE [6]

Preparation	*Stimulant*	*Intravenous ED_{50} (mol kg^{-1})*
Rat: lumen-perfused stomach	histamine	1.37
	pentagastrin	1.40
Dog: Heidenhain pouch	histamine	1.70
	pentagastrin	2.00

drugs normally metabolized by this enzyme are administered simultaneously with cimetidine, prolonged and potentially dangerous actions can result. Examples are warfarin and diazepam, since they are normally deactivated by this enzyme system. Cimetidine has also been reported to cause confusional states in some elderly patients.



(5)

An improved H_2-antagonist would have to have a more selective action and enhanced potency, and a longer duration of action would also be advantageous. Modification of cimetidine undertaken by the group at Bristol Laboratories resulted in etintidine (5), which is approximately twice as active as cimetidine in animal tests [33]; however, the compound is not longer-acting.

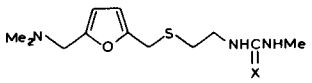
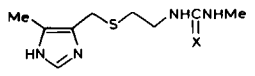
AMINOALKYLFURANS AS H_2-ANTAGONISTS

A common structural feature of the H_2-antagonists discovered by the SKF group was the imidazole ring. It was, in fact, their conclusion that the imidazole ring appeared to have a special importance at H_2-receptors, although the patent literature shows that they did replace the imidazole ring by other basic nitrogen heterocycles such as thiazole and pyridine [34].

We investigated the possibility that the basic heterocyclic ring might not be essential for H_2-blocker activity by replacing it by alternative ring systems to which a basic function was attached as a substituent. We found that replacement of the imidazole ring by furan to which a dimethylaminomethyl substituent was attached afforded molecules of potency comparable with those of the imidazoles [35]. The thiourea (6) and cyanoguanidine (7) derivatives are only slightly less active than the imidazoles metiamide and cimetidine. The nitroethene derivative (8, ranitidine) was substantially more active than its imidazole derivative (9) as an inhibitor of acid secretion in the rat. It was thus apparent that structure-activity relationships in the H_2-antagonists vary with the nature of the heterocycle concerned (*Table 6.4*).

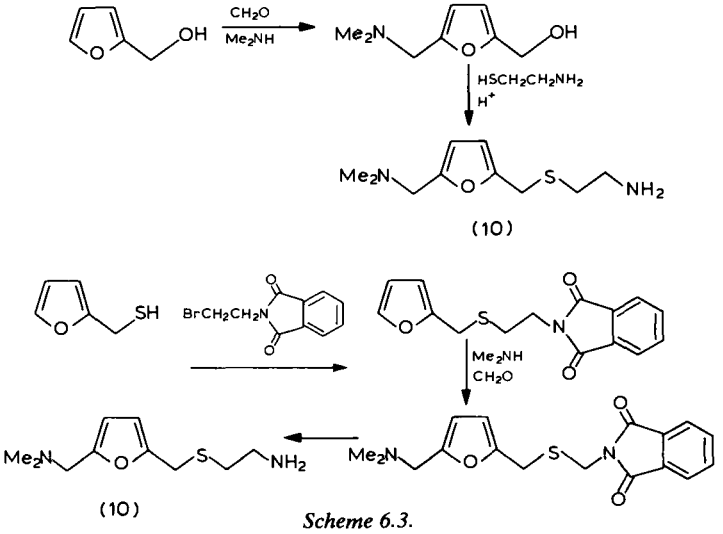
The diamine (10) required for preparing the above derivatives (6–8) has been synthesized in two ways and these two routes have been used generally

Table 6.4. FURAN AND IMIDAZOLE COMPOUNDS [35(a)]

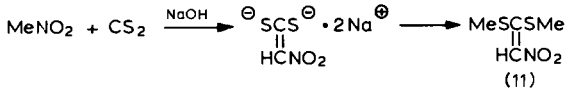
		*i.v. ED_{50} value**
	(6) X = S	2.32
	(7) X = NCN	1.39
	(8) X = $CHNO_2$	0.18
	(3) X = S	0.52
	(4) X = NCN	1.12
	(9) X = $CHNO_2$	1.75

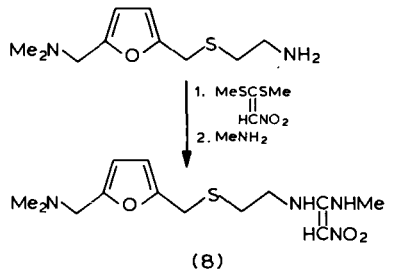
* For *Tables 6.4–6.9*, the i.v. ED_{50} values (in mg kg^{-1}) are for inhibition of acid secretion following infusion of histamine to the anaesthetized rat.

(*Scheme 6.3*). The 2,5-disubstitution pattern of the product arose from the fact that 2-substituted furans readily take part in the Mannich reaction to afford products of this type. The thiourea (6) and cyanoguanidine (7) were prepared by standard conditions analogous to those shown previously [36]. The nitroethene derivative was prepared from 1,1-bis(methylthio)-2-nitroethene (11), the synthesis of which has been described by Gomper and Schaefer [37] (*Scheme 6.4*). A range of compounds with other end-groups was prepared, but



Scheme 6.3.





Scheme 6.4.

none had a potency as an H_2-antagonist as high as that of ranitidine [35] (*Table 6.5*).

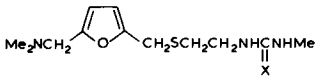
EFFECT OF CHANGING RING SUBSTITUTION PATTERNS

As we discussed earlier, the 2,5-disubstituted pattern arose because 2-substituted furans are readily available and the Mannich reaction introduces the basic function selectively in the 5-position. Thus, for reasons of accessibility, the first compounds prepared had this particular substitution pattern. There are five other substition patterns possible, but when the other members of the series were synthesized the original arrangement proved to be by far the most active (*Table 6.6*).

