PROGRESS IN MEDICINAL CHEMISTRY 28

G. P. ELLIS
G. B. WEST
EDITORS

Progress in Medicinal Chemistry 28

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Edited by

G.P. ELLIS, D.SC., PH.D., F.R.S.C.

School of Chemistry and Applied Chemistry,
University of Wales,
P.O. Box 912,
Cardiff, CF1 3TB,
United Kingdom

and

G.B. WEST, B. PHARM., D.SC., PH.D., F.I.BIOL.*

Institute of Health & Rehabilitation,
Polytechnic of East London,
Romford Road,
London, E15 4LZ,
United Kingdom

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Preface

In this volume, we have pleasure in presenting six reviews which cover a wide range of medicinal chemical topics. In Chapter 1, the 8-aminoquinolines are analyzed for their antimalarial activity following the success of primaquin over the past 25 years. The process of chelation of tripositive elements, described in Chapter 2, is of particular importance as one of the uses of chelators is to remove toxic metals from the body.

Chapter 3 describes the use of computers in defining total parenteral nutrition, thereby providing all the needs of seriously-ill patients. This is a completely new and promising approach to the problem of maintaining the chemical balance in the body and of controlling the characteristics of the parenteral fluid in order to maximize its efficacy and to minimize adverse effects.

Antiulcer drugs of plant origin are discussed in Chapter 4, linking traditional remedies with modern chemicals extracted from them. Chapter 5 reviews the field of heterosteroids and the many biologically active synthetic and natural compounds in this group.

Finally, Chapter 6 is a survey of biologically active receptor-specific opioid peptides, all arising from three distinct precursor molecules.

We thank our authors for surveying and summarizing the considerable literature of each topic. We also offer our thanks to owners of copyright material which we have included, and to the staff of our publishers for their continuing help and encouragement.

G.P. Ellis

June 1990

G.B. West

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In Memoriam

Geoffrey Buckle West, 1916-1990

Dr. G.B. West

It is with deep regret that we record the death on 5th December 1990 of Dr. Geoffrey West, who had been co-editor of this Series since its inception in 1961. He graduated in the University of London (School of Pharmacy) in 1938, gained a Ph.D. in 1942 and a D.Sc. in 1954 from the same university. In 1948 he joined the staff of his *alma mater* as Lecturer in Pharmacology. Two years later he moved to the Clinical Pharmacology Department, University of St. Andrews at Dundee. He began his work on endogenous amines and discovered that mast cells were the major site of histamine stores in the body. For this discovery and the work that followed, he was awarded the 1956 Annual Prize Essay of the American Dermatological Association. His old university recalled him to London in 1955 as Reader in Pharmacology. The role of histamine in allergy was his main interest at this time. He and his colleagues showed that one strain of rats behaved quite differently from others when injected with dextran. One strain was immune to the anaphylactoid response which humans and most rats exhibit. For the great volume of innovative and important work which Geoffrey and his team published on this subject, he was given the Biology Award of the New York Academy of Sciences in 1964. He was for many years the Secretary-General of the European Histamine Research Society, and Chief Editor of the International Archives of Allergy. In 1981 he retired from the post of Reader in Pharmacology at the North East London Polytechnic, which he had held for 12 years.

To his editorial work Geoffrey brought a wealth of experience as the author of many well-written papers and reviews. He gave meticulous care to each manuscript and expected a high standard of accuracy and clarity from the author – qualities which are not as often to be found today as when this Series began.

Geoffrey had a friendly, kind and sympathetic personality with a highly developed sense of humour, and was a 'real gentleman'. After working together for so long, I shall miss his wise counsel and guidance on pharmacological matters.

On behalf of all our authors, the Publisher and for myself, I offer his wife, Jean, and the family our sincere and deeply felt sympathy.

G.P. Ellis
December 1990

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Progress in Medicinal Chemistry – Vol. 28, edited by G.P. Ellis and G.B. West

1 Antimalarial Activity of the 8-Aminoquinolines

EDWARD A. NODIFF, B.A.[1], SANKAR CHATTERJEE, Ph.D.[1] and HIKMAT A. MUSALLAM, Ph.D.[2]

[1] Franklin Research Center, Arvin Calspan Corporation, Valley Forge Corporate Center, 2600 Monroe Boulevard, Norristown, PA 19403 and [2] Department of Medicinal Chemistry, Division of Experimental Therapeutics, Walter Reed Army Institute of Research, Walter Reed Army Medical Center, Washington, DC 20307-5100, U.S.A.

INTRODUCTION

Malaria is the world's most ravaging infectious disease. Rampant throughout much of the tropics and some of the temperate areas, its numbers beggar the imagination. It threatens a third of the world's population, presently afflicts hundreds of millions of people, causes several million deaths annually and may generate as many as 92 million new clinical cases each year [1, 2]. Its socio-economic drain is enormous.

The resurgence of this pestilence during the past two decades has stimulated the search for a vaccine but, despite a prodigious research effort and some cautious optimism, an effective vaccine is still far from fruition [3–10]. In the interim, there must be continued reliance on drugs for prophylaxis and therapy. Research leading to the currently available antimalarial agents has been detailed in a number of comprehensive reviews [11–29]. Unfortunately, most of these agents are obsolescent because of the facility with which the malarial parasite produces drug-resistant mutants [30]. The need for more effective antimalarials is therefore critical. In a search for such drugs, a small group of investigators has returned to an old, very heavily worked and seemingly exhausted mine, the 8-aminoquinolines. The evolution of an extremely promising series of new, broad-spectrum, antimalarial 8-aminoquinolines is described in this chapter. The new drugs are unique in their dual efficacy against the blood and tissue forms of the disease.

THE PARASITE

Malaria is caused by protozoan parasites of the genus *Plasmodium*. There are four species of Plasmodia pathogenic to man: *P. falciparum* (malignant tertian malaria), *P. vivax* (benign tertian malaria), *P. malariae* (quartan malaria) and

P. ovale. *P. falciparum* and *P. vivax*, together, cause 95% of human malaria. *P. ovale*, the rarest of the four species, occurs mainly in West Africa and is much like *P. vivax*. *P. malariae* causes a generally mild but extraordinarily tenacious form of the disease which can persist in the blood, with or without symptoms, for a lifetime.

The life cycle of the parasite has three phases: exoerythrocytic (tissue) and erythrocytic (blood) in man; sporogony in the mosquito.

EXOERYTHROCYTIC PHASE

Each species begins its cycle with the injection of thread-like, motile organisms (sporozoites) into the host's bloodstream during the bite of an infected female anopheles mosquito. Within an hour, the sporozoites leave the bloodstream, enter the liver and invade hepatocytes. Most of the sporozoites develop into primary tissue schizonts (schizogony) which undergo repeated asexual division to form thousands of merozoites. At maturity, the merozoites burst from the hepatocytes into the bloodstream, attack red blood cells and begin the erythrocytic phase of the cycle. Some of the sporozoites of *P. vivax* and *P. ovale* remain in the liver as latent forms called hypnozoites [31]. The hypnozoites become active intermittently, for years, releasing new merozoites into cleared blood and causing relapses. *P. falciparum* and *P. malariae* have no hypnozoite stage and, therefore, no potential for relapse.

ERYTHROCYTIC PHASE

On invading an erythrocyte, the merozoite assumes a ring shape called a trophozoite and begins to feed on haemoglobin. The globin fraction is metabolized and the haem fraction is deposited in the tissues as granules called haemozoin or malaria pigment. When the trophozoite matures and begins to divide, it becomes a blood schizont. Division continues causing cell lysis and the discharge of new merozoites which invade fresh red cells for another asexual cycle. It is the periodic rupture of erythrocytes, with the expulsion of merozoites, their waste products and cellular debris, which is responsible for the paroxysmal nature of malaria. Some of the merozoites enter red cells and develop into trophozoites but form no schizonts. Instead, their nuclei remain intact and they differentiate into sexual forms, male and female, known as gametocytes. The gametocytes undergo no further development in man but circulate in the bloodstream until ingested during an anopheline blood meal.

SPOROGONY

In the mosquito midgut, all stages of the parasite except the gametocytes are destroyed. On maturation, the sperm-like male (microgamete) fertilizes the egg-like female (macrogamete) forming a zygote. The zygote becomes a motile, saclike structure called an ookinete which penetrates the stomach wall and forms an oocyst beneath the wall's outer membrane. Development of the oocyst leads to the formation of large numbers of infectious sporozoites which escape and migrate to the mosquito's salivary glands. With the insect's next bite, the parasite's life cycle is renewed. Sporogony takes from 2 to 3 weeks, depending on the species.

THE DISEASE

Malaria becomes manifest with a general malaise followed by a series of recurrent, three-stage paroxysms of shaking chills, a temperature which can rise as high as 106°F and a drenching sweat. The paroxysms, which signal the completion of each asexual cycle, can recur at regular intervals – about 48 h for *P. vivax*, *P. ovale* and *P. falciparum* and about 72 h for *P. malariae*. With the non-falciparum malarias, some degree of immunity eventually develops, parasitemia is diminished and, despite relapses and considerable debilitation, death is uncommon, even without treatment. However, an untreated falciparum infection frequently leads to a fulminating, pathophysiologic cascade (non-responsive hypotension; anaemia, hyperpyrexia; hypoglycaemia; lactic acidosis; fluid and electrolyte disturbances; septicemia; hemoglobinuria; pulmonary oedema; failure of hepatic, cerebral and renal function; shock; coma) and death may occur soon after onset of symptoms [32, 33]. The mortality rate from the cerebral form of severe falciparum malaria may be as high as 50% [34]. It is mainly the rapid geographic spread of extremely dangerous, multi-drug-resistant *P. falciparum* which makes the need for new antimalarial drugs so urgent.

CLASSIFICATION OF ANTIMALARIAL DRUGS

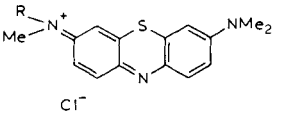
The aim of antimalarial chemotherapy is a cheap, safe, stable drug which could be administered in a single, well-tolerated, long-lasting, oral dose and which would interrupt all of the stages in the parasite's life cycle without the selection of resistant strains. No such drug exists. Because of their relatively narrow activity spectra, the presently available antimalarials are classified according to

their mode of attack. Thus, drugs which destroy the intrahepatic parasites, before their emergence into the circulation, are termed pre-erythrocytic or primary tissue schizontocides or causal prophylactics. True prophylactics, which would act on the sporozoites during the brief interval between anopheline injection and hepatic sequestration, are unknown. Destruction of the asexual blood forms of the relapsing malarias, with the resulting elimination of clinical symptoms, is effected by agents called suppressive drugs or blood schizontocides. However, complete elimination of the relapsing malarias (radical cure) requires a second drug which can eradicate the persistent liver forms. Such drugs are known variously as radically curative drugs, antirelapse agents, secondary tissue schizontocides or hypnozoitocides. Radical cure of malariae and falciparum malaria, which have no persistent liver forms, can be achieved with blood schizontocides. The parasites may also be attacked during their sexual development in the host (gametocytocide) or mosquito (sporontocide), thereby preventing transmission.

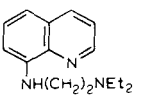
The most pressing present needs in malaria chemotherapy are a safe antirelapse drug and a blood schizontocide which can eliminate resistant *P. falciparum* without selecting new troublesome mutants. Thus far, there is no single clinical drug which can carry out both functions. Among the drugs and drug combinations in current use as blood schizontocides are chloroquine, amodiaquine, amopyroquine, quinine, proguanil, chlorproguanil, cycloguanil, pyrimethamine, trimethoprim, sulfadoxine, sulfalene, dapsone, mefloquine, halofantrine, qinghaosu and its derivatives, doxycycline, tetracycline, pyrimethamine-sulfadoxin (Fansidar), pyrimethamine-sulfadoxine-mefloquine (Fansimef), pyrimethamine-sulfadoxine-amodiaquine, pyrimethamine-dapsone (Maloprim), quinine-quinidine-cinchonine (Falcimax) and quinine-tetracycline. In stark contrast, there are presently only two drugs in clinical use as tissue schizontocides (radical curative or antirelapse drugs); those drugs are the 8-aminoquinolines, primaquine and, to a lesser extent, quinocide.

HISTORY

The germinal work in the evolution of primaquine occurred a century ago when Guttmann and Ehrlich treated malaria with methylene blue (1) after noting the dye's selective staining of intraerythrocytic plasmodia *in vitro* [35]. Schulemann and colleagues subsequently found that the activity of methylene blue could be improved by substituting the basic dialkylaminoalkyl chain, $-(CH_2)_2NEt_2$, for one of the dye's methyl groups to give compound (2) [36]. After attachment to a number of diverse structures, the chain found its way to an 8-aminoquinoline producing the avian antimalarial (3) [37]. The activity of (3), and



(1) R = Me
(2) R = $(CH_2)_2NEt_2$

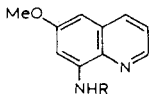


(3)

Germany's need to find a substitute for the quinine interdicted by World War I, triggered a massive investigation of the 8-aminoquinolines which led through thousands of variants to the first practical synthetic antimalarial, pamaquine (4) [38–45]. Most of the original German research was never published and, despite some interesting detective work by Steck [14], the exact sequence, from methylene blue in 1891 to pamaquine in 1926, remains somewhat nebulous.

Unfortunately, the early enthusiasm for pamaquine's ability to cure vivax infections [46–48] was soon tempered by the frequency with which it caused methaemoglobinemia and acute haemolytic anaemia [14]. When World War I ended, research on the 8-aminoquinolines became less intense [49–58] and at the beginning of World War II, pamaquine was still the only antirelapse drug known. When malaria's terrible toll on military personnel created an urgent need for an antirelapse drug, a number of investigators re-evaluated pamaquine. They confirmed its activity but found a level of toxicity which precluded its use [59–62].

In an effort to improve the therapeutic index of pamaquine, the U.S. Office of Scientific Research and Development (OSRD) supported the synthesis of hundreds of new 8-aminoquinolines [63–80]. By the end of World War II, this programme had produced pentaquine (5) [81–84]. Following the war, the U.S. Public Health Service sponsored a programme from which emerged isopentaquine (6), SN-3883 (7) and primaquine (8). Although compounds (5)–(8) were all better than pamaquine, it was the pressure of another war, the Korean conflict (1950–1953), which ultimately established primaquine (8) as the drug of choice for the radical cure of the relapsing malarias [85–93].



(4)	R = $CHMe(CH_2)_3NEt_2$	pamaquine (*Plasmochin*)
(5)	R = $(CH_2)_5NHCHMe_2$	pentaquine
(6)	R = $CHMe(CH_2)_3NHCHMe_2$	isopentaquine
(7)	R = $(CH_2)_4NH_2$	SN - 3883
(8)	R = $CHMe(CH_2)_3NH_2$	primaquine
(9)	R = $(CH_2)_3CHMeNH_2$	quinocide

Quinocide (9), an isomer of primaquine, is used in Eastern Europe and the USSR but it is more toxic than primaquine [94–101]. Primaquine is presently the preferred radical curative drug in most parts of the world. The work leading from pamaquine to primaquine has been detailed in a number of reviews [11, 12, 62]. Primaquine has a broad range of antimalarial activity. In addition to its radical curative activity, primaquine is a causal prophylactic, a gametocytocide and a sporontocide. It can also be produced at low cost and has been relatively slow to select resistant strains [102–104]. Despite these attributes, primaquine is far from the ideal antimalarial. It is a poor blood schizontocide and, although it is the best of the 8-aminoquinolines, its clinical use as a tissue schizontocide is still limited by serious side-effects such as haemolysis, methaemoglobinemia and gastrointestinal distress. Particularly prone to haemolytic reactions are patients with a genetic deficiency of glucose-6-phosphate dehydrogenase (G-6-PD) [105]. Also troublesome is the need to administer primaquine in divided doses over an extended period (15 mg daily for 14 days) since, in addition to its toxicity, it is rapidly absorbed, metabolized and eliminated. Clinicians have therefore been using primaquine with some trepidation and with the desire for a safer, more effective congener.

The most recent surge in antimalarial chemotherapeutic research started with the disturbing discovery in 1960 that *P. falciparum* was becoming resistant to the major blood schizontocide, chloroquine [106–108]. In 1963, the U.S. Army Medical Research and Development Command (USAMRDC) initiated the Antimalarial Drug Development Program because resistant *P. falciparum* was endangering U.S. forces in Vietnam. This monumental program, coordinated through the Walter Reed Army Institute of Research (WRAIR), Washington, DC, has resulted in the primary screening of over 300,000 compounds. From these compounds emerged a number of amino alcohol blood schizontocides, most notably mefloquine, which are relatively safe and presently effective against resistant *P. falciparum*. Amazingly, even though mefloquine is not yet in general use, resistant cases have been observed in Thailand [109] and in Tanzania [110]. Thus, after 20 years of development, and while still in field trials, this cream of the blood schizontocidal crop is already under a cloud.

The Vietnam experience also stressed the need for a better radical curative drug than primaquine [111–114]. Accordingly, in 1968, the Army shifted its emphasis to the discovery of more effective, less toxic 8-aminoquinolines. In 1975, the Army's effort was bolstered by the initiation of the research program of the Scientific Working Group (SWG) on the Chemotherapy of Malaria (CHEMAL), organized by the UNDP/WORLD BANK/WHO Special Programme for Research and Training in Tropical Diseases (TDR) [115]. A third

source of funds, the U.S. Agency for International Development, has concentrated its support on vaccine development. In the interval since 1968, the USAMRDC has supported the synthesis of a great number of new aminoquinolines [116–145]. Another intensive effort to improve primaquine is under way in the People's Republic of China [146–159]. The USAMRDC program has produced new primaquine derivatives which, in animal models, combine low toxicity and a remarkable dual efficacy as blood and tissue schizontocides. This research is continuing.

METHODS FOR ANTIMALARIAL DRUG EVALUATION

The methods devised to screen candidate compounds for antimalarial activity have been described in a number of detailed reviews: avian [11, 12, 160–164], rodent [165–169], simian [170–173], human [174–176] and *in vitro* [177–180].

An early avian model, *P. relictum* in canaries, led to the development of pamaquine [38]. Other avian plasmodia, *P. gallinaceum* in chicks and *P. lophurae* in ducklings, were used for the mass screening of potential antimalarials in the World War II programme and helped in the development of pentaquine [11, 12]. Although the avian plasmodia had provided some valuable information, they are quite different from mammalian species and they ultimately fell into disrepute as uncertain predictors of drug effects in humans.

A major advance occurred with the isolation, in 1948, of a rodent plasmodium, *P. berghei*, in a wild African rat [181]. This parasite readily infected laboratory rats and mice and was soon the basis for a number of more reliable, standardized tests [182–185]. One of the tests, the Rane screen, [185] has been used by the USAMRDC as a primary screen for over 300000 candidate antimalarials and has generated a number of promising new leads [29]. Another important stride was the demonstration, by Schmidt in 1947, that infection with sporozoites of *P. cynomolgi* in rhesus monkeys is the biological and chemotherapeutic counterpart of similarly induced infections with *P. vivax* in humans [24, 62, 186]. It was the *P. cynomolgi*-rhesus screen which pointed to primaquine as a superior weapon against *P. vivax* [187]. The subsequent discovery that the South American owl monkey (*Aotus trivirgatus*) and the squirrel monkey (*Samiri sciureus*) were susceptible to *P. falciparum* and *P. vivax* made it possible to carry out *in vivo* preclinical studies on human malaria [188–190]. However, the price and scarcity of monkeys have stimulated the search for *in vitro* screens which may eventually eliminate the need for preclinical testing in animals [177–180].

With antimalarials, as with all drugs designed for human use, the ultimate

screening animal is man. From 1917 to 1939, antimalarials were screened in volunteers and in patients with neurosyphilis who received therapeutic malaria for the beneficial effect of the induced fever [191]. The advent of penicillin caused a drastic drop in the number of neurosyphilitics and minimized the utility of malariotherapy as a screening device. Since 1939, clinical trials have been carried out in volunteers; particularly helpful during World War II and the conflicts in Korea and Vietnam were prison inmates and military personnel [11, 19, 62, 192–196]. Public concern caused the discontinuation of the prison programme in 1975 and clinical trials are now conducted with paid civilian volunteers.

The screens most relevant to the work discussed in this chapter are the Rane blood schizontocidal test (*P. berghei*-mouse) [185] and the Schmidt radical curative test (*P. cynomolgi*-rhesus) [23, 197, 198]; these are the preclinical screens most commonly used by the U.S. Army Antimalarial Drug Development Program. None of the promising new 8-aminoquinolines has reached the clinical stage.

RANE BLOOD SCHIZONTOCIDAL SCREEN (*P. BERGHEI* INFECTION IN MICE)

This simple model has served as the principal primary screening test for blood schizontocidal activity since 1964. It benefits from the genetic homogeneity of laboratory mice, their resistance to interfering bacterial and viral pathogens, their small body size, ready availability, ease of handling and low cost [199]. The Rane screen is highly reproducible and responds to a broad spectrum of chemical classes. It is based on a comparison of responses by groups of treated and control mice, five in each group, after infection with *P. berghei* KBG 173. Utilizing young ICR/HA Swiss mice and a standard inoculum of *P. berghei*, it is possible to produce a uniform disease fatal to 100% of untreated animals, within 6 to 8 days, with a mean survival time of 6.2 days. Test animals of approximately the same age and weight are housed in metal-topped plastic cages, given a standard laboratory diet and water *ad libitum*. The animals receive an intraperitoneal injection of 0.5 ml of 1 : 100 dilution of heparinized heart's blood with a minimum of 90% parasitized cells (4×10^7 cells), drawn from donor mice infected 1 week earlier with *P. berghei*. The donor strain is maintained by weekly passages in separate groups of mice inoculated with 0.5 ml of 1 : 500 dilution of heparinized heart's blood. Test compounds are administered after dissolution or suspension in peanut oil. A single dose is given subcutaneously 72 h after the mice are infected with *P. berghei*. At this time a 10–15% parasitemia has developed; the disease is well established, but has not produced sufficient debility to alter the response of the host to any toxic effects

of the test compound. Since treatment is withheld for 3 days and death occurs in untreated controls within 6–8 days, this system presents a candidate drug with the maximum challenge. In order to check factors such as changes in the infectivity of *P. berghei* or in the susceptibility of the host or to detect technical errors, a group of infected animals treated with pyrimethamine at dose levels producing definite increases in survival time is included as a positive control in every experiment. In each experiment, test compounds are administered in graded doses; 640, 160 and 40 mg/kg in the first series and, if activity warrants, 640, 320, 160, 80, 40 and 20 mg/kg in the second series. If activity is still evident, the dose is halved, stepwise, until activity disappears. With highly active compounds, increases in dose levels are usually followed by increases in the survival time of the treated mice. However, if an active drug is toxic for the host, its toxicity may become a limiting factor; a continued increase in dose levels also increases the toxic effects and may result in the diminution of survival times. Deaths prior to the 6th day, when untreated controls begin to die, are regarded as non-parasitic and become the basis for toxicity evaluation. A mean survival time twice that of the controls is evidence of activity and after 60 days survivors are considered cured. Toxic deaths and 60-day survivors are not included in calculating mean survival time.