ADDITIONAL SUBSTITUTION OF THE FURAN RING

In the imidazole series, the effect of a methyl group in the ring adjacent to the side-chain was shown to enhance the potency in that series. This suggested to

Table 6.5. VARIATION OF THE END-GROUP [35(b)]



X	*i.v. ED_{50} value*
S	2.32
NCN	1.39
NCO_2Et	> 10
NSO_2Me	3.80
NSO_2Ph	3.40
$CHNO_2$	0.18
NSO_2CF_3	> 10

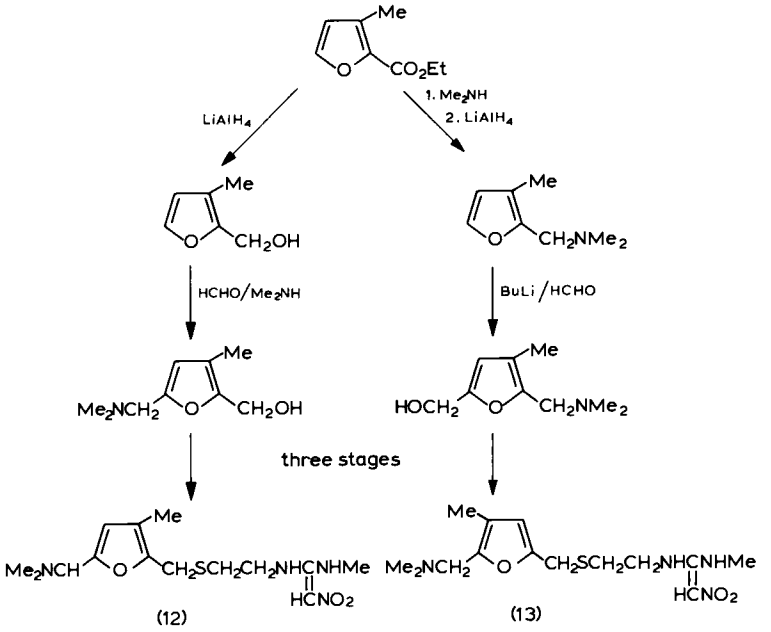
Table 6.6. CHANGES IN SUBSTITUTION PATTERN ON FURAN

$R^1 = CH_2SCH_2CH_2NHCSNHMe$
$R^2 = CH_2NMe_2$

R^1	R^2	*i.v. ED_{50} value*
2	5	2.3
2	3 or 4	> 10
3	2, 4 or 5	> 10

us that the 3-methylfuran analogue might be more active than the corresponding 4-methyl isomer.

We devised two routes (*Scheme 6.5*) which allowed the preparation from the readily available ester of the two aminoalcohols which were converted into the required products using standard conditions. It was a surprise to us when we found that the 3-methyl derivative (12) proved to be inactive, whereas the 4-isomer (13) was shown to be a highly active compound (*Table 6.7*).



Scheme 6.5.

Table 6.7. EFFECT OF CHANGING THE FURAN RING

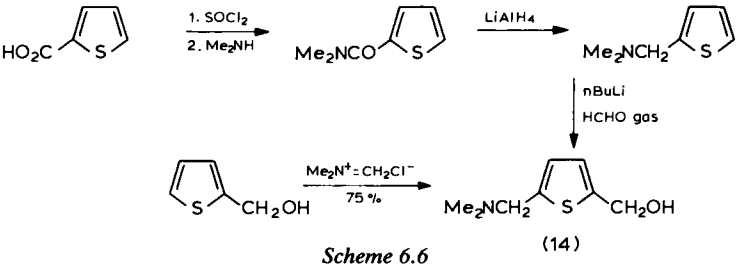
$Me_2NCH_2XCH_2SCH_2CH_2NHCNHMe$
$HCNO_2$

Ring X	*i.v. ED_{50} value*
Furan-2,5-	0.18
3-Methylfuran-2,5-	> 10.0
4-Methylfuran-2,5-	0.25
Thiophen-2,5-	0.50
Benzen-1,3-	0.93

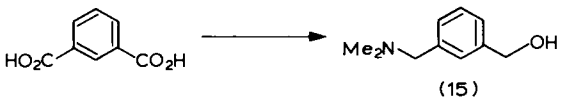
REPLACEMENT OF THE FURAN RING

It was naturally of interest to prepare molecules in which the furan ring was replaced by other non-basic ring systems with an appropriate dimethylaminomethyl substituent. Under conventional Mannich reaction conditions, it is known that thiophen reacts only sluggishly; hence we first prepared the key amino alcohol intermediate (14) in three stages starting from thiophen-2-carboxylic acid. We later found that treatment of thiophen-2-methanol, when treated with the iminium salt ('magic' Mannich), gave the required tertiary amine in a single operation (*Scheme 6.6*).

The appropriate disubstituted benzene (15) was prepared by standard methods from isophthalic acid. The two alcohols were then converted by the same methods employed for the furans to afford the analogues.



Scheme 6.6



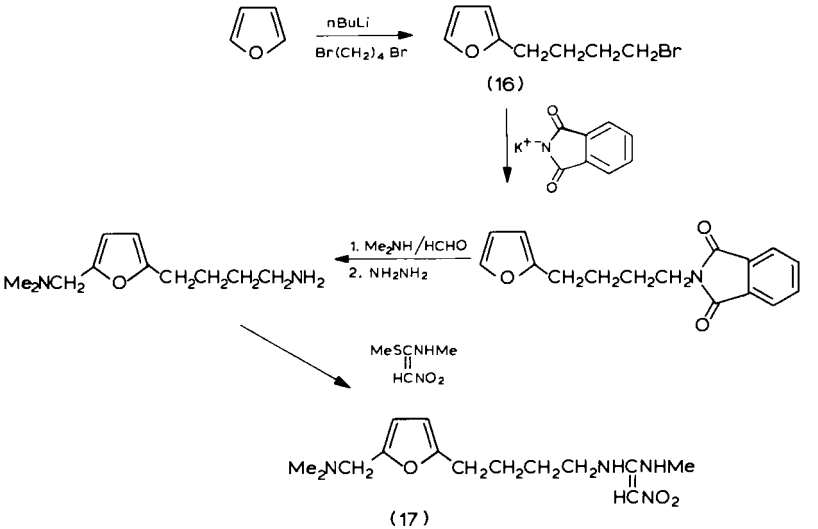
It may be seen in *Table 6.7* that the thiophen derivative was found to be less active than the corresponding furan and the benzene derivative still less active. It may well be that increasing the lipophilicity was reponsible for the decrease in histamine H_2-blocking activity in these cases.