SCHMIDT RADICAL CURATIVE SCREEN (*P. CYNOMOLGI* INFECTION IN RHESUS MONKEYS)

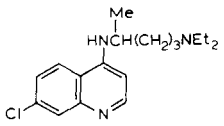
As noted earlier, sporozoite-induced cynomolgi malaria in rhesus monkeys (*Macaca mulatta*) is much like a vivax infection in man. *P. cynomolgi*'s persistent tissue forms, resembling human hypnozoites, make it an excellent model for the evaluation of candidate radical curative drugs. Well-conditioned Indian rhesus monkeys of either sex, weighing 2–4 kg, are utilized. *P. cynomolgi* (Bastianelli strain) sporozoites are prepared by grinding heavily infected anopheles balabacensis salivary glands in 1 : 1 monkey serum-saline vehicle. Monkeys are infected by i.v. injection of 10^6 freshly isolated *P. cynomolgi* sporozoites on day 0. A rapidly rising parasitemia develops after a 7–9-day prepatent period, and administration of the test drug is initiated when the rising parasite count exceeds 5000 per mm^3 (typically day 10–12). Test drugs are administered orally, by nasogastric intubation, once daily for 7 consecutive days in aqueous solution or, if insoluble, in suspension in 0.3% methylcellulose solution. Chloroquine diphosphate (3.1 mg/kg base orally per day) is always administered concurrently with the test drug for 7 days to eliminate blood schizonts. Thus, any tissue schizontocidal activity of the test drug will always be apparent even if it lacks blood schizontocidal activity. A vehicle control monkey and a positive

drug control (primaquine) monkey are included in each group of inoculated monkeys. The effect of the test drug is determined by counting blood parasites. Parasite counts are made daily through day 20 and every 2 days thereafter. Initially, a clearance of blood parasites is observed due to the blood schizontocidal action of chloroquine. If exoerythrocytic parasites (tissue schizonts) survive the action of the test drug, because it is inactive or marginally active, there will be a relapse of blood parasites. If there is no relapse within 20 days of the initial clearance of parasitemia, parasitemia is followed for an additional 80 days. If there is no relapse within this period, the experiment is terminated and the monkey is considered cured. Primaquine diphosphate cures 90% of monkeys in this test system when administered at a dose of 1.3 mg/kg per day for 7 days (1.0 mg/kg free base) in combination with chloroquine.

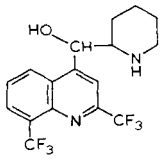
In an early version of this screen, at the Southern Research Institute in Birmingham, Alabama, Schmidt used set drug doses of 10, 1, 0.75, 0.5, 0.25, 0.125 and 0.0625 mg/kg. A subsequent version directed by John Brown and Frank Chapple of the SEATO Medical Research Group, Bangkok, Thailand, used doses of 10, 3.16, 1.0, 0.316 and 0.1 mg/kg. The same doses are at present being used by M.M. Dhar in the screen's most recent home in the Central Drug Research Institute, Lucknow, India.

STRUCTURE–ACTIVITY RELATIONSHIPS

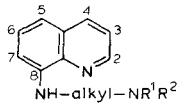
Primaquine was itself the culmination of more than a half century of intensive research in the U.S.A., the United Kingdom, Germany, France and Russia. The determination of the U.S. Army Medical Research and Development Command to improve on primaquine therefore presented a daunting challenge. Fortunately, the earlier avian work [11, 12] had provided a useful pad from which to launch the new programme. Despite the multiplicity of avian models and the differences between avian and mammalian malarias, it was cautiously assumed that among the 8-(aminoalkylamino)quinolines (10):



chloroquine



mefloquine



(10)

(a) A methoxy group at position 6 would elevate both activity and toxicity; 2-, 4- and 5-methoxy groups would be less beneficial than the 6-methoxy group.

(b) The hydrogen, hydroxy, ethoxy, *n*-butoxy and hydroxyethoxy groups would be less effective at position 6 than the methoxy group.
(c) The 2,6- and 5,6-dimethoxyquinolines and 5,6-methylenedioxyquinoline would be less active and less toxic than the corresponding 6-methoxy derivative, with toxicity decreasing more than activity for an increase in therapeutic index.
(d) A 5-phenoxy group would reduce toxicity much more than activity, thus producing a high therapeutic index; a 5-phenyl group would eliminate activity.
(e) One or more halogens at positions 3, 4 or 6 would diminish activity and toxicity.
(f) The 5-, 6- and 7-methyl groups would contribute little of value; the 4-methyl group would diminish toxicity without affecting activity; a pair of methyls at positions 2 and 4 would have about the same effect as a single methyl at position 4.
(g) An amino or anilino group at position 5 of a 6-methoxyquinoline would reduce toxicity more than activity.
(h) A 4-methylthio group or a 4-benzyl group would reduce activity and toxicity.
(i) Saturation of the quinoline ring would reduce efficacy.
(j) A primary terminal amino group in the side-chain would be less toxic than a secondary or tertiary amino group; the aromatic 8-amino group should be secondary.
(k) The number of methylene groups separating the 8-amino nitrogen atom and the terminal nitrogen atom of the side-chain should be greater than three to avoid the severe irreversible neurotoxicity of antimalarials like rhodoquine (plasmocid); the optimal number of methylenes between nitrogen atoms would probably be 4–6.

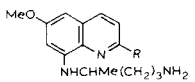
On this tentative basis and with the support of the new mammalian screens, major work on the design and synthesis of improved 8-aminoquinoline antimalarials was undertaken in 1968, by the American teams of Blanton (University of Georgia, Athens, GA), Carroll (Research Triangle Institute, Research Triangle Park, NC), LaMontagne (Ash Stevens, Inc., Detroit, MI) and Nodiff (Franklin Research Center, Philadelphia, PA). Additional synthetic contributions were provided by Archer (Rensselaer Polytechnic Institute, Troy, NY), Cheng (Midwest Research Institute, Kansas City, MO), Klayman (Walter Reed Army Institute of Research, Washington, DC) and Werbel (Warner-Lambert/ Parke-Davis, Ann Arbor, MI). During the past decade a number of Chinese investigators have also entered the field (Institute of Parasitology and the Second Military Medical College, Shanghai; Military Academy of Medical

Sciences, Beijing). The new programme placed primary emphasis on 8-aminoquinolines in which the 6-methoxy group and the primaquine side-chain were held constant while the nature, number and position of ring substituents were varied. Extensive synthesis of side-chain variants of primaquine, in earlier programs, had produced none with a better therapeutic index than primaquine itself.

2-SUBSTITUTED ANALOGUES OF PRIMAQUINE

Blanton and co-workers assumed that the activity of the 8-aminoquinolines stemmed from their metabolic conversion to the labile 5,6-quinones and that these quinones could be stabilized by appropriate substitution at position 2 of the quinoline nucleus [122]. This group therefore embarked on a program which produced compounds (11)–(25) in *Table 1.1.* Also included in *Table 1.1* are derivatives prepared in the laboratories of the Franklin Research Center (26) [140] and the Second Military Medical College, Shanghai (27–37) [151].

Table 1.1. 2-SUBSTITUTED ANALOGUES OF PRIMAQUINE



(11) - (37)

Compound No.	*R*	*Compound No.*	*R*
11	$PhCH_2O$	25	Me_2N
12	$4\text{-}FC_6H_4CH_2O$	26	Me
13	$4\text{-}ClC_6H_4CH_2O$	27	$3\text{-}ClC_6H_4CH_2O$
14	$2,4\text{-}Cl_2C_6H_3CH_2O$	28	$3,4\text{-}Cl_2C_6H_3CH_2O$
15	$3\text{-}CF_3C_6H_4CH_2O$	29	$4\text{-}BrC_6H_4CH_2O$
16	$4\text{-}CF_3C_6H_4CH_2O$	30	PhO
17	$4\text{-}MeOC_6H_4CH_2O$	31	$4\text{-}FC_6H_4O$
18	$PhCH_2S$	32	$4\text{-}ClC_6H_4O$
19	$4\text{-}ClC_6H_4CH_2S$	33	$2,4,5\text{-}Cl_3C_6H_2O$
20	MeO	34	$4\text{-}BrC_6H_4O$
21	Cl	35	$4\text{-}MeC_6H_4O$
22	Et	36	piperidino
23	$CH_2 = CH$	37	pyrrolidino
24	NH_2		

In the Rane blood schizontocidal screen (*P. berghei*, mouse), the Blanton group's lead compound (11) was slightly active and non-toxic at doses up to 640 mg/kg [122]; primaquine was toxic at 160 mg/kg in the same screen. On learning that (11) was also active in the radical curative screen (*P. cynomolgi*; rhesus), Blanton's group extended its work to include the substituted benzyloxy derivatives (12)–(17) and their sulphur isosteres (18, 19) [119]. All of the new compounds had toxic doses greater than 640 mg/kg in the Rane screen, but only (12), (14) and (19) had even marginal activity in the same model. In the radical curative screen, the best of the new analogues, namely, the 4-fluoro (12) and 2,4-dichloro (14) derivatives were slightly less potent than primaquine. A final attempt to optimize this series provided Blanton with compounds (20)–(25) [118]. Unfortunately, the methoxy (20), chloro (21), vinyl (23) and dimethylamino (25) derivatives had lost all of primaquine's radical curative activity while retaining its toxicity; no data were available for the amine (24). The ethyl derivative (22), a weak, non-toxic blood schizontocide, was also inactive in the radical model. Addition of a 2-Me group to primaquine to give (26) resulted in a small increase in radical curative activity and an attenuation of acute toxicity (1/5 toxic deaths (*T*) at 320 mg/kg vs. 2/5*T* at 160 mg/kg for primaquine). Stimulated by this work, Xu's group prepared compounds (27)–(37) and resynthesized (11) and (13) [151]. The most effective member of the Chinese series, the phenyl ether (30) was only as effective as primaquine for the radical cure of cynomolgi malaria in the monkey. Thus, introduction of a lone substituent at position 2 of primaquine offers, at best, a small improvement in therapeutic index for the radical cure of malaria.

3-SUBSTITUTED ANALOGUES OF PRIMAQUINE

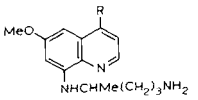
There is a dearth of information on primaquine with a single substituent at position 3. 3-Methylprimaquine [140] is a little more toxic than primaquine (1/5*T* at 80 mg/kg vs. 2/5*T* at 160 mg/kg) and considerably more toxic than 2-methylprimaquine (1/5*T* at 320 mg/kg) [140]; it has insignificant activity in the blood schizontocidal screen and is slightly better than primaquine in the tissue schizontocidal model (2/2 cures (*C*) at 1.0 mg/kg vs. 1/2*C* for primaquine at the same dose). 3-Methylprimaquine is a powerful causal prophylactic in the mouse model [23].

4-SUBSTITUTED ANALOGUES OF PRIMAQUINE

4-Methylprimaquine ((38); *Table 1.2*), a promising antimalarial originally prepared by Elderfield, *et al.* in the World War II programme [80] was resynthe-

sized by Nodiff's group for evaluation in the new mammalian screens. This analogue was somewhat superior to primaquine as a radical curative and blood schizontocidal agent and less acutely toxic in mice [140]. However, the subacute oral toxicity of 4-methylprimaquine in dogs and monkeys was prohibitively greater than that of primaquine [200] and work on (38) was discontinued. In an effort to improve the therapeutic index of (38), additional 4-monosubstituted primaquines (*Table 1.2*) were prepared by Carroll *et al.* [128]

Table 1.2. 4-SUBSTITUTED ANALOGUES OF PRIMAQUINE



(38) – (66)

Compound No.	*R*	*Compound No.*	*R*
38	Me	53	MeO
39	Et	54	MeS
40	$CH_2 = CH$	55	NH_2
41	Pr	56	CH_3CONH
42	Bu	57	MeNH
43	$EtCH(Me)CH_2$	58	OH
44	$C_6H_{11}CH_2$	59	$4\text{-}ClC_6H_4O$
45	$C_6H_{11}(CH_2)_3$	60	$4\text{-}MeOC_6H_4O$
46	$MeCH = CH$	61	$3,4\text{-}Cl_2C_6H_3O$
47	$EtCH = CH$	62	$3\text{-}CF_3C_6H_4O$
48	$C_6H_{11}CH = CH$	63	$4\text{-}ClC_6H_4NH$
49	*cis*-$MeCH = CH$	64	$4\text{-}ClC_6H_4S$
50	$4\text{-}FC_6H_4SCH_2$	65	$4\text{-}MeOC_6H_4S$
51	$4\text{-}ClC_6H_4S(CH_2)_2$	66	$4\text{-}ClC_6H_4CH_2O$
52	$4\text{-}MeOC_6H_4S(CH_2)_2$		

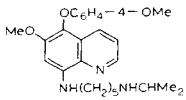
(39–52), and LaMontagne *et al.* [139] (53–66). Of moderate interest among these compounds were (39), (40) and (59). In the blood schizontocidal screen, the ethyl (39) and vinyl (40) derivatives were slightly less active than primaquine at doses up to 80 mg/kg and were less toxic than primaquine at the higher doses. The ethyl derivative, the better of the two, was non-toxic over the range 20–640 mg/kg and it was 40% and 100% curative at 320 mg/kg and 640 mg/kg, respectively. In the radical curative test, the ethyl derivative was as active as primaquine and the vinyl compound was slightly less active. The

4-ClC_6H_4O derivative (59) was 60% curative in the Rane screen at 160 mg/kg and 100% curative at 320–640 mg/kg. However, in contrast to the ethyl (39) and vinyl (40) analogues, the phenoxy derivative (59) was devoid of radical curative activity at the highest dose tested (10 mg/kg).

It now seemed clear that monosubstitution on the pyridine ring of primaquine would, at best, attenuate acute toxicity without the desired concomitant enhancement of radical curative activity. Additional work at positions 2, 3 and 4 was therefore held in abeyance.

5-SUBSTITUTED ANALOGUES OF PRIMAQUINE

The most effective 8-aminoquinoline to emerge from the earlier avian compilation [12] was the 5-aryloxy derivative (67). This compound had a thera-

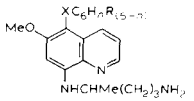


(67)

peutic index of 177 compared with 57 for pentaquine (5) and 30 for primaquine (8). With (67) as a lead, Nodiff's group prepared the various 5-phenoxy-, 5-phenylthio- and 5-anilinoprimaquines (68–88) [142, 144, 145] shown in *Table 1.3*. Prompted by this work, Zheng *et al.* resynthesized (69), (70), (71) and (74) and added the new analogues, (89)–(92) [159]; Xu *et al.* resynthesized (68), (69), (70), (73), (89) and (90) and introduced (93)–(98) [149, 156].

All of the compounds in *Table 1.3* were less toxic than primaquine in the murine blood schizontocidal screen. With the exception of (72), none produced acute lethality at the highest dose tested (640 mg/kg); (72) caused a single death at this dose. The most active blood schizontocides in *Table 1.3* were the fluorine-containing derivatives, 4-fluoro (70) and 3-trifluoromethyl (71), which were completely curative at 320 and 640 mg/kg, respectively. The compound containing both 4-F and 3-CF_3 (77) was less active (2/5*C* at 640 mg/kg) than either of the single substituted derivatives. The 4-MeO derivative (73), closely related to the lead compound (67), was almost equipotent (4/5*C* at 640 mg/kg) with (71). The best of these compounds in the radical curative test (70), (71) and (77) were slightly more active than primaquine. The most interesting compound among the Chinese contributions was the resynthesized analogue (70). Zheng *et al.* [159] reported that, against *P. yoelii* in the mouse, (70) was 20-times less toxic and 4–5-times less effective than primaquine. According to

Table 1.3. 5-PHENOXY, 5-PHENYLTHIO AND 5-ANILINO ANALOGUES OF PRIMAQUINE



(68) - (98)

Compound No.	*X*	*R*	*Compound No.*	*X*	*R*
68	O	H	84	S	3,4-Cl_2
69	O	4-Cl	85	S	2,5-Cl_2
70	O	4-F	86	S	4-MeO
71	O	3-CF_3	87	S	3-CF_3
72	O	4-CF_3O	88	S	3,4-benzo
73	O	4-MeO	89	O	4-Br
74	O	2,4-Cl_2	90	O	4-Me
75	O	3,4-Cl_2	91	O	4-I
76	O	3,5-$(CF_3)_2$	92	O	2-Cl
77	O	4-F-3-CF_3	93	O	3-F
78	O	4-MeCONH	94	O	3-Cl
79	NH	4-Cl	95	O	3-Br
80	NH	3-CF_3	96	O	3-Me
81	S	2-Cl	97	O	3,4-Me_2
82	S	3-Cl	98	O	3,5-Me_2
83	S	4-Cl			

Zheng *et al.* the fluorophenoxy (70) was also less effective than primaquine against *P. cynomolgi* in the monkey.

All of the anilino and phenylthio derivatives (79)–(88) were nontoxic and inactive or very weakly active at 640 mg/kg in the blood schizontocidal screen. In the radical curative screen, the entire phenylthio series (81)–(88) was curative at 10 mg/kg but only the 4-chloro (83) and 3-trifluoromethyl (87) compounds were curative at 1 mg/kg. The anilino derivative (80) was inactive even at 10 mg/kg. Thus, acute toxicity of primaquine can be diminished by introduction of various phenoxy, phenylthio or anilino groups at position 5. The phenylthio and anilino analogues were consistently less active than their oxygen isosteres, but all three series were unimpressive. There is a notable difference between the 4- and 5-phenoxyprimaquines. While the 5-phenoxy derivatives showed moderate radical curative activity in the monkey, the corresponding 4-phenoxy derivatives were inactive at the highest dose tested. In contrast, the

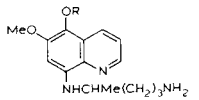
4-phenoxy derivatives were more potent blood schizontocides than their 5-phenoxy counterparts. Neither group was active in the mouse prophylactic screen.

The lead compound (67) was resynthesized by Kalidas and Blanton [120]. It is ironic that this compound, which had seemed so promising in the avian model and which had inspired so much additional research, was a total failure in the mouse and monkey screens.

5-Methoxyprimaquine (99) had a similar radical curative activity to primaquine and was slightly less toxic; it lacked blood schizontocidal activity. With continuing interest in substitution at position 5, Nodiff's group prepared the higher homologues of (99) shown in *Table 1.4* [140, 141]. The best combination of blood schizontocidal activity and nontoxicity occurred in the *n*-decyl derivative (107). However, this compound was relatively unimpressive with only two cures at 640 mg/kg. On moving from R = decyl down through the homologous series, acute toxicity gradually increased until, when R was butyl or smaller, toxicity was greater than that for primaquine itself. The toxicity increase at the higher doses (80–640 mg/kg) was not accompanied by any elevation in activity at the lower end of the dosage range. With the exception of the *n*-decyloxy derivative (107), which was radically curative only at 10 mg/kg, all of the compounds in *Table 1.4* were completely curative at 1.0 mg/kg. Among these, the propoxy (102), butoxy (103) and pentoxy (104) derivatives were also curative at 0.316 mg/kg, making them somewhat more active than primaquine (1/2*C* at 1.0 mg/kg).

At this juncture, the 5-phenoxyprimaquines seemed more promising than their excessively toxic 5-alkoxy analogues.

Table 1.4. 5-ALKOXY DERIVATIVES OF PRIMAQUINE



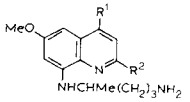
(99) – (107)

Compound No.	*R*	*Compound No.*	*R*
99	Me	104	n-C_5H_{11}
100	Et	105	n-C_6H_{13}
101	CF_3CH_2	106	n-C_8H_{17}
102	n-Pr	107	n-$C_{10}H_{21}$
103	n-Bu		

2,4-DISUBSTITUTED ANALOGUES OF PRIMAQUINE

Recognizing the limited ability of a single substituent to improve primaquine's therapeutic index, various investigators introduced more than one. The 2,4-disubstituted analogues (108)–(110), prepared by Carroll *et al.* [127] and shown in *Table 1.5*, were less toxic and less active than primaquine in the blood schizontocidal screen (no cures or toxic deaths at 640 mg/kg). Primaquine and (108) were equally active as tissue schizontocides.

Table 1.5. 2,4-DISUBSTITUTED ANALOGUES OF PRIMAQUINE

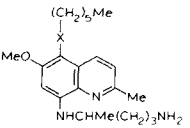


(108) – (110)

Compound No.	R^1	R^2
108	Me	Et
109	Me	$CH_2 = CH$
110	Et	Me

2,5-DISUBSTITUTED ANALOGUES OF PRIMAQUINE

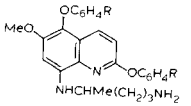
Nodiff and co-workers found that the 2-methyl derivative of 5-(*n*-hexyloxy)-primaquine (111) had slightly greater blood schizontocidal activity than its parent compound (105), at doses from 20 to 160 mg/kg (3*C* vs. 1*C* at 160 mg/kg) and was more toxic at 320–640 mg/kg (3*T* vs. 1*T* at 640 mg/kg). The radical curative difference between (105) and (111) was slight [140]. It is of interest that 5-(*n*-hexylthio)-2-methylprimaquine (112), the sulphur isostere of (111), was a somewhat better blood schizontocide than (111) with 3*C* at 160 mg/kg and 5*C* at 320–640 mg/kg (unpublished data).



(111) X = O
(112) X = S

Xu and Xu [149] and Zheng and Cheng [152] prepared the series of 2,5-disubstituted primaquines (113–119) shown in *Table 1.6*. The fluorine derivative (113) showed some activity against sporozoite-induced infection by *P. yoelii* in mice.

Table 1.6. 2,5-DISUBSTITUTED ANALOGUES OF PRIMAQUINE



(113) - (119)

Compound No.	*R*	*Compound No.*	*R*
113	4-F	117	4-Me
114	4-Br	118	4-MeO
115	4-Cl	119	H
116	3-CF_3		

4,5-DISUBSTITUTED AND 2,4,5-TRISUBSTITUTED ANALOGUES OF PRIMAQUINE

After the extensive, above-detailed exploration of peripheral veins by various groups, the teams of Nodiff and LaMontagne seemed to strike the mother lode (or guiding principle). These investigators found that addition of a 4-methyl group to unimpressive 5-aryloxy- or 5-alkoxyprimaquines produced dramatic antimalarial enhancement. As detailed below, some of these 4-methyl compounds are unique in their combination of very low acute toxicity and wide-ranging activity as blood schizontocides, radical curative drugs and causal prophylactics.

4-Alkyl-5-(aryloxy)primaquines

Table 1.7 includes various members of this series prepared by LaMontagne *et al.* (120)–(124) [134, 136], Nodiff *et al.* (125)–(131) [142], (129)–(131) (unpublished data), Carroll *et al.* (132)–(137) [125], Deng *et al.* (138)–(140) [153] and Wang and Xu (141) [150]. Deng resynthesized (120), (121) and (123) and Wang resynthesized (124). Blood schizontocidal and radical curative data for the most effective compounds in this series are compared (in *Table 1.8*) with

Table 1.7. 4-ALKYL-5-(ARYLOXY)PRIMAQUINES[a]

(120) - (141a)

No.	R^1	R^2	*No.*	R^1	R^2
120[b]	3-CF_3	4-Me	132	3-CF_3	4-Et
121	2,4-Cl_2	4-Me	133	3-CF_3	4-CH_2 = CH
122	3,4-Cl_2	4-Me	134	3-CF_3	4-CH_2OH
123	4-MeO	4-Me	135	3-CF_3	4-$MeOCH_2$
124	4-F	4-Me	136	3-CF_3	4-$(MeO)_2CH$
125	H	4-Me	137	3-CF_3	4-(3-$CF_3C_6H_4OCH_2$)
126	4-F-3CF_3	4-Me	138	4-Me	4-Me
127	4-F-3CF_3	3-Me	139	4-Me_3C	4-Me
128	3,5-$(CF_3)_2$	4-Me	140	2,4,5-Cl_3	4-Me
129	4-EtO	4-Me	141	3-F	4-Me
130	4-(*n*-C_5H_{11})	4-Me	141a[c]	3-CF_3	4-Me
131	4-PhO	4-Me			

[a] In compounds (120)–(141), R^3 = H; in compound (141a), R^3 = MeO.
[b] WR-225,448.
[c] WR-238,605.

corresponding data for primaquine. All of the 4,5-disubstituted derivatives in *Table 1.8* were considerably less toxic than primaquine and much more active in both the blood schizontocidal and radical curative screens. The 3-CF_3 and 4-F derivatives (120, 124) were equally active in the blood screen (multiple *C* at 10 mg/kg) and in the radical screen (2/2*C* at 0.316 and 1.0 mg/kg; no *C* at 0.1 mg/kg) and displayed little toxic lethality below 640 mg/kg. The 3,4-Cl_2 analogue (122) was a more effective, less toxic blood schizontocide (80% curative at 5 mg/kg; 100% curative at 10–640 mg/kg) than (120, WR-225,448) or (124) but it was slightly less active than these compounds in the radical model (1/2*C* at 0.316 mg/kg). The phenyl-unsubstituted derivative (125) was more toxic than the other members of this series (3/5*T* at 320–640 mg/kg) but it was the only one to effect any radical cures at the low dose of 0.1 mg/kg. Most of the high blood schizontocidal and radical curative activities and the low toxicity of (120) were retained when its 4-methyl group was replaced by an ethyl (132) or methoxymethyl (135) group. However, replacement of the methyl of (120) with a CH_2 = CH (133), CH_2OH (134), $(MeO)_2CH$ (136) or

Table 1.8. ANTIMALARIAL ACTIVITY AND TOXICITY OF 4-METHYL-5-(ARYLOXY)-PRIMAQUINES
See formula in *Table 1.7.*

		Blood schizontocidal activity[a] P. berghei, *mouse; dose, mg/kg; cures (C)*[b], *toxic deaths (T)*[c] *or* ΔMST[d]								*Radical curative activity*[e] P. cynomolgi, *rhesus; dose, mg/kg; cures/No. of animals*		
No.	R^1	*5*	*10*	*20*	*40*	*80*	*160*	*320*	*640*	*0.1*	*0.316*	*1.0*
(8) (Primaquine)				4.0	5.0	9.4	2*T*	5*T*	5*T*	0/2	0/2	1/2
120[f]	3-CF_3	6.9	2*C*	5*C*	5*C*	5*C*	5*C*	5*C*	1*C*, 4*T*	0/2	2/2	2/2
122	3,4-Cl_2	4*C*	5*C*	5*C*	5*C*	5*C*	5*C*	5*C*	5*C*	0/3	1/2	3/3
123	4-MeO	5.5	5.9	1*C*	5*C*	5*C*	5*C*	5*C*	2*C*, 3*T*	0/2	2/2	3/3
124	4-F	7.0	3*C*	4*C*	5*C*	5*C*	5*C*	4*C*, 1*T*	3*C*, 2*T*	0/3	2/2	3/3
125	H		8.7	4*C*	3*C*	4*C*	4*C*	3*T*	3*T*	1/2	1/1	2/2

[a] Activities were determined at the Rane Laboratory, University of Miami, Florida, with five mice per group, via the method of Osdene *et al.* [185].
[b] The number of mice surviving at 60 days post-infection.
[c] Deaths prior to the 6th day.
[d] Increase in mean survival time over controls (MST of control group, 6.1 days).
[e] Tests were carried out by the SEATO Medical Research Laboratory, Bangkok and the Central Drug Research Institute, Lucknow, according to the procedure of Schmidt *et al.* [197].
[f] R^2 = 4-Me and R^3 = H for compounds (120), (122)–(125).

3-$CF_3C_6H_4OCH_2$ (137) banished radical curative activity and either eliminated or severely depressed blood schizontocidal activity.

On the basis of blood and tissue data, (120) was judged the most promising member of the series and was selected by the USAMRDC for additional preclinical evaluation [134, 136]. In a repetition of the Rane blood schizontocidal test, which used more than the usual five mice at several concentrations, (120) effected 10/10*C* at 160 mg/kg and 20 mg/kg and 15/15*C* at 40 mg/kg but only 2/10*C* (4 toxic deaths) at 640 mg/kg. Primaquine and (120) were also compared for suppressive activity against *P. cynomolgi* in the rhesus. This test differs from the radical curative test in that parasitemia is induced by intravenous inoculation of parasitized blood rather than by sporozoites and that chloroquine is not co-administered with the test drug [201]. Primaquine was suppressive but did not completely clear blood schizonts at doses from 1.0 to 31.6 mg/kg per day (× 7); (120) was 100% curative at 1.0 mg/kg per day (× 7) and was suppressive but not curative at doses as low as 0.0316 mg/kg.

As mentioned earlier, the toxicity of primaquine precludes administration of a single radically curative dose; in order to achieve radical cure of *P. vivax* in man, multiple doses are required coupled with the blood schizontocide, chloroquine. Accordingly the radical curative screen (*P. cynomolgi* – rhesus) was modified to establish whether a single dose of (120) was effective as both a tissue and blood schizontocide and whether the co-adminstration of chloroquine was necessary. This test showed that primaquine was effective in two of four monkeys at 3.5 mg/kg (× 1) but permitted 2/2 relapses at 1.75 mg/kg; its fully curative dose was 14 mg/kg (× 1). Primaquine and (120) were about equally active at 3.5 mg/kg without chloroquine, but in the presence of chloroquine the fully curative dose of (120) dropped to 0.875 mg/kg (× 1) compared to 14 mg/kg (× 1) for primaquine; (120) permitted 4/4 relapses at 0.4375 mg/kg. Acute toxicity evaluation in rats and guinea-pigs indicated that (120) was less toxic than primaquine by factors of 1.5–3.2.

Causal prophylaxis, originally attributed to residual blood schizontocidal activity, was confirmed in the Most model [25, 183]. Against a trophozoite-induced vivax infection in the *Aotus trivirigatus* monkey, (120) was 100% curative at a total dose of 12 mg/kg, while primaquine was non-curative even when the total dose was raised to 160 mg/kg [201].

However, the elation elicited by the accumulating positive data for (120) was short-lived. In subacute (60 day) studies with rats, (120) was more toxic than primaquine. Clinical chemistry alterations and tissue lesions were induced by (120) which were not seen when primaquine was administered at equal doses [25]. Of particular concern was the haematotoxicity of (120). A general deficiency of the 8-aminoquinolines is their tendency to produce methaemoglobinemia [202–210]. This condition is characterized by a concentration of methaemoglobin (MHb) in erythrocytes which is greater than the usual 1 to 2%. In methaemoglobin, the normal ferrous iron has been oxidized to the ferric state and is incapable of transporting oxygen. Methaemoglobinemia can thus lead to anoxia and death. According to Anders *et al.* [211], (120) induced in dogs a peak methaemoglobin level which was 4-times higher than that produced by primaquine (25.3% vs. 6.3%; *Table 1.9*).

Seeking to improve (120), LaMontagne *et al.* returned to the old avian literature and Mislow's report that a 2-methoxy substituent reduced the toxicity of primaquine [64]. This led to the synthesis of (141a, WR-238,605), the 2-methoxy derivative of (120) (*Table 1.9*) [132]. Compound (141a) had better blood and radical curative activities than its 2-demethoxy parent with no change in acute toxicity but with a third less methaemoglobin induction (16.0% vs. 25.3%; *Table 1.9*) [132, 133, 211]. It was also significantly less toxic than (120) in the rat either by oral or intraperitoneal administration [132, 133].

Table 1.9. EFFECT OF THE 2-METHOXY GROUP ON THE ACTIVITY, ACUTE TOXICITY AND METHAEMOGLOBIN INDUCTION OF (120)
See formula in Table 1.7.

	Blood schizontocidal activity[a] P. berghei, *mouse; dose, mg/kg; cures (C)*[b], *toxic deaths (T)*[c] *or ΔMST*[d]						*Radical curative activity*[e] P. cynomolgi, *rhesus; dose, mg/kg; cures/No. of animals*			
No.	*20*	*40*	*80*	*160*	*320*	*640*	*0.1*	*0.316*	*1.0*	*Peak % MHb*[f]
(8) (Primaquine)	4.0	5.0	9.4	2*T*	5*T*	5*T*	0/2*C*	0/2*C*	1/2*C*	6.3
(120)[g]	1*C*	4*C*	5*C*	5*C*	5*C*	4*C*, 1*T*	0/2*C*	2/2*C*	2/2*C*	25.3
(141a)	3*C*	5*C*	5*C*	5*C*	5*C*	4*C*, 1*T*	2/4*C*	4/4*C*	2/2*C*	16.0

[a-e] See *Table 1.8*.

[f] Anders *et al.* [211] performed these evaluations on beagle dogs at the Walter Reed Army Institute of Research, Washington, DC.

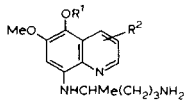
[g] The data reported by LaMontagne for (120), for this comparison [132], were somewhat different from those reported by LaMontagne for the same compound in an earlier study (Cpd (120) in *Table 1.8*) [136].

Against trophozoite-induced infections of the Chesson strain of *P. vivax* in the *Aotus* monkey, (141a) was slightly better than (120) (2/2*C* vs. 1/3*C* at 1 mg/kg × 3) but it was somewhat less effective than (120) as a causal prophylactic against sporozoite-induced *P. berghei yoelii* in the mouse (0/5*C* vs. 6/15*C* at 2.5 mg/kg, subcutaneously) [133]. Replacement of the 2-methoxy of (141a) with Cl, OH, 4-ClC_6H_4O or 4-$ClC_6H_4CH_2O$ gave compounds which were inactive and non-toxic in the Rane screen and were not examined further [132, 133]. Compound (141a) is at present completing advanced preclinical evaluation.

4-Alkyl-5-(alkoxy)primaquines

Concurrently with their research on the 4-Alkyl-5-(aryloxy)primaquines, Nodiff and co-workers prepared the substituted 5-(alkoxy)primaquines included in *Table 1.10* (142–156, 158–170) [140, 141]. Additional analogues were synthesized by LaMontagne *et al.* (157, 171–174) [137] and Carroll *et al.* (175, 176) [124]. R^1 in *Table 1.10* encompasses *n*-alkyl, isoalkyl, cycloalkyl, alkenyl, cycloalkylmethyl, phenoxyalkyl, alkoxyalkyl, benzyloxyalkyl and phenylalkyl groups; work on the phenylalkoxy series (167)–(170) has not yet been published.

Table 1.10. 4-METHYL-5-(ALKOXY)PRIMAQUINES



(142) – (176)

No.	R^1	R^2	*No.*	R^1	R^2
142	*n*-Pr	4-Me	159	$PhO(CH_2)_6$	4-Me
143	*n*-Bu	4-Me	160	$PhO(CH_2)_8$	4-Me
144	*n*-C_5H_{11}	4-Me	161	$C_6H_{11}CH_2$	4-Me
145	*n*-C_6H_{13}	4-Me	162	Cyclopentyl	4-Me
146	*n*-C_7H_{15}	4-Me	163	$CH_2=CH(CH_2)_2$	4-Me
147	*n*-C_8H_{17}	4-Me	164	$Me(CH_2)_3O(CH_2)_2$	4-Me
148	*n*-C_9H_{19}	4-Me	165	$MeO(CH_2)_6$	4-Me
149	*n*-$C_{10}H_{21}$	4-Me	166	$PhCH_2O(CH_2)_6$	4-Me
150	*n*-$C_{11}H_{23}$	4-Me	167	$Ph(CH_2)_4$	4-Me
151	*n*-$C_{12}H_{25}$	4-Me	168	$Ph(CH_2)_5$	4-Me
152	*n*-C_6H_{13}	3-Me	169	$Ph(CH_2)_6$	4-Me
153	*n*-C_6H_{13}	2-Me	170	$Ph(CH_2)_7$	4-Me
154	2-C_6H_{13}	3-Me	171	Me	4-Me
155	3-C_6H_{13}	3-Me	172	Me	2,4-Me_2
156	*n*-C_6H_{13}	2,4-Me_2	173	5,6-OCH_2O–	4-Me
157	*n*-C_6H_{13}	2-MeO,4-Me	174	5,6-$O(CH_2)_2O$–	4-Me
158	$PhO(CH_2)_4$	4-Me	175	Me	4-$CH_2=CH$
			176	Me	4-(*m*-CF_3PhOCH_2)

Among these compounds, introduction of a 4-methyl group to the shorter chain (C_3–C_8) 5-alkoxyprimaquines (142–147) generally produced a pronounced blood schizontocidal increase in the lower part of the dosage range and elevated acute toxicity at the higher doses (160–640 mg/kg). There was a progressive diminution in toxicity with increased chain length; the C_3 derivative (142) caused three toxic deaths at 40 mg/kg, while the C_8 derivative (147) caused only two deaths at 640 mg/kg. The long-chain members of the series (C_9–C_{12}; 148–151) were responsible for no toxic deaths even at 640 mg/kg. Among the latter group, activity was inversely related to chain length, with the nonyl derivative (148) producing 2/5*C* at 10 mg/kg while its dodecyl homologue (151) only provided multiple cures (4/5*C*) at 160 mg/kg. The most promising members of this series were 4-methyl-5-(*n*-pentoxy)primaquine (144) and 4-methyl-5-(*n*-hexyloxy)primaquine (145). Both of these compounds were curative in the Rane screen at the extremely low dose of 2.5 mg/kg. Their activity in the radical curative screen was also extraordinary, with the pentoxy

compound (144) curing 3/4 monkeys at 0.1 mg/kg and its hexoxy analogue (145, WR-242,511) curing 5/5 monkeys at the same low dose. Compound (145) also displayed potent activity against *P. berghei yoelii* in the causal prophylactic screen, achieving 5/5*C* at 10 and 40 mg/kg (subcutaneously), 3/5*C* at 2.5 mg/kg and 1/5*C* at 0.63 mg/kg.

Unfortunately, both (144) and (145) were about as acutely toxic as primaquine in the upper half of the dosage range of the Rane screen. Furthermore, the hexoxy derivative, (145) induced a worrisome methaemoglobin level in the dog (48.1% vs. 25.3% for (120) and 16.0% for (141a); *Table 1.9* [211]. Despite these drawbacks, the USAMRDC still considers (145) a viable antimalarial candidate because of its excellent blood schizontocidal activity (5/5*C* at 20 mg/kg) and its unrivaled low-dose efficacy in the radical curative screen (5/5*C* at 0.1 mg/kg). The hexyl ether (145) is at present undergoing intensive preclinical evaluation.

An effort to elevate the therapeutic index of (145), while retaining its dual activity, led to the synthesis of analogues (152)–(176) (*Table 1.10*) and permitted the following structure–activity correlations among the 5-(alkoxy)-primaquines.

Blood schizontocidal activity

(a) The 2-Me and 3-Me groups were almost equivalent and had a lesser effect on activity and toxicity than the 4-Me group (153 and 152 vs. 145).
(b) Paired methyl groups at positions 2 and 4 contributed more to toxicity and less to activity than a single 4-methyl group (156 vs. 145).
(c) Primaquines bearing a branched-chain alkoxy at position 5 were more toxic than their straight-chain isomer (154 and 155 vs. 152).
(d) A 4-methylprimaquine with a 5-(unsaturated alkoxy) was less active and toxic than the corresponding saturated compound (163 vs. 143).
(e) A 5-cycloalkoxy contributed less to activity and toxicity than the corresponding acyclic group (162 vs. 144).
(f) Replacement of a terminal hydrogen, on a 5-alkoxy group, with a phenoxy moiety had little effect on the activity or toxicity of the resulting primaquine (158–160 vs. 143, 145, 147).
(g) Replacement of a terminal hydrogen, on a 5-alkoxy group, with an alkoxy diminished activity and increased toxicity (165 vs. 145).
(h) The 5,6-bridged derivatives were either totally inactive (173) or highly toxic (174).
(i) Surprisingly toxic, with 2/5*T* at 20 mg/kg, was the benzyloxyhexyloxy derivative (166). This suggested the possibility that at least part of the

toxicity of the 5-(alkoxy)primaquines stemmed from metabolic oxidation of the terminal methyl to a hydroxymethyl. With this in mind, Nodiff's team protected the terminal methyl with the phenyl group producing the (167)–(170) series (unpublished work). In each case, introduction of a phenyl group caused a striking reduction in toxicity with little or no decrease in activity. Comparative data for the 4-demethyl-5-(alkoxy)primaquines and the corresponding 4-methyl-5-(alkoxy) and 4-methyl-5-(phenylalkoxy) primaquines are grouped in *Table 1.11*. It should be noted that the 5-(phenylheptyloxy) derivative (170), with 5/5*C* at 10–640 mg/kg and 2*C* at 5 mg/kg, is considerably more effective than mefloquine, the best of the new wave of blood schizontocides, which is curative at 20 mg/kg [212].

(j) As described earlier, LaMontagne *et al.* were able to upgrade a 4-methyl-5-(aryloxy)primaquine (120) to (141a); (*Table 1.9*) by inserting an MeO group at position 2 [132]. An attempt to enhance (145) in a similar fashion was unsuccessful (157 vs. 145, *Table 1.11*) [132]. The corresponding 5-(phenylalkoxy) analogue (169, *Table 1.11*) was a better blood schizontocide than either (157) or (145).

Radical curative activity

(a) In moving through the homologous 4-methyl-5-(*n*-alkoxy)primaquines, from C_6 (145) to C_{12} (151), activity gradually decreased, with the C_{10} (149), C_{11} (150) and C_{12} (151) compounds non-curative at 1.0 mg/kg.

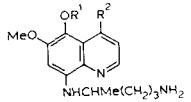
(b) The 2,4-Me_2 analogue (156) of (145) was only half as active as the parent compound (2/4*C* vs. 5/5*C* at 0.1 mg/kg) while the 3-Me (152) and 2-Me (153) analogues produced no cures at 0.1 mg/kg.

(c) The 5-(phenylpentoxy) derivative (168, WR-254,715) and the 5-(phenylhexyloxy) derivative (169) were 100% curative at 0.316 mg/kg (4/4*C*); (168) also effected 1/2*C* at 0.1 mg/kg.

(d) Among the few remaining compounds to achieve cures at 0.1 mg/kg were a 5-(phenoxyoctyloxy)primaquine (160) (1/2*C*), an analogue of (145) in which the 5-(hexyloxy) chain was interrupted by an oxygen atom (164) (1/3*C*) and the 2-MeO derivative (157) of (145) (1/4*C*).

It would thus seem that among the substituted 5-(alkoxy)primaquines, the best combination of low toxicity with high blood and tissue schizontocidal activity resides in 4-methyl-5-(phenylpentoxy)primaquine (168).