MODIFICATION OF THE LINKING CHAIN

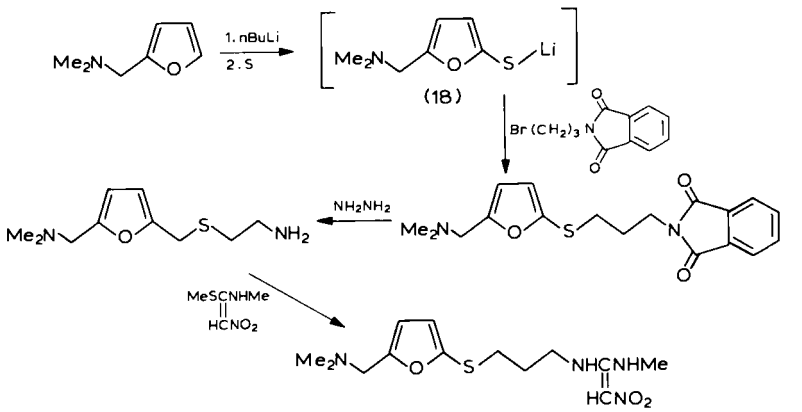
We investigated the possibility that the methylthioethyl chain in ranitidine which links the furan to the end-group may not be the optimum one in this series for H_2-antagonist activity. We first replaced the sulphur atom by a further methylene unit to afford a butylene chain. This was synthesized by preparing the lithio derivative of furan which was readily alkylated with 1,4-

dibromobutane. The bromide (16) was then elaborated by standard reactions to afford the required derivative (17) which was shown to be some 3-times less active than ranitidine (*Scheme 6.7*).

We next investigated the possibility of altering the position of the sulphur atom within the side-chain. The compound in which the sulphur atom is attached directly to the furan ring was prepared by the following route (*Scheme 6.8*). This again utilizes the lithiated furan intermediate which was first reacted with sulphur to give the thiolate anion (18) which was alkylated under standard conditions.



Scheme 6.7.



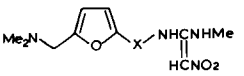
Scheme 6.8.

The isomer of ranitidine was shown to have about one-tenth the potency of the parent compound. Hence, it would seem that the linking chain is of importance in determining the potency of these compounds (*Table 6.8*).

MODIFICATION OF THE AMINE GROUP

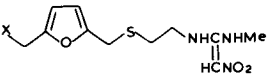
The amine function may readily be modified by carrying out the Mannich reaction using different primary and secondary amines. The basic synthetic routes described for the synthesis of ranitidine were then employed to prepare the compounds shown in *Table 6.9*. It can be seen that the activity is not particularly sensitive to the nature of the basic function. It would appear that neither changes in lipophilicity nor basicity affect the potency. However, in the example in which a cyclic amine is employed there is a reduction in potency.

Table 6.8. VARIATION IN THE SULPHUR SIDE-CHAIN [35(b)]



Ring X	*i.v. ED_{50} value*
$CH_2SCH_2CH_2$	0.18
$(CH_2)_4$	0.55
$S(CH_2)_3$	2.3

Table 6.9. EFFECT OF VARIATION IN THE SUBSTITUENT ON THE FURAN RING



Ring X	*i.v. ED_{50} value*
MeNH	0.23
EtNH	0.52
$CH_2{=}CHCH_2NH$	0.45
CF_3CH_2NH	0.30
Me_2N	0.18
EtMeN	0.48
	2.8

SELECTIVITY OF ACTION OF RANITIDINE IN ANIMALS AND MAN

The prime requirement for any potential drug is selectivity of action to ensure a predictable response and freedom from side-effects. Therefore, substances shown to be H_2-blockers in the tests described earlier (under 'biological test systems') will need to be tested for activity on other receptor systems.

Possibly the first question which could be asked of an H_2-blocker is, does it affect H_1-receptors? Secondly, since it inhibits gastric acid secretion induced by muscarinic agonists, is it anticholinergic? These questions can be answered simply by using the guinea-pig isolated ileum, the classic tool of the pharmacologist. Contractile responses of the ileum can be produced to histamine and acetylcholine alone and in the presence of the test compound. Specific H_2-receptor antagonists will not reduce these responses even at concentrations greatly in excess of those needed to affect H_2-receptors in guinea-pig atrium or rat uterus (*Figure 6.5*). Similarly, lack of activity at β-adrenoceptors, which like H_2-receptors are linked to adenylate cyclase, can be demonstrated using the guinea-pig atrium. Increases in contraction frequency induced by isoprenaline are not affected by specific H_2-blockers [38, 39].

These have been the main pharmacological tests for specificity of H_2-blockers prior to their use in man. Although some other effects were indicated by animal tests, it is clinical experience in man which has revealed some other systems which need to be considered with regard to specificity of action. Metiamide produced a few cases of reversible agranulocytosis [40] which was believed to be linked with the thiourea group in the molecule [6] rather than with H_2-blockade, since cimetidine and ranitidine have not shown this effect. Cimetidine in very high doses was observed to reduce prostate and seminal vesicle weight in developing rats [41]. This anti-androgen effect may be linked with occasional reports of gynaecomastia in men receiving cimetidine therapy. The scientific basis of this observation has been established by studies on binding of cimetidine and ranitidine to androgen receptors [42]. Cimetidine displaces [^{3}H]dihydrotestosterone from androgen receptors, but equivalent concentrations of ranitidine are without effect. Similar results were obtained both *in vitro* and *in vivo* and the authors concluded that the action of cimetidine at H_2-receptors appears truely adventitious and was not necessarily linked with H_2-receptor antagonist activity. This difference has also been confirmed *in vivo*, where ranitidine, unlike cimetidine, does not reduce prostate weight in rats [43, 44].