Table 1.11. COMPARISON OF ANTIMALARIAL ACTIVITY OF 5-ALKOXY AND 5-(PHENYLALKOXY)PRIMAQUINES



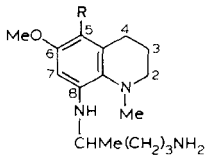
			Blood schizontocidal activity[a] P. berghei, *mouse; dose, mg/kg; cures (C)*[b], *toxic deaths (T)*[c] *or* ΔMST[d]									*Radical curative activity*[e] P. cynomolgi, *rhesus; dose, mg/kg; cures/No. of animals*	
No.	R^1	R^2	*2.5*	*5*	*10*	*20*	*40*	*80*	*160*	*320*	*640*	*0.1*	*0.316*	*1.0*
8	(Primaquine)					4.0	5.0	9.4	2*T*	5*T*	5*T*	0/2	0/2	1/2
38	(4-Me-Primaquine)					3.1	4.9	5.5	9.0	10.0	3*C*		0/4	2/2
103	$Me(CH_2)_3$	H			4.1	6.5	6.7	2*T*	3*T*		5*T*		2/4	2/2
143	$Me(CH_2)_3$	Me			11.0	5*C*	3*C*	4*T*	5*T*	5*T*	5*T*	0/2	2/2	1/1
167	$Ph(CH_2)_4$	Me		7.6	2*C*	4*C*	4*C*	5*C*	3*C*	0.4	0.0	0/3	1/2	4/4
104	$Me(CH_2)_4$	H			4.3	4.7	6.1	4.7	4*T*	5*T*	2*T*	0/2	1/2	1/1
144	$Me(CH_2)_4$	Me	2*C*	5*C*	5*C*	4*C*	1*C*	2*T*	4*T*	5*T*	5*T*	3/4		2/2
168	$Ph(CH_2)_5$	Me		7.9	3*C*	4*C*	5*C*	5*C*	5*C*	3*C*	1*C*	1/2	4/4	2/2
105	$Me(CH_2)_5$	H				3.7	6.5	1*C*	1*C*	0.1	1*T*		0/2	3/3
145	$Me(CH_2)_5$	Me	1*C*	1*C*	1*C*	5*C*	5*C*	4*C*	3*T*	5*T*	5*T*	5/5	3/3	1/1
169	$Ph(CH_2)_6$	Me		5.9	1*C*	3*C*	4*C*	4*C*	3*C*	3*C*	4*C*	0/2	4/4	2/2
157	$Me(CH_2)_5$	2-MeO-4-Me				10.5	2*C*	5*C*	4*C*	5*T*	5*T*	1/4	3/3	2/2
146	$Me(CH_2)_6$	Me	7.9	3*C*	3*C*	5*C*	4*C*	2*C*	1*T*	5*T*	5*T*	0/2	2/2	4/4
170	$Ph(CH_2)_7$	Me		2*C*	5*C*	5*C*	5*C*	5*C*	5*C*	5*C*	5*C*	0/2	0/4	3/3

[a-e] See *Table 1.8.*

MODIFICATION OF THE QUINOLINE HETEROCYCLE

Reduction of the pyridine ring

Encouraged by the high avian antimalarial activity of 8-(5-diethylamino-2-pentylamino)-6-methoxy-1,2,3,4-tetrahydroquinoline (tetrahydropamaquine) [11] Carroll *et al.* prepared the 1,2-dihydroprimaquines (177, 178) and the 1,2,3,4-tetrahydroprimaquine (179) [130]. Mouse and monkey screening data for (177)–(179) suggest that reduction of the pyridine ring in primaquine lessens or eliminates activity.



(177) R = H; 3,4-double bond
(178) R = MeO; 3,4-double bond
(179) R = H

Substitution of naphthalene for quinoline

Archer *et al.* found that several naphthalene analogues (180, 181) of primaquine showed little tissue schizontocidal activity in the *P. cynomolgi* screen [213].

(180) R = H
(181) R = Br

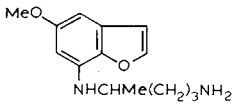
Substitution of acridine for quinoline

Klayman and co-workers synthesized acridine derivatives, which may be considered as a benzoprimaquine (182) and a 4-methylbenzoprimaquine (183) [214]. The demethyl derivative (182) was inactive as a blood schizontocide and curative in the radical model at 10 mg/kg. Both derivatives were ineffective at 3.16 mg/kg and were thus less active than primaquine.

(182) R = H
(183) R = Me

Substitution of benzofuran for quinoline

The benzofuranyl isostere of primaquine (184), synthesized by Johnson and Werbel [215], was ineffective as a blood schizontocide and was never evaluated in the *P. cynomolgi*-rhesus model.



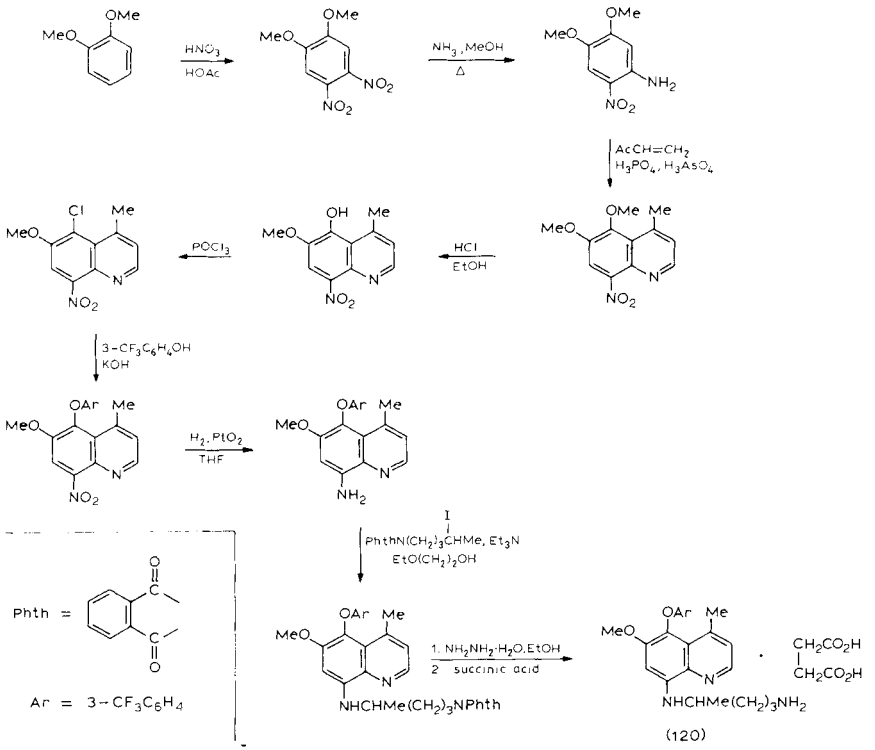
(184)

ENANTIOMERS OF PRIMAQUINE

Primaquine is used clinically as a racemic mixture. It was therefore of interest to compare the (+) and (−) enantiomers with the racemate. After Carroll *et al.* resolved the mixture [129], Schmidt *et al.* studied the comparative antimalarial activities and toxicities of the racemate and its enantiomers in mice and rhesus monkeys [216]. According to the latter authors the (+) enantiomer was 4-times more toxic than the (−) enantiomer in mice but 3–5-times less toxic than the (−) form in the rhesus. The radical curative activity of the racemate and its enantiomers against *P. cynomolgi*-rhesus was essentially the same. Assuming that the rhesus data were more predictive for man than the mouse data, Schmidt *et al.* concluded that the (+) form had a 2-fold advantage in therapeutic index over primaquine and suggested clinical evaluation of this enantiomer against *P. vivax*; this has yet to be done.

CHEMISTRY

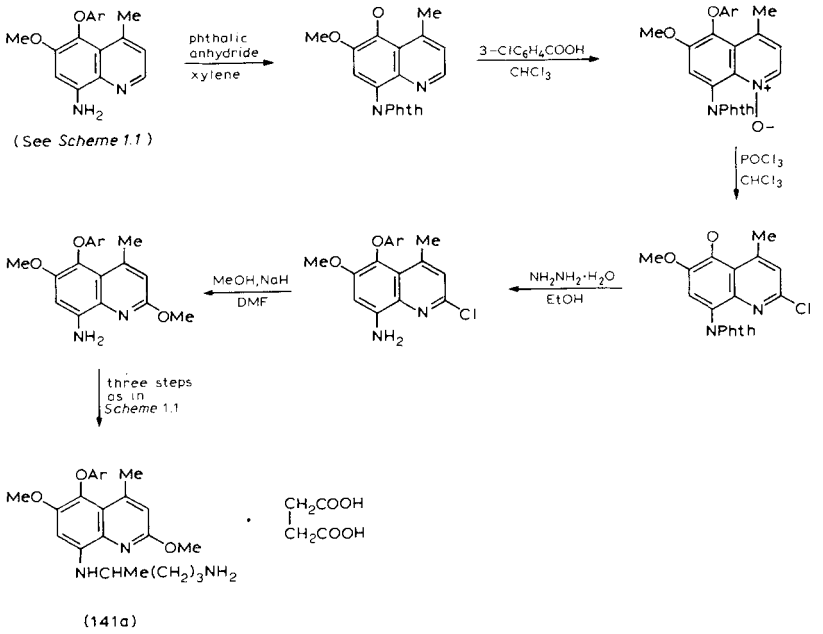
Typical syntheses are outlined in *Schemes 1.1–1.3.*



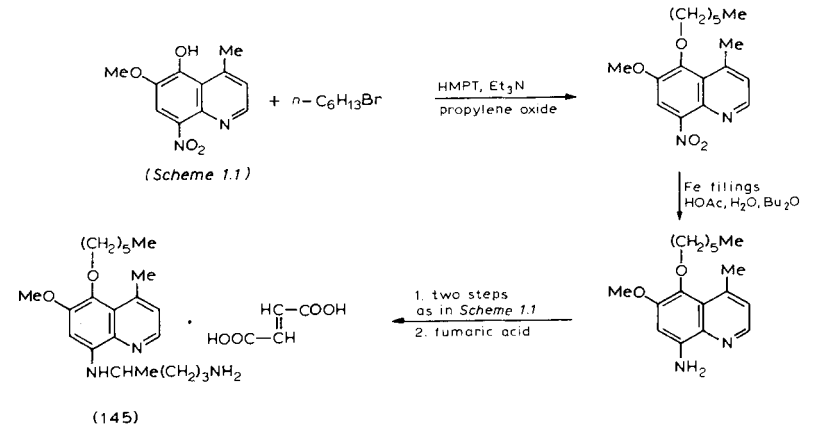
*Scheme 1.1 Compound (120) (*Tables 1.7–1.9*) [134, 136]. For Ar, Phth see lower left corner.*

CONCLUSION

Primaquine, the best available tissue schizontocide, suffers from a number of serious deficiencies. The concentrate of a 25-year search for an improved tissue schizontocide is the trio of new 8-aminoquinolines compared with primaquine in *Table 1.12*. All three are at least 10-times more effective than primaquine in the radical curative screen; (145), the best of the three in this screen, is 100% curative at 0.1 mg/kg. Quite unexpectedly, these compounds are also extremely effective blood schizontocides, with (168) providing multiple cures at 10 mg/kg. (The minimum curative dose for mefloquine, the best of the new blood schizontocides, is 20 mg/kg; mefloquine has no radically curative activity.) Despite some uneasiness over the tendency of these drugs to produce

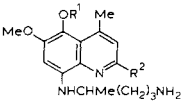


Scheme 1.2 Compound (141a) (Table 1.9) *[132, 133]. Abbreviations as in Scheme 1.1.*



Scheme 1.3 Compound (145) (Tables 1.10, 1.11) *[140, 141].*

Table 1.12. ANTIMALARIAL ACTIVITY, ACUTE TOXICITY AND METHAEMOGLOBIN INDUCTION OF COMPOUNDS (141a), (145), (168) AND PRIMAQUINE



			Blood schizontocidal activity[a] P. berghei, *mouse; dose, mg/kg; cures (C)*[b], *toxic deaths (T)*[c], *ΔMST*[d]							*Radical curative activity*[e] P. cynomolgi, *rhesus; dose, mg/kg; cures/No. of animals*			
No.	R^1	R^2	*10*	*20*	*40*	*80*	*160*	*320*	*640*	*0.1*	*0.316*	*1.0*	*Peak % MHb*[f]
(8)	(Primaquine)			4.0	5.0	9.4	2*T*	5*T*	5*T*	0/2	0/2	1/2	6.3
(141a)	3-$CF_3C_6H_4$	MeO		3*C*	5*C*	5*C*	5*C*	5*C*	4*C*, 1*T*	2/4	4/4	2/2	16.0
(145)	n-C_6H_{13}	H	1*C*	5*C*	5*C*	4*C*	3*T*	5*T*	5*T*	5/5	3/3	1/1	48.1
(168)	$Ph(CH_2)_5$	H	3*C*	4*C*	4*C*	5*C*	5*C*	3*C*	1*C*	1/2	4/4	2/2	

[a-f] See *Table 1.9.*

methaemoglobinemia, (141a) is about to enter Phase I clinical evaluation and an application is being prepared for IND approval of (145). Compound (168) with an excellent primary profile, is still awaiting methaemoglobin evaluation. These broad spectrum antimalarials offer the possibility of a single drug that would be effective against all of the relapsing and non-relapsing malarias.

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2 Clinically Useful Chelators of Tripositive Elements

R.C. HIDER, B.Sc. Ph.D. and A.D. HALL, B.Sc. Ph.D.

Department of Pharmacy, King's College, London University, Manresa Road, London, SW3 6LX, U.K.

Abbreviations: BP-IDA (62), tetrabromo-*o*-cresylphthalein; DCTA (or CDTA) (35), *trans*-1,2-cyclohexylenedinitrilotetraacetate; DFO (33), desferrioxamine B; DOTA (71), 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetate; DTPA (36), diethylenetrinitrilopentaacetate; DTPMP (66), diethylenetrinitrilopentamethylene phosphonate; EDDHA (58), ethylenedinitrilo-*N,N'*-bis(3-hydroxybenzyl)-*N,N'*-diacetate; EDTA (25), ethylenedinitrilotetraacetate; EDTMP (40), ethylenedinitrilotetramethylene phosphonate; EGTA (38), ethylenebis(oxyethylenenitrilo)tetraacetate; 4HBCY (47a), *N,N',N'',N'''*-tetra-(2-hydroxybenzyl)-1,4,8,11-tetraazacyclotetradecane; HBED (44a), *N,N'*-bis(2-hydroxybenzyl)ethylenedinitrilo-*N,N'*-diacetate; HEDP (70), 1-hydroxy-1,1-diphosphonatomethyl succinate; HEDTA (39), *N*-(2-hydroxyethyl)ethylenedinitrilo-*N,N',N''*-triacetate; HEEDTMP (68), *N*-(hydroxyethyl)ethylenedinitrilotrimethylene phosphonate; HMDTP (65), *N*-(hydroxymethyl)diethylenetrinitrilotetramethylene phosphonate; HPED (46), ethylenedinitrilo-*N-N'*-bis(2-hydroxyphenyl)-*N-N'*-diacetate; IDA (27), iminodiacetate; LICAM (55a), *N,N',N''*-tris(2,3-dihydroxybenzoyl)-1,5,10-triazadecane; LICAM-C (55c), *N,N',N''*-tris(4-carboxy-2,3-dihydroxybenzoyl)-1,5,10-triazadecane; 3,4-LICAMS (55b), *N,N',N''*-tris(5-sulpho-2,3-dihydroxybenzoyl)-1,5,10-triazadecane; MDP (53b), methylene diphosphonate; MECAM (31a, $R^1 = H$), 1,3,5-*N,N',N''*-(2,3-dihydroxybenzoylaminomethyl)-benzene; MECAMS (31a, $R^1 = SO_3^-$), 1,3,5-*N,N',N''*-(2,3-dihydroxy-5-sulphobenzoylaminomethyl)benzene; NBTP (69), norbornyl bis(ethylenenitrilodimethylene phosphonate); NTA (26), nitrilotriacetate; NTMP (54), nitrilotrimethylene phosphonate; PIH (57), pyridoxal isonicotinoyl hydrazone; PLED (45a), *N,N'*-bispyridoxylethylenediamine-*N,N'*-diacetate; TACN (48), 1,4,7-triazacyclononane; TA-TACN (48a), 1,4,7-tris(aceto)-1,4,7-triazacyclononane; TS-TACN (48c), 1,4,7-tris(2-mercaptoethyl)-1,4,7-triazacyclononane; TX-TACN (48b), 1,4,7-tris(3,5-dimethyl-2-hydroxybenzyl)-1,4,7-triazacyclononane; TETA (47b), 1,4,8,11-tetraazatetradecane 1,4,8,11-tetraacetate; TPPS (59), 5,10,15,20-tetra(4-sulpho)phenyl-21*H*,23*H*-porphine; TSPC (60), 29*H*,31*H*-tetrasulphophthalocyanine.

INTRODUCTION

Until relatively recently, the clinical use of chelator molecules has been synonymous with the removal of toxic metals, for instance, cadmium and lead [1]. However, with the realization of the central role of iron in so many physiological responses, specific ligands for iron are being widely investigated as potential therapeutic compounds [2,3]. The introduction of diagnostic investigations involving γ-scintigraphy (indium) [4], positron emission tomography (gallium) [5] and nuclear magnetic resonance (gadolinium) [6] has also necessitated the introduction of new chelating agents into medicine. In this review, the coordination chemistries of aluminium, iron, gallium, indium and lanthanum (as a model of gadolinium) are compared. The design and clinical application of selective ligands for each of these metals are discussed in detail. Lanthanum is a Group IIIB metal, aluminium, gallium and indium are Group IIIA metals and, as high spin iron(III) is also spherically symmetrical (*vide infra*), for the purposes of this review these metals will be referred to as Group III cations.

If chelators are to be used in the clinical situation, there are many restrictions on the chemical design of the molecule, in addition to that of selectivity. In general, both the free ligand and the complex should be water-soluble and yet,

if the role of the ligand is to scavenge a metal, it must also be able to penetrate cell membranes. Thus, the ligand should possess no charged functional groups at neutral pH values and the resulting complex should also be neutral, if it is to be capable of effluxing from cells. These properties, together with water solubility, impose considerable constraints on the molecular design [7]. Neutral ligands are likely to be orally active, but if their molecular weight is less than 400, they may also penetrate the blood-brain barrier and thereby trigger undesirable side-effects. Consequently, not only the net charge, but also the molecular weight of the ligand, have considerable influence on its distribution *in vivo*.

As a general rule, oligodentate ligands are more kinetically inert than their bidentate analogues and therefore scavenge metals more efficiently, without causing redistribution. With kinetically labile complexes, the metal can be donated to endogenous high-affinity binding sites, such as those located on the plasma protein apotransferrin. Indeed, if chelators are to be used as imaging agents, then it is essential that the metal complex be kinetically inert, in order to give a well-defined image. In contrast, if the metal complex is used for cell labelling, then the coordinated metal should be selectively donated to intracellular proteins. With these latter prerequisites, the complex should possess a degree of kinetic lability.

Such parameters as affinity constants, kinetic lability, permeability of membranes and interaction with apotransferrin, are systematically discussed for Group III metals in this review.

COMPARATIVE CHEMISTRY OF GROUP III CATIONS

Boron, aluminium, gallium, indium and thallium form Group IIIA of the periodic table. Boron, possessing such a small radius (0.3 Å), lacks cationic chemistry, and thallium readily forms Tl(I) rather than Tl(III) by virtue of the inert electron pair in its valence shell. Consequently, only aluminium, gallium and indium form stable tripositive cations under biological conditions. Group IIIB contains scandium, yttrium and the lanthanide series. Scandium and yttrium form stable tripositive cations with an inert gas configuration, and since the 4*f* electrons are relatively uninvolved in bonding, the lanthanides (which include samarium, europium, gadolinium and terbium) also favour the tripositive oxidation state. The coordination chemistry of scandium and yttrium has not been extensively investigated.

The electron configurations of these six tripositive cations are similar (*Table 2.1*). The *d*-orbitals of aluminium, scandium, yttrium and lanthanum are unoccupied and the *d*-orbitals of gallium and indium are completely filled. As a

Table 2.1 ATOMIC PROPERTIES OF SELECTED TRIVALENT CATIONS

	Electronic structure of M^{3+}	*Ionic radius of M^{3+} (Å)[8]*	*Coordination number of aquo M^{3+}*	*Charge density (e $Å^{-2}$)*	*Free energy of hydration G_f° (kJ mol^{-1})[9]*
Aluminium	[Ne]	0.54	6	0.82	−485
Gallium	[Ar]$3d^5$	0.62	6	0.62	−159
Iron	[Ar]$3d^{10}$	0.65(high spin)	6	0.56	−5
Scandium	[Ar]	0.75	6	0.42	−585
Indium	[Kr]$4d^{10}$	0.80	6	0.37	−98
Yttrium	[Kr]	0.90	6–9	0.29	−693
Gadolinium	[Xe]$5f^7$	0.94	6–9	0.27	–
Lanthanum	[Xe]	1.03	6–9	0.22	−683

result, these cations are spherically symmetrical and interaction with ligands is dominated by electrostatic forces. This contrasts with the coordination chemistry of most trivalent transition metal cations, for instance, cobalt(III) and chromium(III), which possess incompletely filled *d*-orbitals. In such cases, there are strong directional influences in the manner by which ligands coordinate the cations. The *d*-orbitals of iron(III) are half-filled and the cation can exist in either the high-spin ($t_{2g}^3 e_g^2$) or the low-spin (t_{2g}^5) state. Many iron(III) complexes coordinated by oxygen-containing ligands have been demonstrated to be high spin by ESR or X-ray diffraction [10–12]. In the high-spin form of iron(III), all *d*-orbitals are equally occupied and therefore the ion is spherically symmetrical. Thus, high-spin iron(III) is exceptional for transition metals, and possesses coordination properties similar to those of gallium(III). For the purposes of this review, iron(III) is considered as a Group III metal.

By virtue of their high charge density, Group III metals behave as Lewis acids and in a similar manner to protons, form most stable bonds with ligands containing weakly polarisable atoms such as oxygen. There is a good correlation between K_1 values (*Scheme 2.1*) of a wide range of gallium and iron(III)

$$
\begin{aligned}
LH &\rightleftharpoons L^- + H^+ \quad (K_a)\\
LH + Fe^{3+} &\rightleftharpoons [L\cdot Fe]^{2+} + H^+ \quad (K_1)\\
LH + [L\cdot Fe]^{2+} &\rightleftharpoons [L_2\cdot Fe]^{+} + H^+ \quad (K_2)\\
LH + [L_2\cdot Fe]^{+} &\rightleftharpoons [L_3\cdot Fe]^{o} + H^+ \quad (K_3)\\
3LH + 3Fe^{3+} &\rightleftharpoons [L_3\cdot Fe]^{o} + 3H^+ \quad (\beta_3)\\
&\beta_3 = K_1\cdot K_2\cdot K_3
\end{aligned}
$$

Scheme 2.1

complexes (*Figure 2.1*) [13], and a similar relationship exists for indium and iron(III) complexes [14]. Indeed, for many negatively charged oxygen donor ligands, there exists a good linear correlation between $\log K_1$ (^-OH) (*Figure 2.2*) for a wide range of metal ions [15]. This indicates that the affinity of a particular metal ion for an oxygen donor ligand is directly proportional to the affinity of that metal for ^-OH ions. The proportionality constant for such relationships depends only upon the basicity of the oxyanion donor atoms of the ligand [20]. These observations indicate that, for the complexes considered (mainly oxygen ligands), there is little contribution from ligand field stabilization to the stability of iron(III) complexes.

By virtue of the strong interaction between Group III cations and oxygen atoms, all members of the group bind water tightly (*Figure 2.3*), in contrast to either ammonia or hydrogen sulphide. The affinity for both water and OH^-

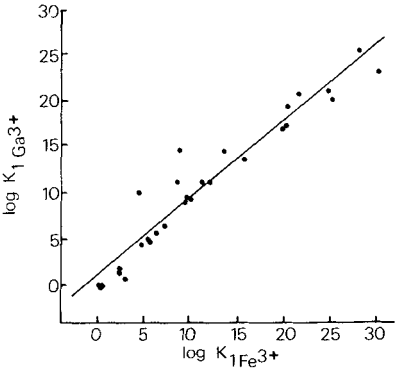


Figure 2.1. Correlation between log $\mathbf{K}_1$ *values of gallium(III) and iron(III) complexes.*

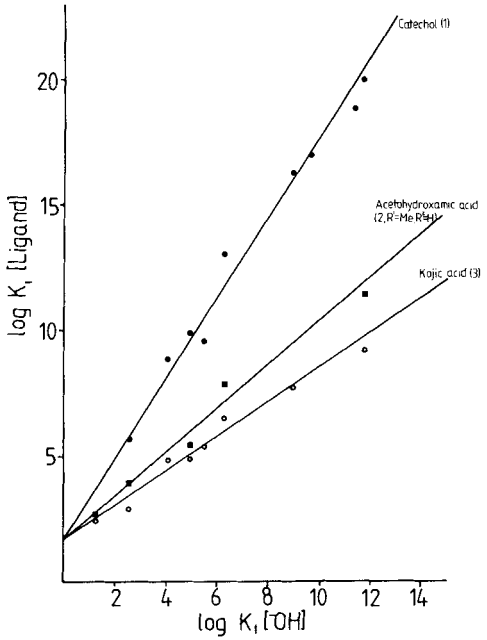
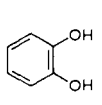
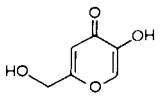


Figure 2.2. Relationship between log $\mathbf{K}_1$ *values for catechol (1), acetohydroxamic acid (2,* R^1 *= Me,* R^2 *= H) and kojic acid (3) ligands versus log* $\mathbf{K}_1$ *[*$^-$*OH] values for a range of metal ions. From [16–19]. The log* $\mathbf{K}_1$ *[*$^-$*OH] axis intercept of 1.75 is the theoretical value of the entropy contribution to the chelate effect.*



(1)

(2)



(3)

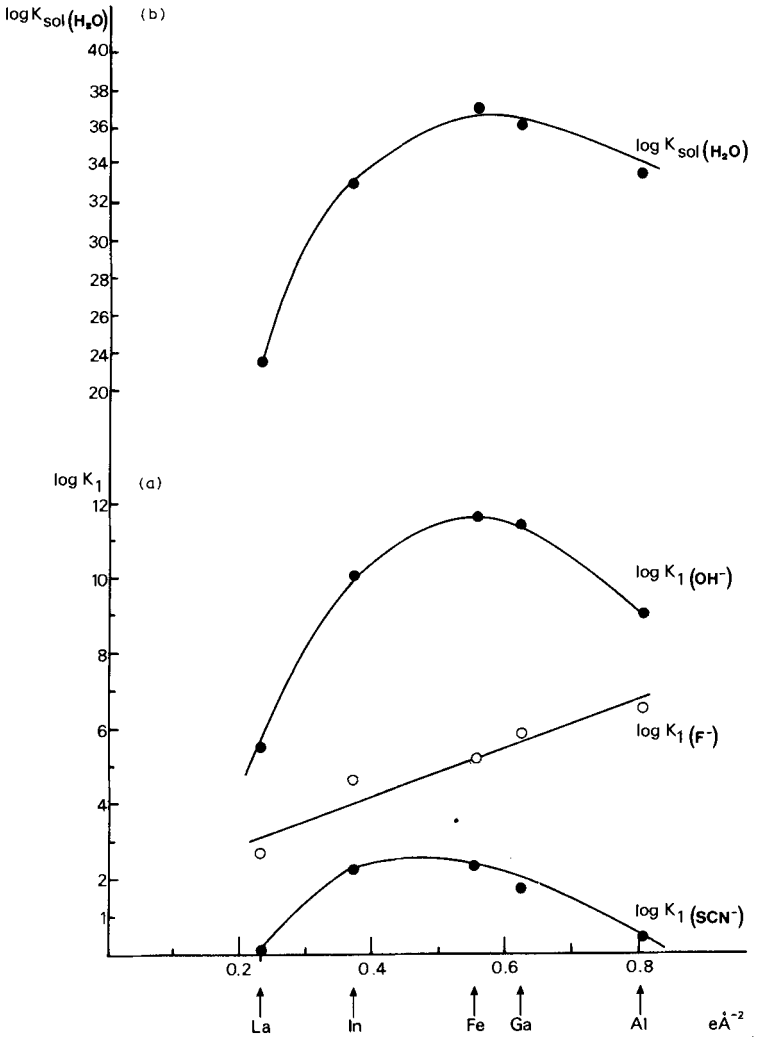


Figure 2.3. Relationship between charge density of M^{3+} cations and (a) log K_1 values of the monodentate ligands, ^-OH, ^-F and ^-SCN; (b) log K_{sol} H_2O. From [16–19].

increases with the charge density of the cation; thus $\log K_1 (OH^-)$ for lanthanum(III) is 5.5, whereas for iron(III) it is 11.8. The affinity of Al^{3+} for OH^- is lower than that of In^{3+}, Ga^{3+} and Fe^{3+}, despite the higher charge density of the cation (*Table 2.1*). This phenomenon, which is largely observed throughout the chelation chemistry of aluminium, is probably related to the difficulty of packing ligand atoms around the extremely small aluminium cation. This small size leads to short ligand–ligand distances and the introduction of associated repulsive forces. Significantly, the general reduction in affinity observed with aluminium is not found with the fluoride anion, which has a smaller ionic radius than oxygen (*Figure 2.3*). A cation of radius 0.58 Å will fit perfectly into a cavity formed from the symmetrical octahedral packing of 6 oxygen atoms of 1.4 Å radius. However, if the octahedron is distorted in a manner similar to that observed for bidentate oxygen-containing ligands, then the size of the cavity increases to a minimum radius of approximately 0.7 Å. Significantly, maximum affinities with water, OH^- and SCN^- are found with iron(III) which possesses a high spin radius of 0.65 Å (*Figure 2.3*). The smaller aluminium cation (r = 0.54 Å) will be unable to form aluminium-oxygen bonds of optimal length in an octahedral field and consequently will form complexes of lower stability.

The pK_a value of the dissociation reaction described by Eqn. (i) is dependent on how strongly the charged metal cation attracts electrons which bind the protons on the hydrate water molecules; the stronger the attraction, the lower the pK_a value.

$$M(H_2O)_6^{3+} + H_2O \overset{K_a}{\rightleftharpoons} M(OH)(H_2O)_5^{2+} + H_3O \qquad \text{(i)}$$

The entire range of values for metals in the periodic table is -2 to $+15$, and when plotted against the coulombic attraction between M^{n+} and OH^-, fall on four straight lines [21]. La^{3+} and Al^{3+} share the same line as Mg^{2+} and Ca^{2+}, and consequently possess comparable 'hardness' to the alkaline earth metals (*Figure 2.4*). In^{3+}, Ga^{3+} and Fe^{3+} share the same line as Na^+ and K^+ and hence are predicted to possess a 'hardness' similar to that of the alkali metals. Significantly, the chemistry of both the alkali and the alkaline earth metals is dominated by electrostatic interactions. Not surprisingly, therefore, the K_1 values for the soft ligand SCN^- are lower than those for OH^- and F^-, the difference being particularly marked for the hardest cations, lanthanum and aluminium (*Figure 2.3*).

All the Group III metals described in this review form hexa-aquo ions $[M(H_2O)_6]^{3+}$ in aqueous solution, these ions being acidic (Eqn. (i)) and extensively hydrolysed by water [22]. The products of hydrolysis are strongly depen-

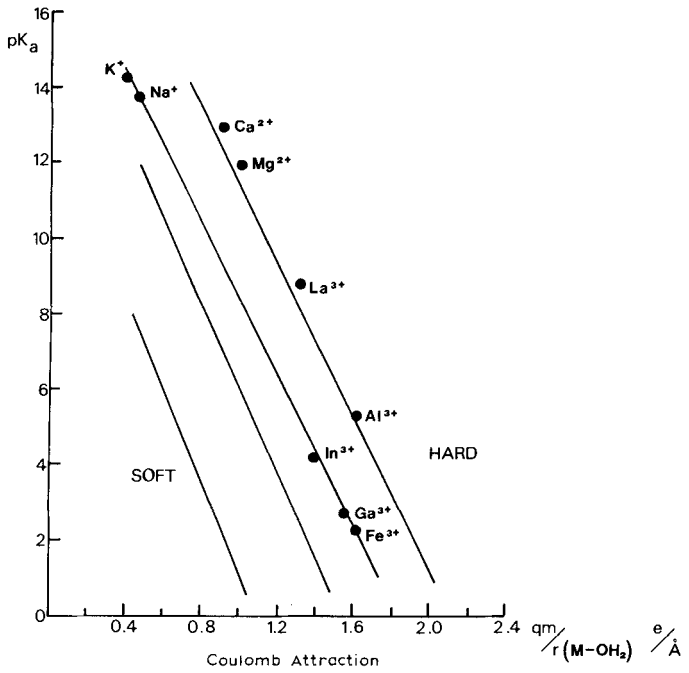
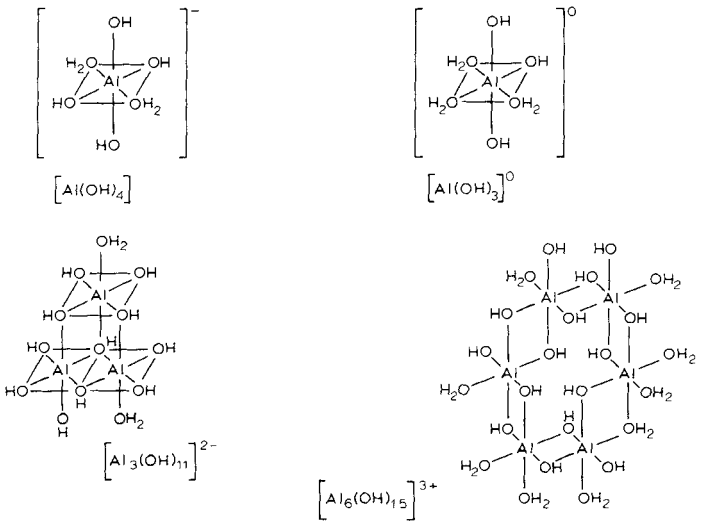


Figure 2.4. pK_a value for metal ion M^{n+} hydrolysis as a function of metal-ion attraction for proton binding electrons on M-OH_2 [21].

dent on the pH value and the metal concentration [23,24]. Thus for aluminium at pH 7.0, the species presented in *Scheme 2.2* are all present, although none exceeds 10^{-8} M [24]. A similar situation occurs with gallium and presumably with indium. Oligonuclear iron(III) oxides, as typified by $[Fe_6(OH)_{12}]^{6+}$, also form in the pH range 4.0–9.0 [25]. Although these structures are precursors to larger iron oxide particles and gels, iron-oligomer structures of the type indicated in *Scheme 2.2* are stabilized in the presence of polyols and carboxylates. It is important to realize that, although the concentration of $[Fe^{III}(H_2O)_6^{3+}]$ is in the region of 10^{-18} M at pH 7.0, the concentration of 'low molecular weight' oligomeric iron(III) is much higher, and may reach micromolar levels. Even the large lanthanum cation forms oligomeric species in aqueous systems [26]. Oligomeric species of aluminium and iron(III) are of considerable biological importance.

Aluminum species



Scheme 2.2

KINETIC LABILITY

Since electrostatic binding forces dominate the interaction between Group III cations and ligands, there is a general trend for those cations with a high charge-density to be less kinetically labile. This trend can be monitored by the water exchange reaction (Eqn. (ii)).

$$M(H_2O)_6^{3+} + H_2O \rightleftharpoons M(H_2O)_5(H_2O)^{3+} + H_2O \qquad \text{(ii)}$$

Although this reaction has no thermodynamic driving force, the rate constants vary over some 17 orders of magnitude [27]. For Group III cations, there is an excellent correlation between charge density of the cation and the rate of water exchange, the difference spanning 10^7 s^{-1} (*Figure 2.5*). Thus, aluminium complexes might be expected to be more kinetically inert than the corresponding complexes of indium and gallium. This is an important factor to be considered when studying metal complexes *in vivo*.

REDOX CHEMISTRY

Of the Group III cations considered in this review, only iron can exist in more than one redox state under physiological conditions. In general, the iron(III)

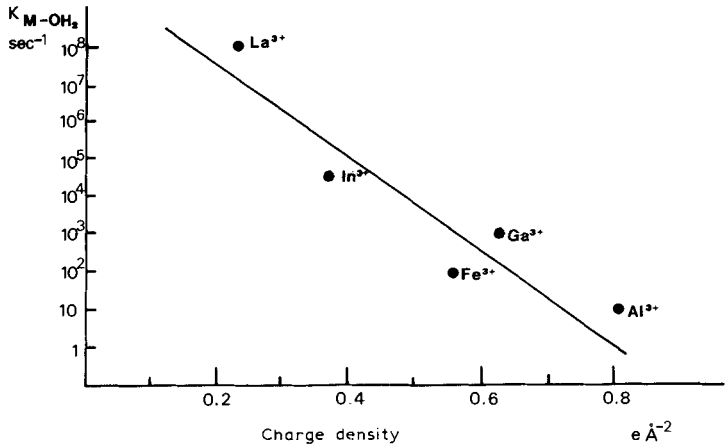


Figure 2.5. Rate constants for exchange of water molecules co-ordinated to M^{3+} cations in aqueous solution [22].

state is more stable under aerobic conditions, although iron(II) will be favoured by nitrogen- and sulphur-containing ligands. The ability of iron to exist in two redox states and also to bind oxygen has rendered the metal a critical status in living systems. Iron is the essential cofactor of numerous enzymes and oxygen-binding proteins. However, the redox activity of iron is also a potential source of toxicity [28,29]. Whereas the activity of iron bound to enzymes is controlled by the protein, iron nonspecifically bound to low-molecular-weight ligands is potentially toxic. A complicated series of reactions, involving the uncontrolled redox cycling of iron, can occur under aerobic conditions (*Scheme 2.3*). This leads to the production of hydrogen peroxide, superoxide and the hydroxyl radical. Of these, the hydroxyl radical is particularly toxic, reacting with most organic molecules with extremely high rate constants; it attacks cell membranes, proteins and nucleic acids.

$$Fe^{3+} + \text{Vit C(red)} \rightleftharpoons Fe^{2+} + \text{Vit C (ox)}$$
$$Fe^{2+} + O_2 \rightleftharpoons Fe^{3+} + O_2^-$$
$$2O_2^- + 2H^+ \rightleftharpoons H_2O_2 + O_2$$
$$Fe^{2+} + H_2O_2 \rightleftharpoons Fe^{3+} + \dot{O}H + OH^-$$
$$\text{Net effect } O_2 \longrightarrow \dot{O}H + H_2O_2$$

Scheme 2.3 O_2^-, superoxide anion; $\dot{O}H$, hydroxyl radical

Although there are protective enzymes such as superoxide dismutase and catalase, which reduce the rate of formation of hydroxyl radicals, these enzymes are unable to cope when iron levels begin to saturate the endogenous binding proteins, apotransferrin and ferritin. Under these conditions, toxicity associated with iron-induced oxygen radical production is initiated [30].

COORDINATION CHEMISTRY OF GROUP III CATIONS

Coordination of cations by ligands involves the displacement of the hydration sphere of water molecules (Eqn. iii).

$$M^{3+}(aq) + 3L^{-} \rightleftharpoons ML_3(aq) \qquad \text{(iii)}$$

The affinity constant of the metal M for the ligand is identical to the equilibrium constant for Eqn. (iii) and depends on the relative stabilities of the hydrated cation and the chelated cation. The stabilities of hydrated Group III cations (ΔG_f°) cover a wide range of values (*Table 2.1*), from iron(III), 5 kJ mol^{-1} to the lanthanide(III) ions > 650 kJ mol^{-1}. The high values for cations with relatively large radii are associated with the greater number of water molecules which are capable of interacting with the cation. The smaller cations, iron, gallium and indium, have correspondingly lower values, the exception being aluminium, which by virtue of its extremely high charge density, interacts more strongly with water molecules.

This range of values offers an explanation for the finding that, although the free energy of aluminium hydroxamates is greater than those of iron(III), the affinity constant is lower by a factor of 10^6 (Van der Helm, personal communication, 1989). The values of the free energy of hydration, (ΔG_f°), must be considered when designing cation selective ligands. The relationships represented in *Figure 2.2* suggest that for a ligand containing oxyanion donors, it will be difficult to alter the established selectivity order for different metal cations away from that imposed by the hydroxide ion affinity [31].

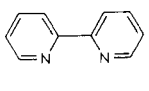
BIDENTATE LIGANDS

Aluminium, iron, gallium, indium and the lanthanides all interact with anions of high charge density, and in particular, oxyanions. Consequently, they have a relatively low affinity for dinitrogen, disulphur and mixed nitrogen-sulphur ligands in aqueous media. This contrasts markedly with the cobaltic ion, which forms a 3 : 1 complex with ethylenediamine of extreme stability ($\log\beta_3 = 49$). Cobalt(III) possesses the electronic structure [Ar]$3d^6$ and consequently lacks

the half-filled *d*-shell of iron(III). Indeed, 2,2′-bipyridyl (4) and 1,10-phenanthroline (5) are considered to be highly selective for iron(II) (electronic structure, $[Ar]3d^6$) over iron(III) (electronic structure, $[Ar]3d^5$). In contrast to these ligands, dioxygen and mixed oxygen-nitrogen and oxygen-sulphur ligands bind iron(III) in aqueous media. In general, compounds with two ligating oxygen atoms have been more widely studied than the other two classes, although 8-hydroxyquinoline (6) has been demonstrated to bind Group III cations tightly (*Figure 2.6*). The affinities of a range of bidentate ligands for the series lanthanum, indium and iron increases with increasing charge density on the cation. However, the trend levels at radii between 0.60–0.65 Å, the affinity of gallium being slightly lower than that of iron (see also *Figure 2.1*). Aluminium(III), has without exception lower $\log K_1$ values than either gallium(III) or iron(III), this trend almost certainly reflecting the relative ease of cation packing within the octahedral field provided by the oxygen atoms (cf. earlier discussion of aluminium coordination by monodentate ligands).

With most bidentate ligands, at least three Group III metal complexes are possible; namely ML, ML_2 and ML_3. A general scheme for a monoprotic ligand is presented in *Scheme 2.1*. The overall equilibrium constant, β_3, is the product of the three constants, K_1, K_2 and K_3. The relative proportions of these three complexes depends on the pH of solution. As protons and the cation compete with each other for the ligand, alkaline pH values favour ML_3 whereas acid pH values favour ML; the precise position of equilibrium depending on the pK_a and β_3 values. The ratio of metal to ligand can also influence the relative population of the three species; thus a high L : M ratio will shift the equilibria to the right-hand side and favour the formation of the ML_3 species. The above are important considerations for *in vivo* behaviour of bidentate ligands.