Cimetidine has also been reported to release prolactin when administered in high intravenous doses [45]; this effect is not seen with equipotent doses of ranitidine [46, 47].

Cimetidine has been reported to retard the metabolism of drugs such as warfarin, antipyrine (phenazone) and diazepam [48, 49]. This has been attributed to the imidazole ring of cimetidine, since many imidazole compounds inhibit hepatic monooxygenase function [50]. This hypothesis is supported by studies in man which have shown that cimitidine, but not ranitidine, inhibits the metabolism of antipyrine and the demethylation of aminopyrine [51]. Confirmatory studies with drug-metabolizing enzymes *in vitro* and *in vivo* have shown that ranitidine, unlike cimetidine, binds poorly to cytochrome *P*-450. This effect was supported by the *in vivo* observation that cimetidine, but not ranitidine, prolonged pentobarbitone-induced sleeping time in rats [52]. These differences are of clinical importance, but they also show that the imidazole-based H_2-blockers can be expected to interact not only with H_2-receptors but also with sites receptive to imidazole. It is therefore of considerable value to compare ranitidine and cimetidine, because only in this way is it possible to distinguish between some nonspecific effects at imidazole sites and those due to an action at H_2-receptors.

CLINICAL EVALUATION OF RANITIDINE

PHARMACODYNAMICS

Studies in volunteers and duodenal ulcer patients have shown that ranitidine inhibits gastric acid secretion induced by a variety of secretagogues. Bohman, Myren and Larsen [53] have shown that submaximal gastric secretory output, induced by a continuous intravenous infusion of histamine, is reduced in a dose-related manner by ranitidine. Ranitidine, infused concurrently with the histamine in doses doubled every 30 min from 0.06 to 0.48 mg $kg^{-1}h^{-1}$, inhibited acid secretion by 21 to 100% and secretion volume by 40 to 82%.

This degree of inhibition by ranitidine was slightly greater than that observed by the same workers when they used a 5-fold greater dose of cimetidine. Other workers [54] compared the inhibitory effects of ranitidine and cimetidine on maximal gastric acid secretion induced by a constant infusion of histamine in duodenal ulcer patients in remission. The dose of ranitidine required for 50% inhibition of histamine-induced maximum acid output was 0.15 mg kg^{-1} h^{-1} and that for cimetidine was 1.3 mg kg^{-1} h^{-1}. Thus ranitidine was found to be 8.7-times more potent than cimetidine on a weight basis.

One of the first human studies [55] examined the effect of ranitidine on sub-maximal gastric acid secretion induced by the intravenous infusion of pentagastrin. Ranitidine was administered i.v. in bolus doses of 20, 40 or 80 mg.

Reduction of the mean volume of gastric secretion ranged from 59% to 75% and reduction in mean acid output ranged from 73% to 95%. This dose-related inhibition of gastric secretion correlated with the ranitidine plasma levels.

Another dose-response study [56] compared the effect of placebo, ranitidine 2.5, 5, 10 and 20 mg i.v. with 25, 50, 100 or 200 mg cimetidine given i.v. on separate occasions to each volunteer. It was found that ranitidine was 13.1- and 11.1-times more potent than cimetidine, on a molar basis, for inhibition of acid output and volume of secretion, respectively. Hagenmuller, Zeitler-Abu-Ishira and Classen [57] compared the effect of 40 mg ranitidine i.v. with that of 200 mg cimetidine i.v. on the sub-maximal response to a continuous i.v. infusion of pentagastrin. Ranitidine inhibited acid secretion by 86%, while the 5-fold greater dose of cimetidine inhibited acid secretion by 81%. Thus ranitidine was shown to be approximately 7-times more potent than cimetidine on a molar basis.

Peden, Saunders and Wormsley [58] have shown that ranitidine inhibits pentagastrin-induced gastric secretion in patients with active duodenal ulcer. Ranitidine, 40 or 80 mg, administered intraduodenally, inhibited the gastric secretory response to intravenously infused pentagastrin by 42 and 69%, respectively. Simon and Kather [59] have shown that oral doses of 25, 50, 75, 100 and 150 mg ranitidine inhibit pentagastrin-induced gastric secretion by 22, 50, 55, 74 and 90%, respectively. Half-maximal inhibition was obtained at a dose of 75 mg. It was observed in this study that ranitidine produced a long-lasting inhibition of gastric acid secretion. The effect of 100 mg ranitidine orally was reduced by only about half after 5 h.

A later study [60] investigated the duration of action of 150 mg ranitidine. Basal secretion was inhibited by 35% at 9 h after ingestion of 150 mg ranitidine. with a non-significant reduction of acid output in response to a maximal dose of pentagastrin.

The effect of ranitidine on gastric acid secretion stimulated by intravenously infused amino acids has also been studied in volunteers [61]. Infusion of pentagastrin stimulated a constant submaximal acid output which was then augmented 47–67% by concurrent amino acid infusion. Administration of 50 mg ranitidine i.v. at the time of commencing the amino acid infusion resulted in a 53% reduction below the initial pentagastrin-stimulated levels. Under the same conditions, cimetidine, 200 mg i.v., produced a 42% reduction.

A number of studies have investigated the ability of ranitidine to inhibit gastric acid secretion induced by food, which is the most natural stimulus. One study [62] monitored intragastric pH over 24 h in seven duodenal ulcer patients allowed to eat and drink normally. On separate occasions, the patients received ranitidine 150 mg b.d., ranitidine 200 mg b.d., placebo or cimetidine

200 mg t.d.s. and 400 mg nocte. The mean intragastric 24 h hydrogen ion concentration was reduced by 69% with a daily dose of 300 mg ranitidine, by 70% with a daily dose of 400 mg ranitidine and by 47% with a daily dose of 1 g cimetidine. Thus, ranitidine 150 mg b.d. was clearly more effective than 1 g cimetidine daily in reducing gastric acidity over a 24 h period in which normal meals and fluids were consumed by an ambulatory patient. Ranitidine was at least 3.3-times more effective than cimetidine, but 200 mg ranitidine b.d. was not significantly more effective than a dose schedule of 150 mg ranitidine b.d.