There is a clear relationship between the pK_a values of the ligands and the $\log K_1$ value for iron(III), again indicating the dominant influence of electrostatic interaction between iron(III) and the ligating atoms (*Figure 2.7*). Thus,



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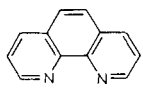

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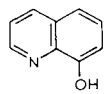
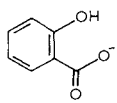


(6)

(7)



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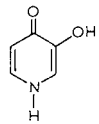

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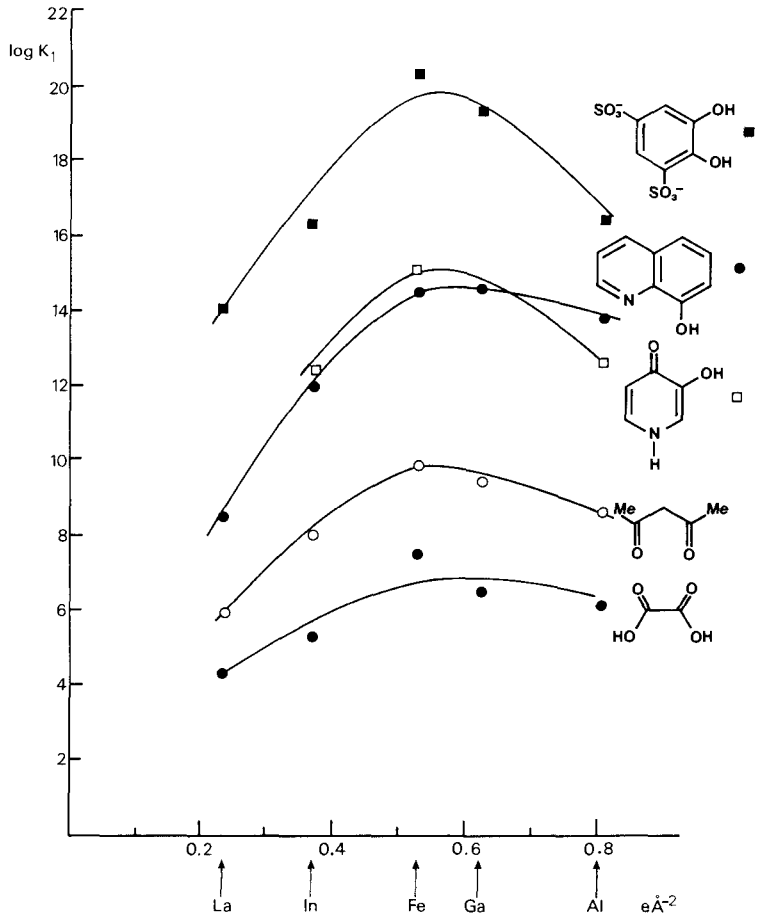


Figure 2.6. Relationship between charge density of M^{3+} cations and log K_1 values of various bidentate ligands. From [16]–[19].

dioxygen ligands with relatively high pK_a values would appear to be ideal ligands for this type of cation, typical examples being catechol (1), hydroxamate (2), tropolone (7), salicylate (8) and 3-hydroxypyridin-4-one (9). Conversely, lactate, which is sometimes used as a ligand for Group III cations, has a low pK_a value (3.8) and consequently is an extremely poor complexing agent (log K_1 for Fe(III), 2.9). Indeed, it has a lower affinity that the monodentate acetate (log K_1 for Fe(III), 3.4).

Although the bidentate ligands identified in *Figure 2.6* possess a high affinity

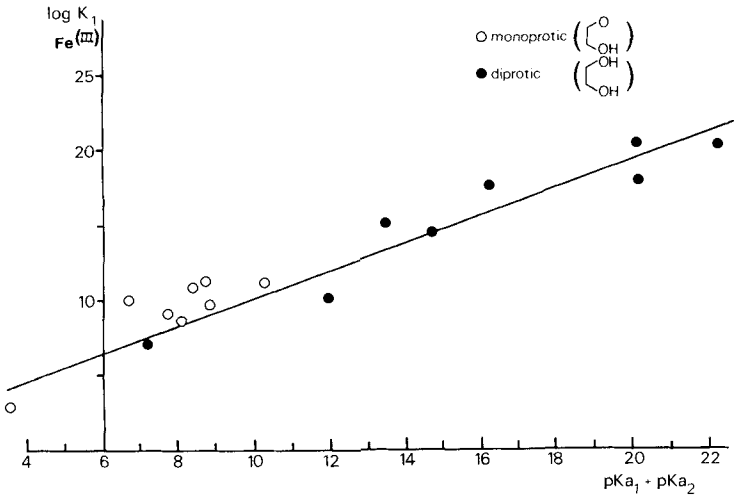


Figure 2.7. Relationship between log K_1 *values for iron(III) complexes with* $\Sigma\ pK_{a1}$ *and* pK_{a2} *for a range of mono- and diprotic oxygen containing bidentate ligands. From [16–19].*

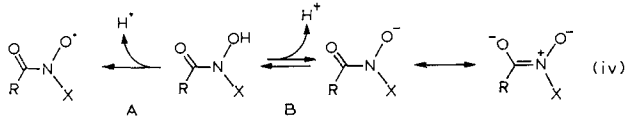
for Group III cations, they also bind divalent transition metals (*Table 2.2*). Of these five classes, the only mixed oxygen-nitrogen ligand, 8-hydroxyquinoline (6), has a lower selectivity. These observations provide a powerful indication as to why microorganisms have selected hydroxamate and catechol functions for siderophore construction [32].

Table 2.2 AFFINITY CONSTANTS (K_1) OF SELECTED BIDENTATE LIGANDS

	log K_1				
	Fe(III)	*Cu(II)*	*Zn(II)*	*Ca(II)*	*Fe(III)/Cu(II)*
3,5-Disulphonatocatechol	20.4	13.3	9.4	4.4	1.53
8-Hydroxyquinoline (6)	14.5	12.6	8.6	3.3	1.16
3-Hydroxypyridin-4-one (15)	15.0	9.4	6.7	–	1.60
Oxalate	7.5	4.8	3.9	1.7	1.56
Acetohydroxamic acid ($2,R^1 = Me,R^2 = H$)	11.4	7.9	5.4	2.4	1.45

Hydroxamate

The hydroxamate moiety (2) is a chelating function which has been widely adopted by bacteria and fungi for siderophore construction [32]. The trend of Group III log K_1 values for hydroxamate is similar to those presented in *Figure 2.6*, namely La^{3+}, 5.2; Fe^{3+}, 11.4 and Al^{3+}, 8.0 [33]. Although these values are much lower than the corresponding values for catechols (*Figure 2.6*), only one oxygen is protonated under physiological conditions (pK_a = 9.35) and therefore competition with protons is less marked. The high electron density on both ligating oxygen atoms results from delocalization of the nitrogen lone pair (Eqn. iv). Indeed, the electron density of the carbonyl oxygen can be further

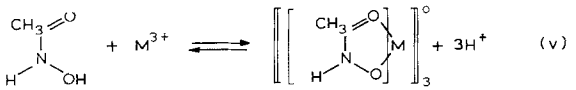


(iv)

enhanced by delocalization of electrons from conjugated side-chains (10). This results in an increase in affinity for cations, and is a strategy used in siderophore design, for instance with mycobactin and some ferrichrome and rhodotorulic acid derivatives [32].

(10)

Speciation plots for iron(III) acetohydroxamic acid demonstrate that the 3 : 1 complex dominates over a wide pH range (*Figure 2.8*). As only 3 protons are displaced from this monobasic ligand on formation of the 3 : 1 complex, the resulting compound is neutral (Eqn. v). In principle therefore, if the octanol/water partition coefficient (K_{part}) can be adjusted to the range (0.1–2),



(v)

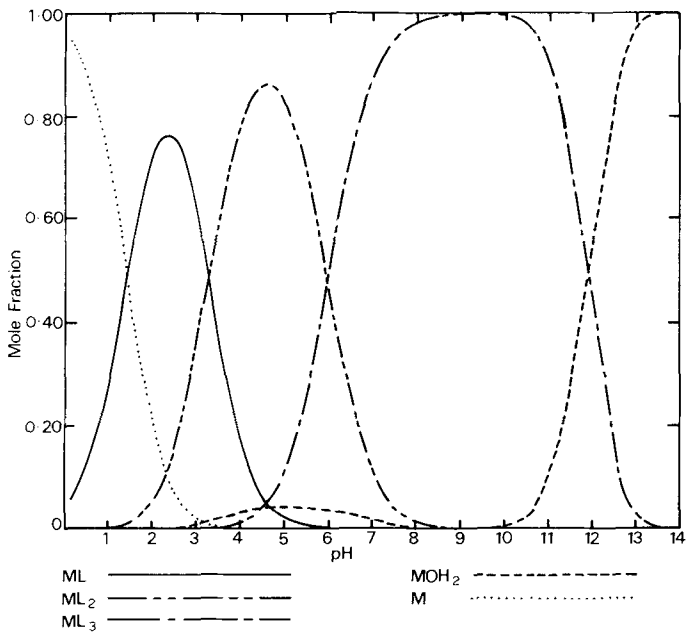


Figure 2.8. Speciation plot of acetohydroxamic acid (2; R^1 = Me; R^2 = H) and iron(III). Concentration of ligand, 4 × 10^{-4} M; concentration of iron(III), 1 × 10^{-4} M. The following parameters were incorporated into the model for this system: log K_{11} ML = 11.3; log K_{12} ML_2 = 9.8; log K_{13} ML_3 = 8.1; pK_a = 9.35; log K (MOH/M · OH) = 11.81; log K $(M(OH)_2/MOH \cdot OH)$ = 10.5; log K $(M_2(OH)_2/M^2 \cdot OH^2)$ = 25.1. The speciation programme MLGEN50 (based on MLGEN11 [33]) was used for this simulation.

the complex is likely to penetrate biological membranes [7]. As discussed earlier, complex formation with iron(III) can be considered as a competition reaction with hydrogen ions (*Scheme 2.1*), and consequently the charged 1 : 1 and 1 : 2 species dominate under acidic conditions. Thus, at pH 4.0, the 1 : 2 species dominates (*Figure 2.9A*), although as an excess of hydroxamate is added, the 1 : 3 species makes an increasing contribution. In contrast, at pH 7.0, the 1 : 3 species dominates as soon as sufficient hydroxamate is present (*Figure 2.9B*). The relative stability of the 1 : 3 complex is further enhanced in the presence of excess ligand (*Figure 2.10*). These trends in stability hold for the interaction of Group III cations with all bidentate ligands.

Hydroxamates, like amides, are hydrolysed in acidic environments and consequently they possess low oral activity [34,35]. Aromatic hydroxamates

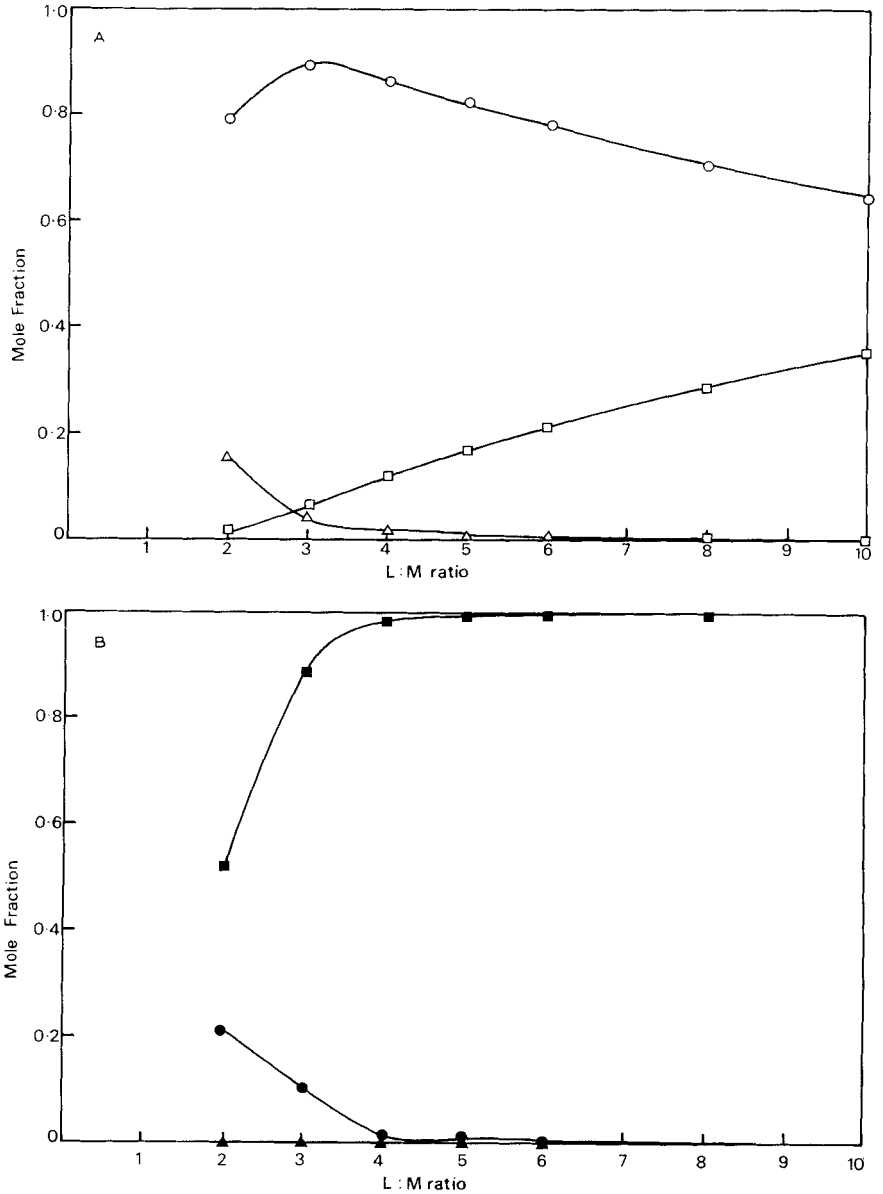


Figure 2.9. The mole fraction of the three iron(III) acetohydroxamic complexes, ML, ML_2 and ML_3 as a function of the M : L ratio. (A) pH 4.0 and (B) pH 7.0. ML, □ and ■; ML_2, ○ and ●; ML_3 △ and ▲. Parameters and speciation programme as Figure 2.8.

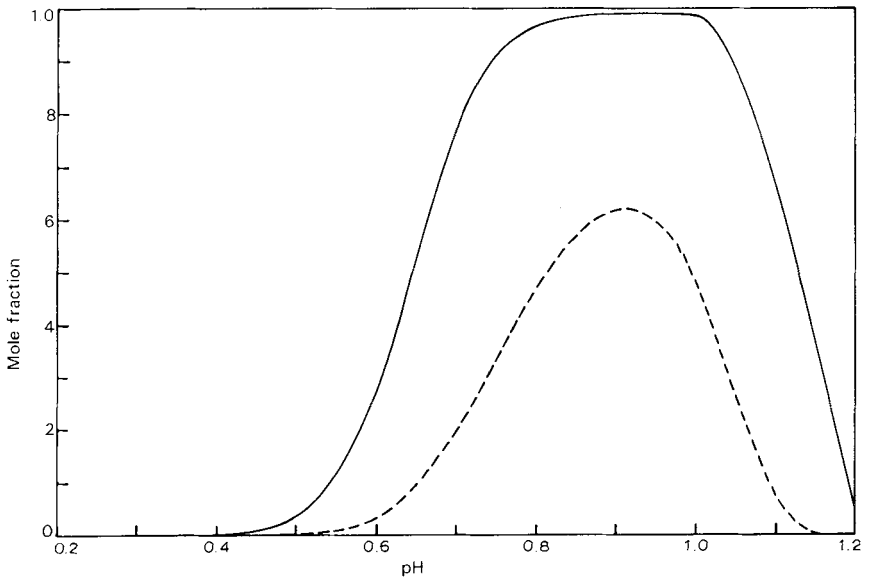
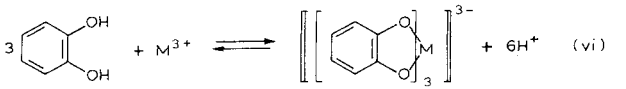


Figure 2.10. Plot depicting the influence of pH on the mole fraction of ML_3 complex of acetohydroxamic acid and iron(III). Iron concentration = 10^{-5} M.———, [L]/[Fe] = 10; - - - - - - -, [L]/[Fe] = 3. Parameters and speciation programme as Figure 2.8.

are capable of forming stable radicals (Eqn. ivA) which may be responsible for toxic effects [36].

Catechol

Catechol (1) binds Group III cations tightly, the maximum affinity being centered on iron(III) (*Figure 2.6*). This extremely strong interaction with tribasic cations results from the high electron density of both oxygen atoms. Unfortunately, this is also associated with a high affinity for protons (pK_a values, 13.0 and 9.2) and therefore, cation binding has a marked pH sensitivity (Eqn. vi). This competition between metal cation and protons leads to the



formation of a range of species at neutral pH values [37]. A formalised speciation plot of iron(III)-catechol shows that the 2 : 1 complex is the dominant form in the pH range 5.5–7.5, the 3 : 1 complex only forming in appreciable quantities above pH 8.0 (*Figure 2.11*). As the other Group III metals under comparison possess lower affinity constants for catechol, competition by protons would be expected to be even more effective. The 2 : 1 iron(III)-catechol complex has a tendency to condense to form 4 : 2 complexes. Both the 3 : 1 [38] and 4 : 2 [39] complexes possess a distorted octahedral geometry. Similar structures might be anticipated for Al^{3+} and Ga^{3+}. Recently, a disubstituted catechol has been reported to form a stable ML_3 species in the pH range 6–10 [40].

The range of complexes likely to form at neutral pH values all bear a net charge, (3 : 1, 3^-; 2 : 1, 1^-; and 4 : 2, 2^-) and consequently, are unlikely to penetrate membranes by simple diffusion. A further problem with catechol-based ligands is their susceptibility towards oxidation (Eqn. vii) [41]. The

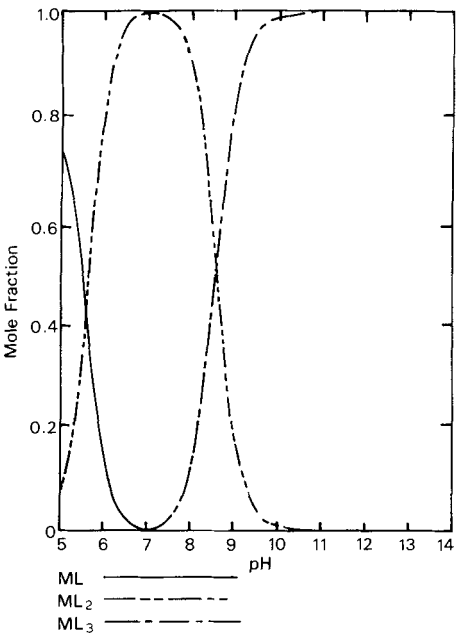
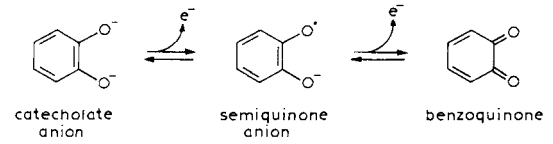
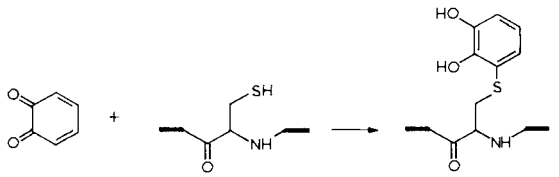


Figure 2.11. Speciation plot of catechol (1) and iron(III). Concentration of ligand, 4 × 10^{-4}M; concentration of iron(III), 1 × 10^{-4}M. The following parameters were incorporated into the model for this system: log K_{11} ML = 20.0; log K_{12} ML_2 = 14.7; log K_{13} ML_3 = 9.0; pK_{a1} = 13.00, pK_{a2} = 9.20. Constants for iron-hydroxy complexes, and speciation program, as Figure 2.8.



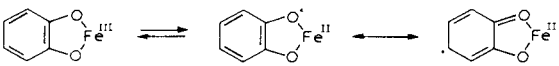
(vii)

oxidation products are also able to coordinate cations, but generally with reduced affinity. Quinones react with protein thiol groups (Eqn. viii) thereby



(viii)

contributing to the toxicity of catechols [42]. The redox activity of catechols is a particular problem in the presence of iron(III), as under mildly acidic conditions, an internal redox reaction occurs (Eqn. ix) [37,43]. The semi-



(ix)

quinone binds iron(II) less tightly and consequently the complex dissociates, permitting the semiquinone to disproportionate to the quinone and catechol. The percentage of iron(II) present depends on the pH and the substituents on the catechol moiety (*Figure 2.12*) [44]. Catechols are metabolised by many

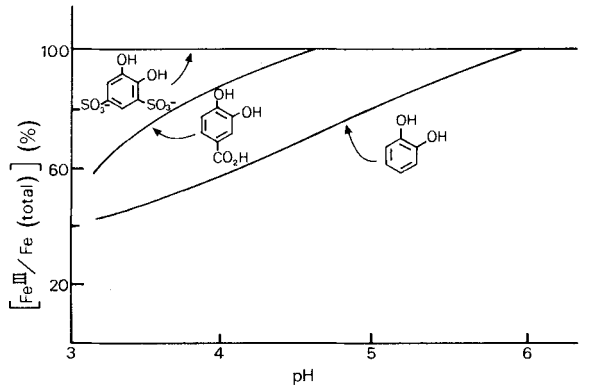


Figure 2.12. Influence of pH on the internal reduction of iron(III) coordinated by bidentate catechols.

enzymes, for instance, tyrosinase [45], catechol-*o*-methyl transferase [46] and catechol oxidase [47], and consequently, they are likely to possess a short biological half-life.

8-Hydroxyquinoline

8-Hydroxyquinoline (6) possesses a high affinity for Group III metals (*Figure 2.6*), but is not selective, as it also binds copper(II) tightly (*Table 2.2*). There is a marked increase in affinity with charge density of the cation for the series, La^{3+}, In^{3+} and Fe^{3+}, but on reaching the apparently critical radius ≈ 0.6 Å, the affinity constants for Ga^{3+} and Al^{3+} do not increase further (*Figure 2.6*). Although dibasic, the pK_a value of the nitrogen is low (4.9), and thus, in the pH range 6–10 the ligand can be considered to be monobasic. As with the hydroxamate function, the 3 : 1 species dominates in the neutral to alkaline pH ranges (*Figure 2.13*). By virtue of the higher affinity for iron(III) ($\log \beta_3$:

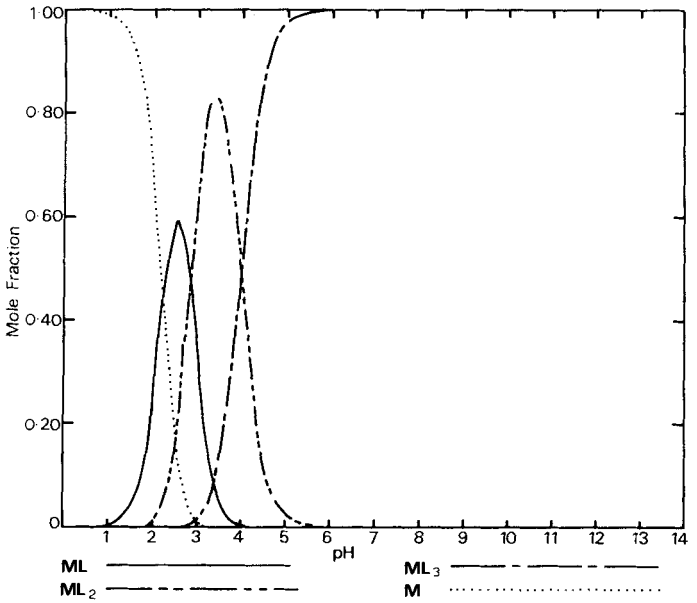
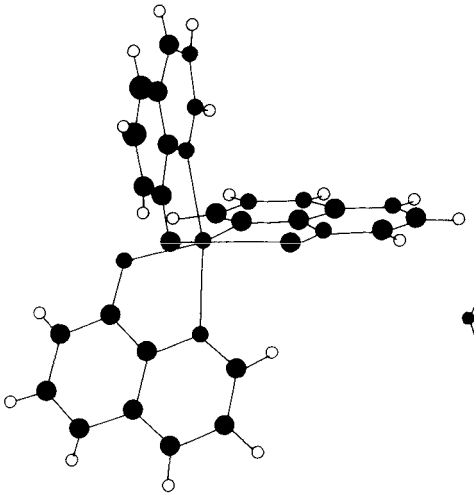


Figure 2.13. Speciation plot of 8-hydroxyquinoline (6) and iron(III). Concentration of ligand, 4 × 10⁻⁴ M, concentration of iron(III), 1 × 10⁻⁴ M. The following parameters were incorporated into the model for this system: log K_{11} *ML = 13.6; log* K_{12} *ML_2 = 12.6; log* K_{13} *ML_3 = 10.7;* $pK_{a1} = 4.95$; $pK_{a2} = 9.65$. *Constants for iron-hydroxy complexes, and speciation programme, as* Figure 2.8.



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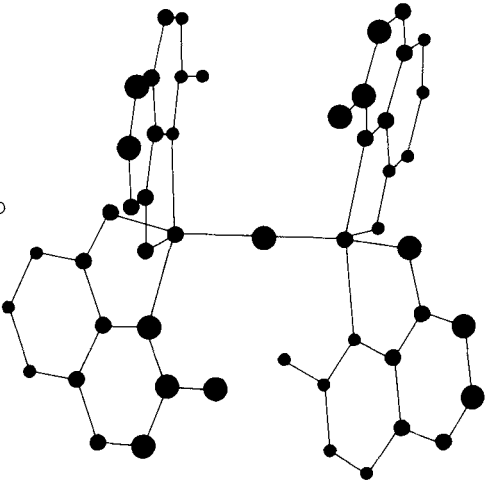


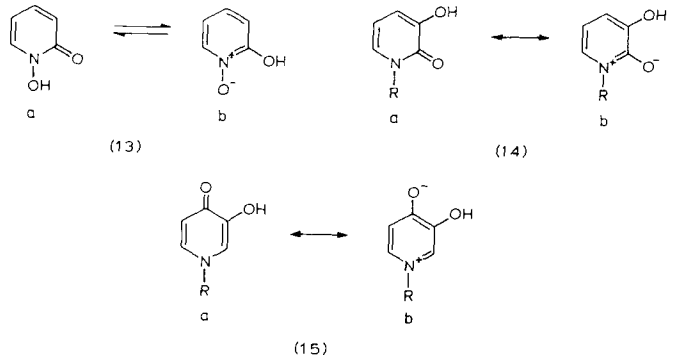
(12)

8-hydroxyquinoline, 37.7; acetohydroxamate, 28.3), the 3 : 1 iron(III) complex is stable even at pH 14, in marked contrast to the hydroxamate (*Figure 2.8*). The 3 : 1 M^{3+} complexes possess a distorted octahedral geometry (11) [48]. With the smaller cations, iron and aluminium, 4 : 2 complexes have also been characterized (12) [49].

8-Hydroxyquinoline is toxic [50], this property being associated with its extreme hydrophobicity. Indeed, because the 3 : 1 complexes possess zero net charge, they are also hydrophobic and tend to accumulate in membranes. 8-Hydroxyquinoline is particularly toxic in the presence of traces of iron [50]. The membrane-bound ligand could possibly act as a binding site for the redox-active iron, thereby generating hydroxy radicals in the presence of oxygen. Although hydroxyquinoline derivatives are still used in many parts of the world for the treatment of diarrhoea [51], their use has been criticised [52,53] due to their extreme toxicity [54,55]. Albert [56] designed quinoline analogues possessing ring nitrogen atoms with a view to reducing the high partition coefficient of this molecular class. However, with the exception of 5-hydroxypyrido[3,4-*b*]pyrazine, most derivatives have a reduced affinity for iron(III) [56].

Hydroxypyridinones

It has been appreciated for over 20 years that hydroxypyridinones and catechols are isoelectronic [57], both molecules ((1) and (9)) possessing the same number of electrons. Consequently, the pyridinone moiety has been used as a substituent for catechol in drug design [58,59]. The three classes of pyridinones (13, 14 and 15), like catechol, form five-membered chelate rings with the two



adjacent oxygen atoms coordinating the metal. Like hydroxamates, the pyridinones are monobasic and consequently form neutral 3 : 1 complexes. Each hydroxypyridinone class possesses several mesomers, and the relative contribution made by each form has a strong influence on metal binding affinities, as exemplified by iron(III) (*Table 2.3*).

Table 2.3 pK_a VALUES AND AFFINITY CONSTANTS FOR IRON(III) OF SELECTED BIDENTATE LIGANDS
Data from [33] and [60–63].

	pK_{a1}	pK_{a2}	$logK_1$	$logK_2$	$logK_3$	$log\beta_3$
1-Hydroxypyridin-2-one (13)	5.78	−0.9	10.3	9.0	7.6	26.9
3-Hydroxypyridin-2-one (14)	8.88	0.1	11.7	9.8	8.1	29.6
3-Hydroxypyridin-4-one (15)	9.01	3.34	14.2	11.6	9.3	35.1
Catechol (1)	13.00	9.22	20.0	14.7	9.1	43.8
Acetohydroxamic acid (2,R^1 = Me, R^2 = H)	9.36	<0	11.4	9.7	7.2	28.3
Maltol (23)	8.61	<0	11.1	9.2	8.1	28.4

1-Hydroxypyridin-2-one (*2-hydroxypyridine*-N-*oxide*). 1-Hydroxypyridin-2-one can exist as an *N*-oxide (13b), and as an *N*-hydroxide (13a), the latter tautomer being equivalent to a cyclic hydroxamate and predominating in solution [64]. 1-Hydroxypyridin-2-ones have been reported to chelate cobalt(III) [65], copper(II) [66], zinc(II), nickel(II) [67] and iron(III) [68,69]. A neutral 3 : 1 iron(III) complex dominates over the pH range 5 to 9 (*Figure 2.14*). By virtue of the low pK_a value of this ligand (5.8), it is particularly effective at iron chelation at acid pH values when compared with catechol (*Figure 2.11*), acetohydroxamic acid (*Figure 2.8*) and 8-hydroxyquinoline (*Figure 2.13*). However, a reduction in proton affinity is associated with a reduction in affinity for Group III metals. Thus, the β_3 values for 1-hydroxypyridin-2-ones (13) are lower than those of acyclic hydroxamates (2) (*Table 2.3*). As the O–O bite distance (2.54 Å) is similar to that of hydroxamates (2.56 Å

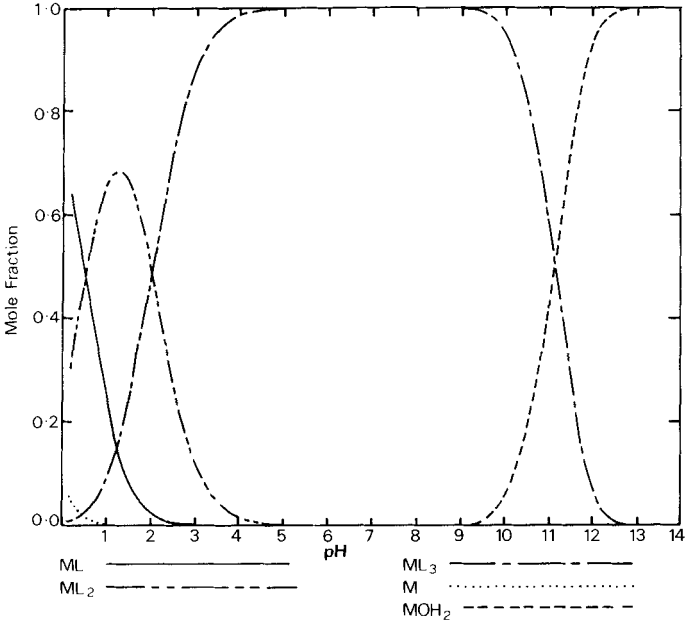
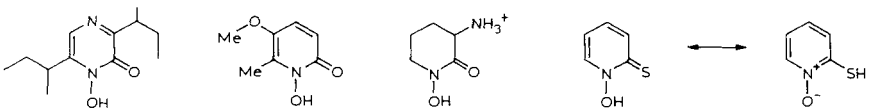


Figure 2.14. Speciation plot of 1-hydroxypyridin-2-one (13) and iron(III). Concentration of ligand, 4×10^{-4} M, concentration of iron(III), 1×10^{-4} M. The following parameters were incorporated into the model for this system: log K_{11} ML = 10.3; log K_{12} ML_2 = 8.9; log K_{13} ML_3 = 6.5; $pK_{a1} = -0.95$; $pK_{a2} = 5.78$. Constants for iron-hydroxy complexes, and speciation program, as Figure 2.8.

[70]), the affinity for La^{3+}, In^{3+} and Al^{3+} would be predicted to be appreciably lower than that for Fe^{3+} (*Figure 2.6*).

A number of pyridine *N*-oxides have been isolated from microorganisms, including aspergillic acid (16) [71] and G1549 (17) [72]. Both compounds bind iron(III) and aluminium(III) tightly [72]. Significantly, these two compounds are toxic and possess antibiotic activity. This property is almost certainly associated with the extreme hydrophobicity of the free ligands. A water-soluble pyridine *N*-oxide (18) is secreted by *Rhodotarula pilimanae* when grown under strongly acidic conditions (pH 2.8) [73]. As this class of molecule possesses good iron-scavenging properties at acidic pH values (*Figure 2.14*), (18) may well be acting as a siderophore. The analogues of this class of molecule are also known, 2-mercaptopyridine *N*-oxide (19) being toxic and a potent bacteriocide and fungicide [74]. Again, this property is probably associated with the extreme hydrophobicity of the molecule and its ability to complex metals.



(16) (17) (18) (19)

3-Hydroxypyridin-2-one. The coordination properties of this class of compound have not been extensively investigated, although members of the group have been reported to bind zinc(II), copper(II) [75] and iron(III) [76]. 3-Hydroxypyridin-2-ones (14) form a 3 : 1 complex with iron(III) which carries zero net charge [61,77]. The partitioning properties of the molecule can be readily modulated by *N*-alkylation [62,77]. The affinity constants for H^+ and Fe^{3+} are similar to those of aliphatic hydroxamates (*Table 2.3*), and consequently, the neutral 3 : 1 complex is the dominant species over the pH range 6.0–10.0 (*Figure 2.15*). The affinity of this ligand type for aluminium, indium and lanthanum is, like catechol (same O–O bite distance [70]), predicted to be lower than that of iron(III). Unlike the hydroxamate ligand, 3-hydroxypyridin-2-ones are stable in the presence of acid. Consequently, they have potential as orally active chelators. As the *N*-derivatized molecules possess a bulky function *ortho* to the ligating oxygen atoms, these molecules are not potent inhibitors of catechol-metabolizing enzymes [78].

3-Hydroxypyridin-4-one. As with 3-hydroxypyridin-2-ones, 3-hydroxypyridin-4-ones have two possible mesomeric forms (15a and 15b). However, unlike the pyridin-2-ones, the zwitterionic form (15b) makes a major contribution [79,80], leading to a much higher pK_a value for the carbonyl oxygen (3.3 as compared

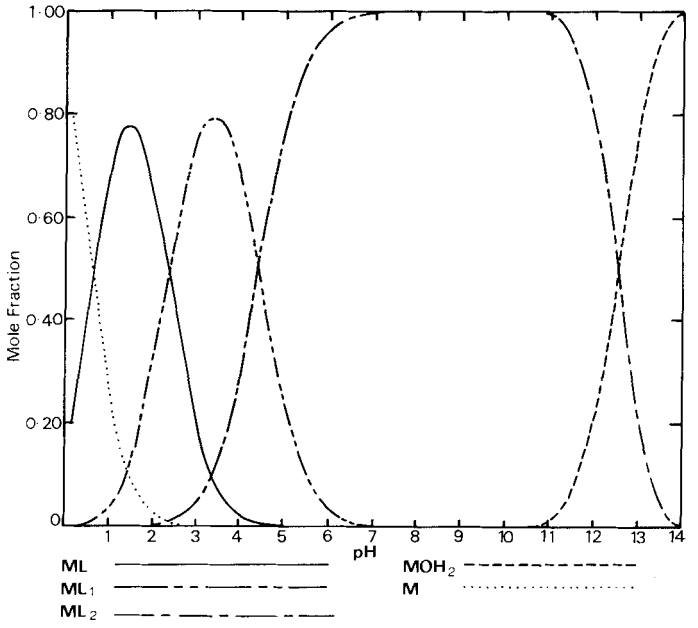


Figure 2.15. Speciation plot of 3-hydroxypyridin-2-one (14) and iron(III). Concentration of ligand, 4×10^{-4} M, concentration of iron(III), 1×10^{-4} M. The following parameters were incorporated into the model for this system: log K_{11} ML = 11.6; log K_{12} ML_2 = 9.9; log K_{13} ML_3 = 8.1; pK_{a1} = 0.12; pK_{a2} = 8.64. Constants for iron-hydroxy complexes, and speciation program, as Figure 2.8.

with 0.1, *Table 2.3*). Thus, the pyridin-4-ones protonate on the carbonyl oxygen and not on the ring nitrogen [81,82]. This enhanced electron density leads to an increased affinity for Group III metals when compared with the other two pyridinone classes (*Table 2.3*). The pyridin-4-ones form a neutral 3 : 1 complex with iron(III) (20) [83], which is stable over a wide range of pH values (*Figure 2.16*). Indeed, under the conditions examined in the speciation plot, the hydroxide anion is unable to compete to any appreciable extent over the entire pH range investigated (0.0–14.0). The 3-hydroxypyridin-4-ones bind indium [84] and aluminium [85,86] with affinities less than that of iron(III) (*Figure 2.6*), and thus fit into the general trend shown by bidentate ligands. These molecules have also been reported to chelate gallium [87,88] and hydrophobic forms have been used to extract a range of metals from acid ore digests [89,90]. The pyridinones are extremely stable in acid, and as with the pyridin-2-ones, their K_{part} values can be readily adjusted to fall within the range 0.1–2.0 by modification of the *N*-alkyl function, rendering them suitable for oral use [77,91].

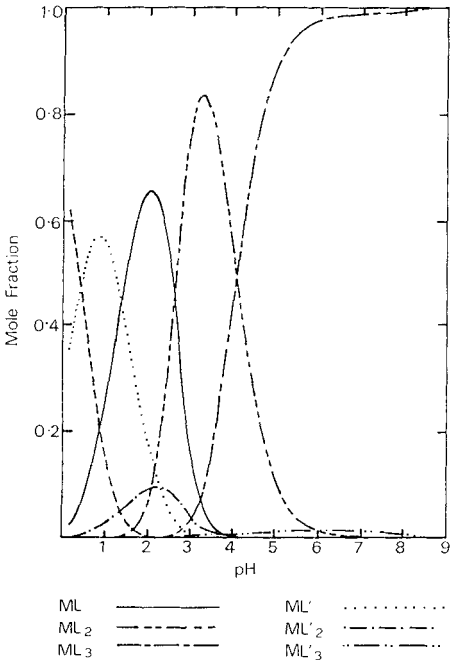
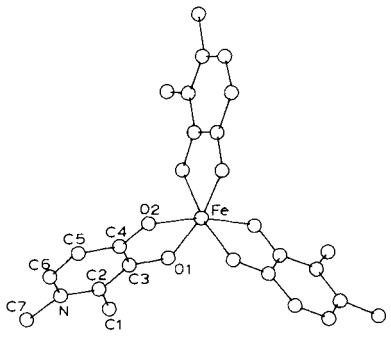


Figure 2.16. Speciation plot of 3-hydroxypyridin-4-one (15) and iron(III). Concentration of ligand, 4×10^{-4} M, concentration of iron(III), 1×10^{-4} M. The following parameters were incorporated into the model for this system: log K_{11} ML = 15.1; log K_{12} ML_2 = 12.1; log K_{13} ML_3 = 9.8; pK_{a1} = 3.58; pK_{a2} = 9.92. Constants for iron-hydroxy complexes, and speciation program, as Figure 2.8.



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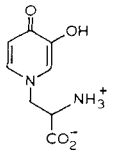

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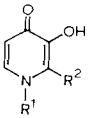

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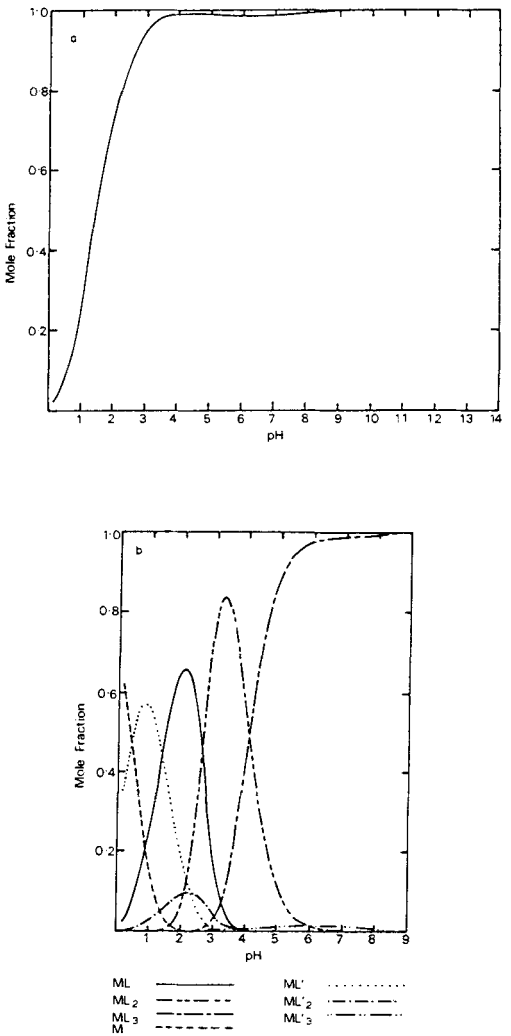


Figure 2.17.(a) Z plot of 3-hydroxypyridin-4-one (15) vs. 3-hydroxypyridin-2-one (14) in the presence of iron(III). Concentration of each of the competing ligands = 4 × 10⁻⁴ M; concentration of iron(III) = 1 × 10⁻⁴ M. A Z plot provides information regarding the distrubution of a metal between various competing species. In this figure the area under the curve represents iron bound to the 3-hydroxypyridin-4-one (ML, ML_2 and ML_3 complexes). (b) Speciation plot of 3-hydroxypyridin-4-one (15) vs. 3-hydroxypyridin-2-one (14) in the presence of iron(III). Concentration of each of the competing ligands = 4 × 10⁻⁴ M; concentration of iron(III) = 1 × 10⁻⁴ M. For parameters incorporated into this model see legends to Figures 2.15 *and* 2.16. *The speciation program MLCOM50 (based on MLCOM3 [33]) was used for this simulation.*

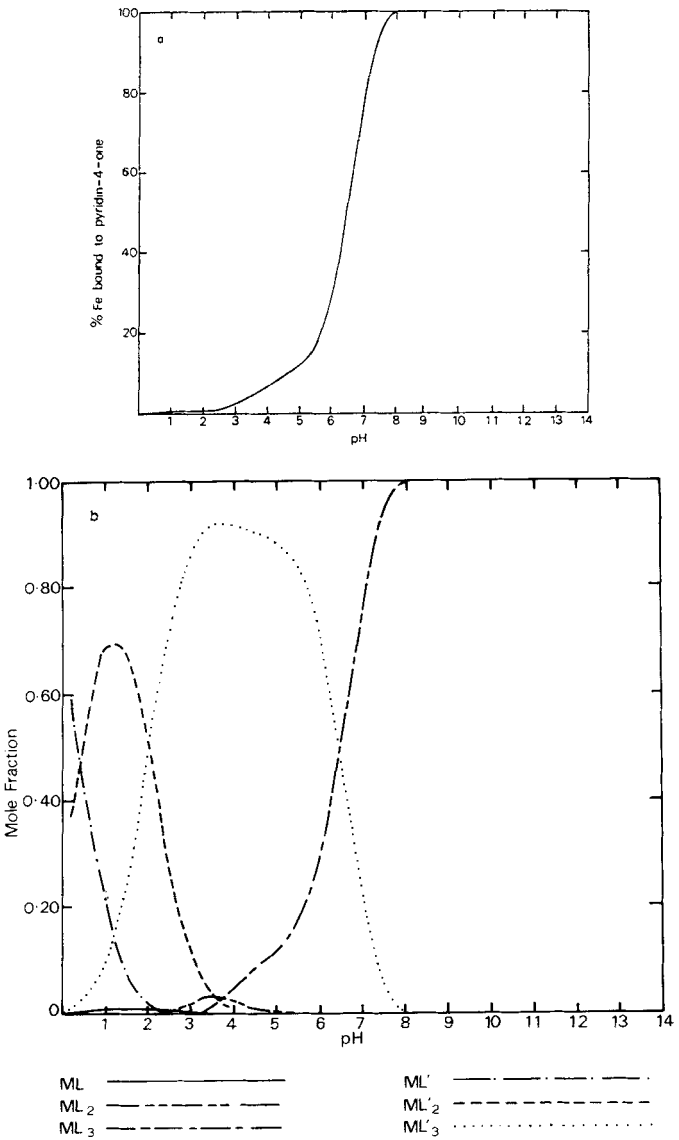


Figure 2.18.(a) Z plot of 3-hydroxypyridin-4-one (15) vs. 1-hydroxypyridin-2-one (13) in the presence of iron(III). Concentration of each of the competing ligands = 4 × 10^{-4} M; concentration of iron(III) = 1 × 10^{-4} M. In this figure, the area under the curve represents only iron bound to the 3-hydroxypyridin-4-one (15) (ML, ML_2 and ML_3 complexes). (b) Speciation plot of 3-hydroxypyridin-4-one (15) (L) vs. 1-hydroxypyridin-2-one (13) (L') in the presence of iron(III). Concentration of each of the competing ligands = 4 × 10^{-4} M; concentration of iron(III) = 1 × 10^{-4} M. For parameters incorporated into this model, see legends to Figures 2.14 *and* 2.16. *Speciation program, as* Figure 2.17.