Hagenmuller, Zeitler-Abu-Ishira and Classen [57] also compared the effects of ranitidine and cimetidine on gastric acid output in response to a peptone test meal in volunteers. Intragastric titration showed a 73% reduction of peptone stimulated gastric acid secretion by 40 mg ranitidine i.v. and 80% reduction by 200 mg cimetidine i.v. They concluded that ranitidine was approximately 7-times more potent than cimetidine on a molar basis.

The effect of ranitidine on gastric acid secretion induced by a liver extract meal in duodenal ulcer patients has been studied by intragastric titration [54]. Ranitidine, 0.5 mg kg^{-1} h^{-1}, caused immediate and almost complete inhibition of the acid response, which remained suppressed even after discontinuation of the ranitidine infusion. Cimetidine, 2 mg kg^{-1} h^{-1}, inhibited acid secretion by 75%, but this effect was not sustained after the drug infusion had been stopped. Neither ranitidine nor cimetidine significantly affected the post-prandial rise in serum gastrin levels.

Berstad, Rydning, Kolstad and Frislid [63] showed that 100 mg ranitidine taken orally at the start of a steak meal maintained median gastric pH above 3.5. In control experiments, the pH fell below 2. They also found that the action of ranitidine was not significantly affected by antacid consumption.

Mignon, Bonfils, Sauvage and Alexandre [64] studied the effect of ranitidine, 50, 100, 150 or 200 mg orally, on the gastric secretory response to a test meal. Measurements were made by intragastric titration and the different doses of ranitidine or placebo were investigated on separate occasions. Ranitidine inhibited gastric acid output by 42, 75, 85 and 95%, respectively. Although ranitidine was clearly effective in reducing gastric acid secretion, it did not modify the gastrin response or gastric emptying.

Another facet of the study [54] mentioned earlier was an investigation of the action of ranitidine on the response to sham feeding in duodenal ulcer patients. The cephalic phase of gastric secretion was invoked by chewing but not swallowing an appetizing meal with checks to ensure that food particles had not been swallowed. Sham feeding induced a secretory response with a peak equivalent to 66% of the maximum pentagastrin-stimulated acid output. Ranitidine, 0.5 mg kg^{-1} h^{-1}, almost completely abolished this response and

cimetidine, 2 mg kg^{-1} h^{-1}, reduced the response by about 70%. The pepsin response was also virtually abolished by ranitidine and reduced to about 50% of the control value by cimetidine.

The effect of oral ranitidine on the secretory response to sham feeding has been investigated in volunteers by Muller-Lissner, Sonnenberg, Eichenberger and Blum [65]. Administration of ranitidine 150 mg orally 2 h before sham feeding caused a 95% reduction in acid secretion relative to those experiments in which placebo was administered.

Peden, Saunders and Wormsley [58] have shown that ranitidine inhibited nocturnal acid secretion in patients, four with duodenal ulcer and three with post-vagotomy recurrences of duodenal ulcer. Ranitidine, 80 mg orally, inhibited nocturnal acid secretion by an average of 95%. An earlier study from the same centre showed that 400 mg cimetidine inhibited nocturnal acid secretion by an average of 66% in duodenal ulcer patients [66].

The various results described above are in accord with predictions from the animal pharmacology regarding the efficacy and potency of ranitidine.

PHARMACOKINETICS

Following an oral dose, ranitidine is absorbed rapidly and produces peak blood levels within 90 min [55]. Oral bioavailability is approximately 50% [55, 67, 68]. Ranitidine is largely excreted as unchanged drug, approximately 70%, with the main metabolite being the *N*-oxide [52, 69, 70]. The plasma half-life is 140 min [55]. Following an oral dose of 150 mg ranitidine, plasma levels remain above the IC_{50} value of 0.285 mol/l [71] for more than 8 h. This permits a twice-daily dose regime which is of considerable convenience to the patient.

THERAPEUTICS

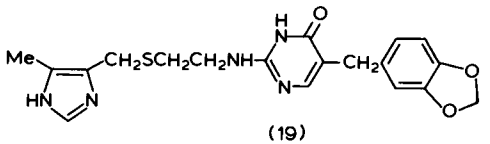
The characteristics described above suggested that ranitidine would be useful in the treatment of clinical conditions associated with hypersecretion of gastric acid. This prediction has been confirmed in many clinical trials. Only a few of the many published studies can be cited in this brief review. More detailed information can be found elsewhere [72].

Healing rates of 79–94% have been obtained in duodenal ulcer patients receiving 150 mg ranitidine twice daily for 4 weeks [73, 74]. Ranitidine has also proved effective in healing gastric ulcer, with success rates of 47–70% [75, 76]. Higher healing rates, 83–93%, have been obtained with a longer treatment period [74, 77]. Ranitidine has also been shown to be beneficial in the treatment of reflux oesophagitis [72], in prophylaxis of Mendelson's syndrome [78,

80], acute upper gastrointestinal bleeding [81, 82] and the Zollinger-Ellison syndrome [83].

OTHER COMPOUNDS AS H_2-RECEPTOR ANTAGONISTS

The development of ranitidine has provided an H_2-receptor antagonist which is effective in healing the consequences of acid peptic disease. It is well tolerated by patients and its highly selective action has resulted in a low incidence of side-effects [72]. Such a profile of action does not leave much scope for improvement.



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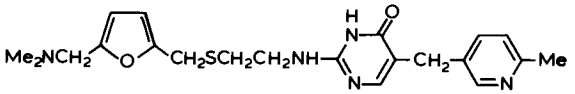
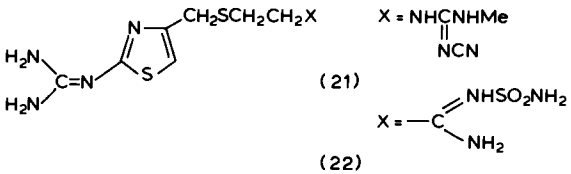


(20)

SKF 92994 (oxmetidine, 19) was reported to have a longer duration of action than cimetidine in the anaesthesized rat, but there was no prolonged action in the conscious dog [84] or man [85]. The furan analogue SKF 93479 (20) has been reported to show a sustained inhibition of histamine-induced gastric acid secretion in the dog [86]. Studies in man suggest that its maximum antisecretory effect is sustained for more than 3 h after an oral dose and a significant effect for longer than 8 h [87]. However, it should be noted that the normal therapeutic dose of ranitidine in man has also been shown to produce inhibition of basal acid secretion over this period [60, 88].



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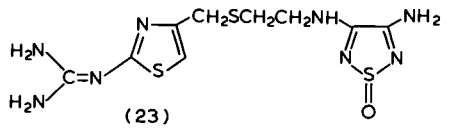
(22)

The guanylthiazole derivative (tiotidine, 21) has been reported to be longer acting than ranitidine, but has had to be withdrawn from clinical development due to toxicity in animal experiments. It significantly inhibited the secretory response of duodenal ulcer patients to a test meal 10–12 h after a dose [89]. However, this may be a reflection of potency as well as duration of action, because the dose of tiotidine (150 mg) tested was only half that of the dose of cimetidine employed in the study and animal studies have shown that tiotidine is 10-times more potent than cimetidine on a weight basis [90].

Another thiazole with a novel sulphonylamidine end-group (YM-11170, 22) [91, 92] has been reported to be much more potent (38.3-times) than cimetidine and to be 1.3- to 1.5-times longer acting in the dog.

Ranitidine has the advantage over cimetidine of requiring the patient to take a tablet only twice daily. A genuinely longer acting compound might be useful in allowing once-daily dosing. A dose at bedtime would inhibit nocturnal acid secretion, which is a problem in duodenal ulcer patients, but allow some acid secretion during the day when ingesting meals. Two distinct classes of compound have been described which hold out real hope of providing a once-a-day treatment in the future. Both classes of compound have a heterocyclic ring system as the end-group. The compound first reported by Bristol Laboratories, BL-6341A (23), has been claimed to be the most potent H_2-antagonist yet produced and to be intrinsically longer acting [93, 94, 96].



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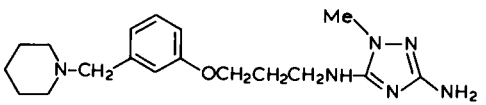

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More recently the triazole (AH22216, 24), has been reported by workers at Glaxo to have a very long duration of action [95]. This compound is a potent and highly selective H_2-receptor antagonist. It is 8-times more potent than ranitidine as an inhibitor of gastric acid secretion in the dog. While ranitidine exhibits an antisecretory action for 8 h in the dog, AH22216 is still active after 24 h (*Table 6.2*). This may be of benefit in requiring only a single daily dose for gastric or duodenal ulcer patients. It may also be of value in resistant acid-peptic disease states such as the Zollinger-Ellison syndrome [97].

Table 6.10. STRUCTURAL FEATURES OF H_2-ANTAGONIST DRUGS

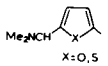
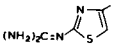
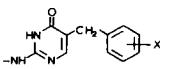
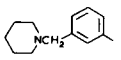
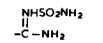
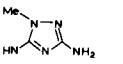
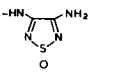
Left-hand side	*Chain*	*Right-hand side*
	$CH_2SCH_2CH_2$	
	$CH_2CH_2CH_2CH_2$	
	$OCH_2CH_2CH_2$	
		
		
		
		

Table 6.10 gives a summary of the structural features which appear in the most important H_2-antagonists reported so far.

It can be seen from the diversity of compounds described here that the structural requirements for molecules to act as antagonists at the H_2-receptor are not strict. Much work remains to be done before a full understanding of the structure-activity relationships becomes known.

REFERENCES

1. B.I. Hirschowitz, Annu. Rev. Pharmacol. Toxicol., 19 (1979) 203.
2. D. Bovet and A.M. Staub, C.R. Seances Soc. Biol. Paris, 124 (1937) 547.
3. B. Folkow, K. Haeger and G. Kahlson, Acta Physiol. Scand., 15 (1948) 264.
4. A.S.F. Ash and H.O. Schild, Br. J. Pharmacol. Chemother., 27 (1966) 427.
5. J.W. Black, W.A.M. Duncan, C.J. Durant, C.R. Ganellin and E.M. Parsons, Nature (London), 236 (1972) 385.
6. C.R. Ganellin, J. Appl. Chem. Biotechnol., 28 (1978) 183.
7. O. Arunlakshana and H.O. Child, Br. J. Pharmacol. Chemother., 14 (1959) 48.
8. M.E. Parsons, D.A.A. Owen, C.R. Ganellin and G.J. Durant, Agents Actions, 7 (1977) 31.
9. B.Y.C. Wan, J. Physiol. (London), 266 (1977) 327.
10. K.T. Bunce and M.E. Parsons, J. Physiol. (London), 258 (1976) 453.

11. J.A. Angus, J.W. Black and M. Stone, Br. J. Pharmacol., 68 (1960) 413.
12. B.L. Tepperman, B. Schofield and F.S. Tepperman, Can. J. Physiol. Pharmacol., 53 (1975) 1141.
13. P. Holton and J. Spencer, J. Physiol. (London) 255 (1976) 465.
14. R. Hearn and I.H.M. Main, J. Physiol. (London), 251 (1975) 11P.
15. D. Fromm, J.H. Schwartz and R. Quijano, Gastroenterology, 69 (1975) 453.
16. J.G. Forte, G.M. Forte and T.E. Machen, J. Physiol. (London), 244 (1975) 15.
17. P. Scholes, A. Cooper, D. Jones, J. Major, M. Walters and C. Wilde, Agents Actions, 6 (1976) 677.
18. D.N. Croft and F.J. Ingelfinger, Clin. Sci., 37 (1969) 491.
19. A.H. Soll, J. Clin. Invest., 61 (1978) 370.
20. D.R. Bristow, J.R. Hare, J.R. Hearn and L.E. Martin, Br. J. Pharmacol., 72 (1981) 547 P.
21. A.H. Soll, J. Lechago and J.H. Walsh, Gastroenterology, 70 (1976) 975.
22. W.P. Burkard, Eur. J. Pharmacol., 50 (1978) 449.
23. P. Devoto, A.M. Marchisio, E. Carboni and P.F. Spano, Eur. J. Pharmacol., 63 (1980) 91.
24. D.B. Norris, T.J. Rising, S.E. Warrander and T.P. Wood, Br. J. Pharmacol., 72 (1981) 548P.
25. I.R. Smith, M.T. Cleverley, C.R. Ganellin and K.M. Metters, Agents Actions, 10 (1980) 422.
26. S.E. Svensson, J. Physiol. (London), 207 (1970) 329.
27. K.T. Bunce and M.E. Parsons, Agents Actions, 7 (1977) 507.
28. C.F. Code, Fed. Proc., 24 (1965) 1311.
29. R.L. McIsaac, Ph.D. Thesis, University of London, 1976.
30. M.J. Daly, J.M. Humphray, K.T. Bunce and R. Stables, Agents Actions, 11 (1981) 160.
31. C.R. Ganellin, G.J. Durant and J.C. Emmett, Fed. Proc., 35 (1976) 1924.
32. S. Rendic, V. Sunjic, R. Toso and F. Kaijfez, Xenobiotica, 9 (1979) 555.
33. A.A. Algieri, Abstr. 183rd ACS National Meeting, Las Vegas 1982, MED 008.
34. G.J. Durant, J.C. Emmett, C.R. Ganellin and H.D. Prain, Belg. Pat., 814, 941 (1973); Ger. Offen. 2,423,813; Chem. Abstr., 82 (1975) 120943.
35.(a) R.T. Brittain, D. Jack and B.J. Price, Trends Pharm. Sci., 2 (1982) 310. (b) J. Bradshaw, M.E. Butcher, J.W. Clitherow, M.D. Dowle, R. Hayes, D.B. Judd, J.M. Mckinnon and B.J. Price, in: Chemical Regulation of Biological Mechanisms, Royal Society of Chemistry, Special Publication No. 42, ed. A.M. Creighton and S. Turner (1982).
36. G.J. Durant, J.C. Emmett, C.R. Ganellin, P.D. Miles, M.E. Parsons, H.D. Prain and G.R. White, J. Med. Chem., 20 (1977) 901.
37. R. Gomper and H. Schaefer, Chem. Ber., 100 (1967) 591.
38. M.E. Parsons, in: Cimetidine – Proceedings of the Second International Symposium on Histamine H_2-receptor Antagonists, eds. W.L. Burland and M.A. Simkins (Excerpta Medica, Amsterdam-Oxford) (1977) pp. 13-23.
39. M.J. Daly, J.M. Humphray and R. Stables, Br. J. Pharmacol., 72 (1981) 49.
40. J.A.H. Forrest, D.J.C. Shearman, R. Spence and L.R. Celestin, Lancet, i (1975) 392.
41. G.B. Leslie and T.F. Walker, in: Ref. 38, pp. 24-37.

42. P. Pearce and J.W. Funder, Clin. Exp. Pharmacol. Physiol., 7 (1980) 442.
43. R.T. Brittain, M.J. Daly and M. Sutherland, J. Pharm. Pharmacol., 32 suppl. (1980) 76p.
44. R.T. Brittain and M.J. Daly, Scand. J. Gastroent., 16 suppl. 69 (1981) 1.
45. H.E. Carson and A.F. Ippoliti., J. Clin. Endocrinol. Metab., 45 (1977) 367.
46. G.F. Nelis and J.G.C. van de Meene, Postgrad. Med. J., 56 (1980) 478.
47. K.J. Graef, D.V. Kleist and K.H. Meiyer zum Bueschenfelde, Acta Endocrinol., 94 suppl. 234 (1980) 4.
48. M.J. Serlin, R.G. Sibeon, S. Mossman, A.M. Breckenridge, J.R.B. Williams, J.L. Atwood and J.M.T. Willoughby, Lancet, ii (1979) 317.
49. U. Klotz and I. Reimann, N. Engl. J. Med., 302 (1980) 1012.
50. C.F. Wilkinson, K. Hetnarski and L.J. Hicks, Pesticide Biochem. Physiol., 4 (1973) 299.
51. D.A. Henry, I.A. Macdonald, G. Kitchingman, G.D. Bell and M.J.S. Langman, Br. Med. J., 2 (1980) 775.
52. J.A. Bell, A.J. Gower, L.E. Martin, E.N.C. Mills and W.P. Smith, Biochem. Soc. Trans., 9 (1981) 113.
53. T. Bohman, J. Myren and S. Larsen, Scand. J. Gastroenterol., 15 (1980) 183.
54. S.J. Konturek, W. Obtulowicz, N. Kwiecien, E. Sito, E. Mikos and J. Oleksy, Gut, 21 (1980) 181.
55. E.P. Woodings, G.T. Dixon, C. Harrison, P. Carey and D.A. Richards, Gut, 21 (1980) 187.
56. K-Fr. Sewing, A. Billian and H. Malchow, Gut, 21 (1980) 750.
57. F. Hagenmuller, A. Zeitler-Abu-Ishira and M. Classen, Gut, 20 (1979) A905.
58. N.R. Peden, J.H.B. Saunders and K.G. Wormsley, Lancet, i (1979) 690.
59. B. Simon and H. Kather, Dtsch. Med. Wochenschr., 104 (1979) 1676.
60. H.G. Damman, J. Kather, H.J. Augustin and B. Simon, Dtsch. Med. Wochenschr., 105 (1980) 603.
61. J. Weingart, H. Kunert and R. Ottenjann, Br. J. Clin. Pharmacol., 10 (1980) 174.
62. R.P. Walt, P.J. Male, J. Rawlings, P. Torrie, R.H. Hunt, J.J. Misiewicz and G.J. Milton-Thompson, Gut, 20 (1979) A904.
63. A. Berstad, A. Rydning, B. Kolstad and K. Frislid, Scand. J. Gastroent., 16 suppl. 69 (1981) 67.
64. M. Mignon, S. Bonfils, M. Sauvage and C. Alexandre, Hepato-Gastroenterology, 27 (1980) 72.
65. S.A. Muller-Lissner, A. Sonneberg, P. Eichenberger and A.L. Blum, Hepato-Gastroenterology, 27 (1980) 377.
66. J.H.B. Saunders, J.M. Cargill and K.G. Wormsley, Digestion, 15 (1977) 452.
67. D.C. Garg, D.J. Weidler, N. Baltodano and F.N. Eshelman, Clin. Pharmacol. Ther., 29 (1981) 247.
68. K. Bogues, G.T. Dixon, P. Fowler, W.N. Jenner, J.G. Maconochie, L.E. Martin and B.A. Willoughby, Br. J. Pharmacol., 73 (1981) 275P.
69. G.W. Mihaly, O.H. Drummer, A. Marshall, R.A. Smallwood and W.J. Louis, J. Pharm. Sci., 69 (1980) 1155.
70. P.F. Carey, L.E. Martin and P. Owen, Biochem. Soc. Trans., 9 (1981) 112.
71. N.R. Peden, D.A. Richards, J.H.B. Saunders and K.G. Wormsley, Lancet, ii (1979) 199.

72. Clinical Use of Ranitidine, 2nd International Symposium. eds. J.J. Misiewicz and K.G. Wormsley (Medicine Publishing Foundation, Oxford, 1982).
73. G. Dobrilla, L. Barbara, G. Bianchi-Porro, M. Felder, G. Mazzacca, L.. Migliolo, A. Pera, M. Petrillo, F. Sabbatini and G. Verme, Scand. J. Gastroenterol., 16 suppl. 69 (1981) 101.
74. T. Takemoto, Y. Okazaki, K. Okita, M. Namiki, M. Ishikawa, S. Oshiba and K. Kurokawa, Scand. J. Gastroenterol., 16 suppl. 69 (1981) 125.
75. J.P. Wright, I.N. Marks, L.S. Mee, A.H. Girdwood, P.C. Bornman, N.H. Gilinsky, P. Tobias and W. Lucke, S. Afr. Med. J., 61 (1982) 155.
76. M.R. Ashton, C.D. Holdsworth, F.P. Ryan and M. Moore, Br. Med. Jr., 1 (1982) 467.
77. P. Barbier, A. Dumont and M. Adler, Acta Gastro-Enterol. Belg., 42 (1979) 268.
78. J.R. Johnston, W. McCaughey, P.J. Wright, J.A.S. Gamble and J.W. Dundee, Br. J. Anaesth., 53 (1981) 664P.
79. A.D. Andrews, J.G. Brock-Utne and J.W. Downing, Anaesthesia, 37 (1982) 22.
80. R.N. Francis and R.S.H. Kwik, Anaesth. Analg., 61 (1982) 130.
81. J. Dawson and R. Cockel, Gut, 22 (1981) A878.
82. A. Nowak, Cz. Sadlinksi, Z. Gorka, E. Nowakowska, J. Rudzki and K. Gibinski, Hepato-Gastroenterology, 28 (1981) 267.
83. S. Bonfils, M. Mignon, Th. Vallot and S. Mayeur, Scand. J. Gastroent., 16 suppl. 69 (1981) 119.
84. R.C. Blakemore, T.H. Brown, G.J. Durant, J.C. Emmett, C.R. Ganellin, M.E. Parsons and A.C. Rasmussen, Br. J. Pharmacol., 70 (1980) A105P.
85. J.G. Mills, M.A. Melvin, R. Griffiths, H. Hunt, W.L. Burland and G.J. Milton-Thompson, Gut, 21 (1980) A462.
86. R.C. Blakemore, T.H. Brown, G.J. Durant, C.R. Ganellin, M.E. Parsons, A.C. Rasmussen and D.A.R. Rawlings, Br. J. Pharmacol., 74 (1981) 200P.
87. W.L. Burland, A.C. Clancy, R.H. Hunt, J.G. Mills, D. Vincent and G.J. Milton-Thompson, Gut, 22 (1981) A426.
88. H.G. Damman and B. Simon, Scand. J. Gastroent., 16 suppl. 69 (1981) 39.
89. C.T. Richardson, M. Feldman, C. Brater and J. Welborn, Gastroenterology, 80 (1981) 301.
90. T.O. Yellin, S.H. Buck, D.J. Gilman, D.F. Jones and J.M. Wardleworth, Life Sci., 25 (1979) 2001.
91. M. Takeda, T. Takagi and H. Maeno, Jap. J. Pharmacol., 31 (1981) suppl. 222P.
92. T. Takagi, M. Takeda and H. Maeno, Archs. Int. Pharmacodyn., 256 (1982) 49.
93. W.C. Lumma, P.S. Anderson, J.J. Baldwin, W.A. Bolhofer, C.N. Habecker, J.M. Hirshfield, A.M. Pietruszkiewicz, W.C. Randall, M.L. Torchiana, S.F. Britcher, B.V. Clineschmidt, G.H. Denny, R. Hirschmann, J.M. Hoffman, B.T. Phillips and K.B. Streeter, J. Med. Chem., 25 (1982) 207.
94. A.A. Algieri, G.M. Luke, R.T. Standridge, M. Brown, R.A. Partyka and R.R. Crenshaw, J. Med. Chem. 25 (1982) 210.
95. B.J. Price, 183rd ACS National Meeting, Las Vegas 1982.
96. R.L. Cavanagh, J.J. Usakewicz and J.P. Buyniski, Fed. Proc., 40 (1981) 693.
97. R.T. Brittain, M.J. Daly, J.M. Humphray and R. Stables, Br. J. Pharmacol., 1982 (76) 195P.

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