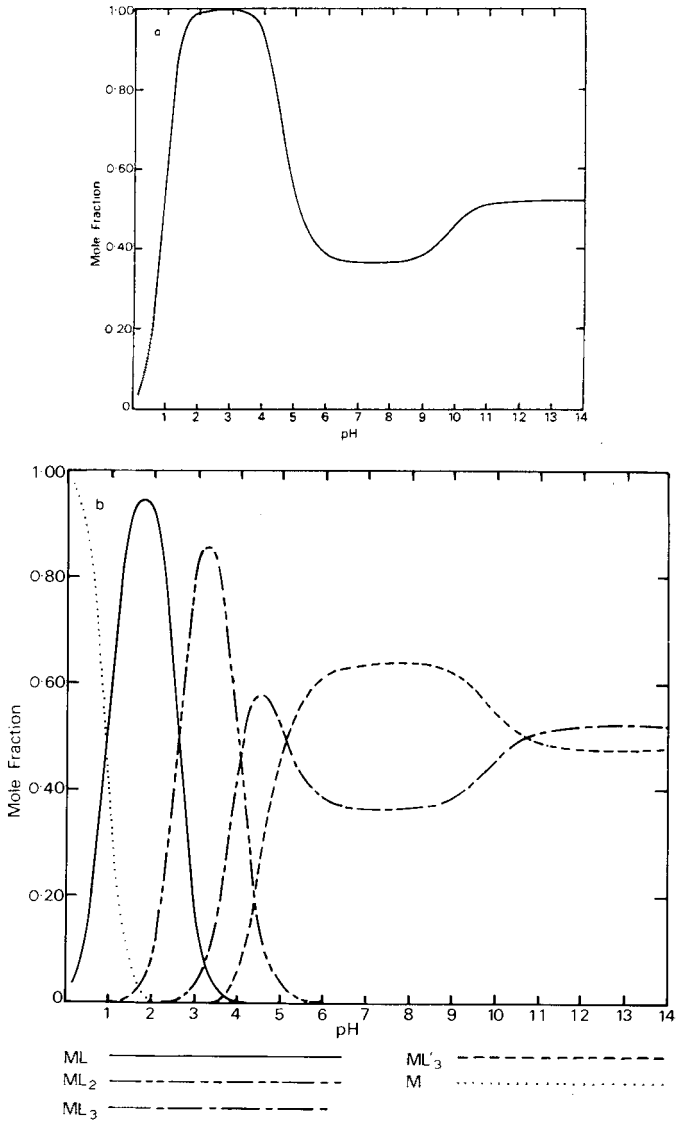


Figure 2.19.(a) Z plot of 3-hydroxypyridin-4-one (15) vs. 8-hydroxyquinoline (6) in the presence of iron(III). Concentration of each of the competing ligands = 4×10^{-4} M; concentration of iron(III) = 1×10^{-4} M. In this figure, the area under the curve represents only iron bound to the 3-hydroxypyridin-4-one (15) (ML, ML_2 and ML_3 complexes). (b) Speciation plot of 3-hydroxypyridin-4-one (15) (L) vs. 8-hydroxyquinoline (6) (L') in the presence of iron(III). Concentration of each of the competing ligands = 4×10^{-4} M; concentration of iron(III) = 1×10^{-4} M. For parameters incorporated into this model, see legends to Figure 2.13 *and* 2.16. *Speciation program, as* Figure 2.17.

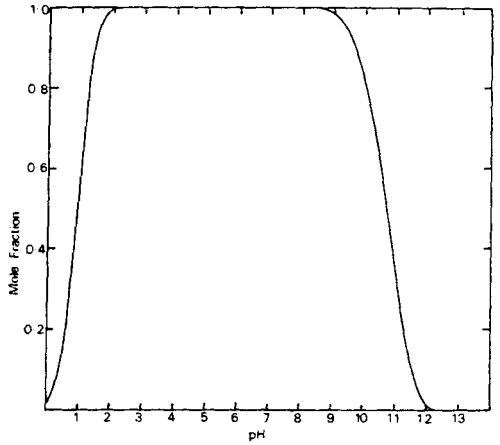


Figure 2.20. Z plot of 3-hydroxypyridin-4-one (15) vs. catechol (1) in the presence of iron(III). Concentration of each of the competing ligands = 4 × 10⁻⁴ M; concentration of iron(III) = 1 × 10⁻⁴ M. In this figure, the area under the curve represents only iron bound to the 3-hydroxypyridin-4-one (15) (ML, ML_2 and ML_3 complexes). For parameters incorporated into this model, see legends to Figures 2.11 *and* 2.16. *Speciation program, as* Figure 2.17.

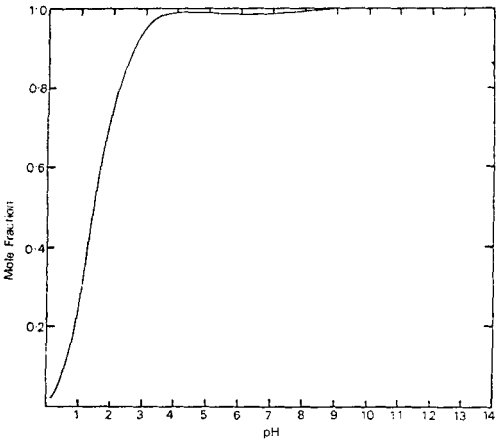
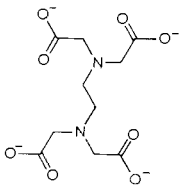


Figure 2.21. Z plot of 3-hydroxypyridin-4-one (15) vs. EDTA (25) in the presence of iron(III). Concentration of each of the competing ligands = 4 × 10⁻⁴ M; concentration of iron(III) = 1 × 10⁻⁴ M. In this figure, the area under the curve represents only iron bound to the 3-hydroxypyridin-4-one (15) (ML, ML_2 and ML_3 complexes). Parameters for EDTA (25): log K_{11} ML = 25.1; pK_{a1} = 2.00; pK_{a2} = 3.70; pK_{a3} = 6.20; pK_{a4} = 10.20. Parameters for 3-hydroxypyridin-4-one as in legend to Figure 2.16. *Speciation program, as* Figure 2.17.



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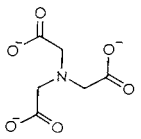


(26)

TRIDENTATE AND TETRADENTATE LIGANDS

In principle, both tridentate and tetradentate ligands can form 1 : 1 and 2 : 1 complexes with Group III metals (*Scheme 2.4*). This is demonstrated with nitrilotriacetate (NTA) (26) (*Figure 2.22a*), which, despite a high affinity for iron, does not compete effectively with OH^- at neutral and alkaline pH values unless a large molar excess of ligand is present (*Figure 2.22b*). The relative affinities of the different Group III cations for this ligand class (*Figure 2.23*) follows the same trend as that found with bidentate ligands (*Figure 2.6*). The higher affinity for NTA as compared to citrate and IDA is probably associated with NTA being a tetradentate ligand, as compared to the tridentate nature of the other two ligands [107]. Aroyl hydrazones (27) [108] and desferrithiocin (28) [109] are also good tridentate ligands for Group III metals. The stereochemistry of *cis*-inositol is such that three axial hydroxy functions can coordinate Group III metals. Erni and co-workers [110] have synthesised tri-amino derivatives, for instance TTCI (29), which possess relatively low pK_a values (8.0 and 13), and therefore, a high affinity for iron(III). The stereochemistry of some potential tetradentate ligands is such that they form 3 : 2 complexes (*Scheme 2.4*) with iron and presumably with other Group III metals. Such structures occur with rhodotorulic acid [111] and bipyridinones [112].

A serious problem with tri- and tetradentate ligands is that there is also the possibility of forming polymeric structures (*Scheme 2.4*). Consequently, they are not ideal for the chelation of Group III cations. The formation of polymeric structures is improbable with bidentate ligands, and hence they find greater application in the clinical situation.

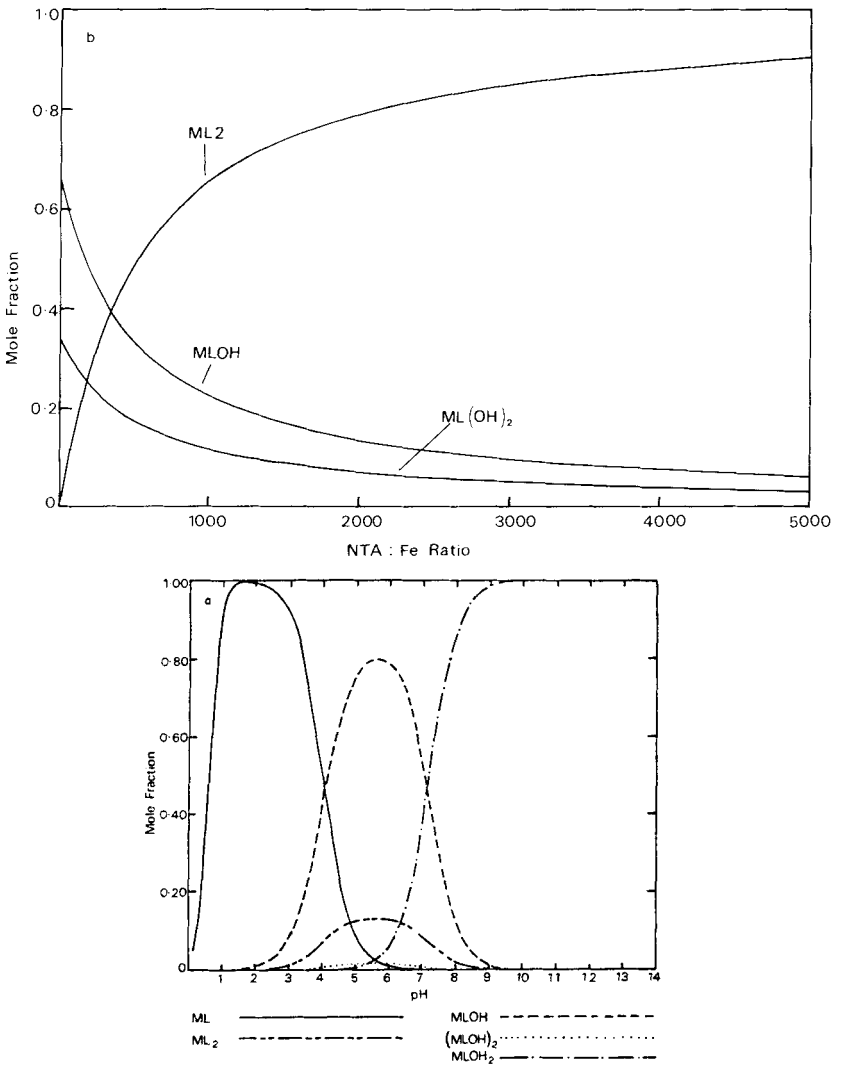


Figure 2.22.(a) Speciation plot of NTA (26) and iron(III). Concentration of ligand, 4×10^{-4} M, concentration of iron(III), 1×10^{-4} M. The following parameters were incorporated into the model for this system: log $\mathbf{K}_{11}$ *ML = 15.8; log* $\mathbf{K}_{12}$ *ML_2 = 7.4; log pK_{a1} = 1.9; log pK_{a2} = 2.5; pK_{a3} = 9.7; log* **K** *(MOHL/ML · OH) = 9.9; log* **K** *$(M(OH)_2L/MOHL \cdot OH)$ = 6.1; log* **K** *$(MOHL)_2/(MOHL_2)$ = 4.0; log* **K** *$((MOHL)_2)/(ML)^2 \cdot OH^2)$ = 21.6. Constants for iron-hydroxy complexes, and speciation program, as* Figure 2.8. *(b) The mole fraction of the three iron(III) NTA complexes, ML_2, MLOH and $ML(OH)_2$ as a function of the L : M ratio. Concentration of iron, 10^{-5} M; pH = 7.4. Parameters and speciation program in (a).*

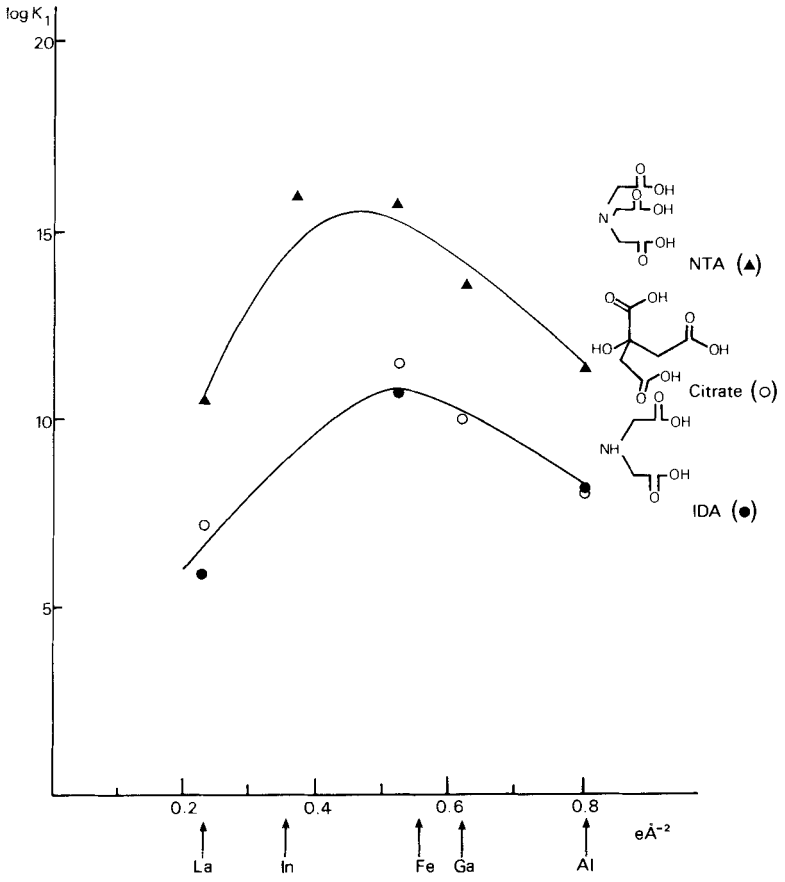
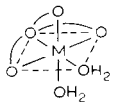
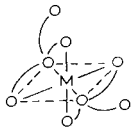


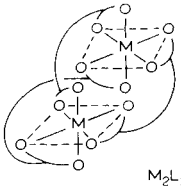
Figure 2.23. Relationship between charge density of M^{3+} cations and log K_1 values of tridentate and tetradentate ligands. From [16–19].



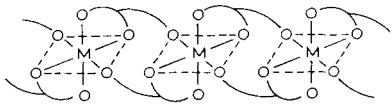
ML



ML_2

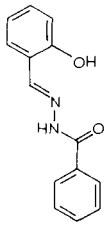


M_2L_3



$(ML_2)_n$

Scheme 2.4



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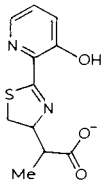

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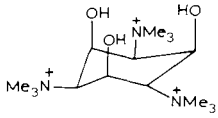
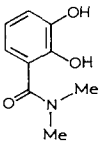


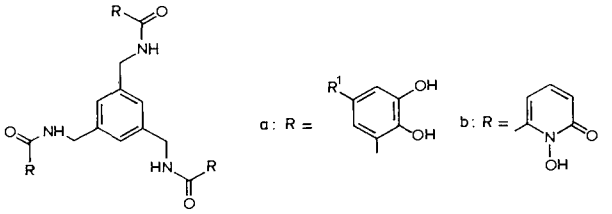
(29)

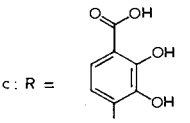
HEXADENTATE LIGANDS

For Group III cations, donor-acceptor bond energies largely determine the enthalpy contribution to the stability of the complex, but entropy can also make

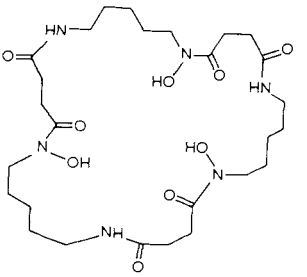


(30)





a considerable contribution with multidentate ligands. A favourable entropic contribution from displacing coordinated water molecules on going from a tris(bidentate) to a hexadentate complex can account for an increase in the formation constant of up to 6 log units [113]. Precisely this increment is observed when the formation constants of tris(*N*, *N*-dimethyl-2,3-dihydroxybenzamide) iron(III) (30) and the synthetic tricatechol ligand MECAM (31a, R^1 = H) are compared. The log K_1 values are 40.2 [114] and 45.9 [115], respectively. A similar transition is observed for hydroxamate ligands, although the difference is usually not so marked, for instance the log affinity constants of acetohydroxamic acid and desferrioxamine-E (32) for iron are 28.3 and 32.5,



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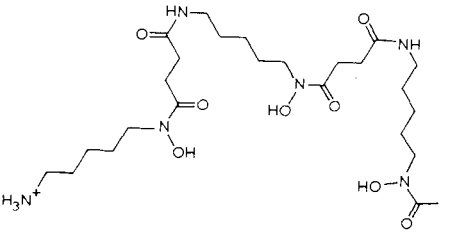
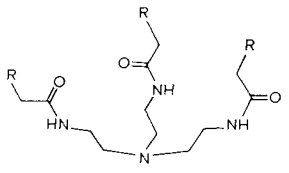


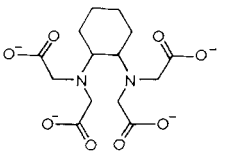
(33)

respectively [33]. With desferrioxamine-B (DFO) (33) the differential is even less, indicating that although chelation provides a favourable entropic contribution, hexadentate formation can involve molecular strain, which in turn gives rise to unfavourable enthalpic contributions. The influence of steric strain on complex formation has recently been reviewed by Hancock and Martell [116]. In addition to the higher affinity constant, hexadentate ligands bind to Group III metals more tenaciously at low concentrations ($\leq$mM). This difference results from the concentration of the hexadentate complex having first-order dependence on ligand concentration as compared with the concentration of the trisbidentate complex, which has third-order dependence. Thus, at low concentrations, the hexadentate ligand competes progressively more effectively for metal cations than the trisbidentate complex [62]. By virtue of their multidentate nature, hexadentate ligands form kinetically stable complexes; thus, the kinetic exchange of iron(III) between two such ligands can be extremely slow. The $T_{1/2}$ for ^{59}Fe exchange between desferrioxamine-B (33) and a closely related hexadentate hydroxamate, ferrichrome, is over 200 h at pH 7.4 [117], although

the rate can be greatly increased by acidification. Thus, iron(III) chelates possessing small differences in thermodynamic stability can have very different kinetic stabilities. For instance, whereas the half-time for donation of iron(III) to apotransferrin is less than 5 min from bidentate 3-hydroxy-1-alkylpyridin-2-ones (14) ($\log \beta_3 \approx 30$), the half-life from the corresponding hexadentate pyridin-2-one (34a) ($\log K_1 = 28.8$) is so long (> 24 h), that it is unlikely to have any relevance *in vivo*. It is for these reasons that the majority of siderophores, the iron-scavenging molecules produced by microorganisms, are hexadentate [32].

Many hexadentate complexes exist as enantiomeric pairs. Furthermore, with asymmetric ligands such as hydroxamate and pyridinone both geometric and optical isomers are possible: Δ-*cis*, Δ-*trans*, Λ-*cis* and Λ-*trans* [118]. However, many structures are stereochemically limited to the *cis* isomer; ferrichrome, for instance, crystallizes with Λ-*cis* stereochemistry [119].





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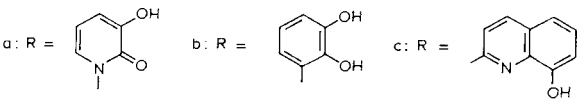


(34)

Aminocarboxylates

Molecules bearing aminocarboxylate groups can be considered as the prototypes of synthetic oligodentate chelators, EDTA (25) being the classic example. As a class, they all possess a high affinity for the Group III cations (*Figure 2.24*), the selectivity being shifted towards the larger cations, and in particular indium(III), when compared with monodentate (*Figure 2.3*) and bidentate ligands (*Figure 2.6*). Presumably, this change in selectivity results from the larger cavity generated by the oligodentate ligands. However, EDTA is not sufficiently large to completely envelop the coordinated cation, the iron(III)

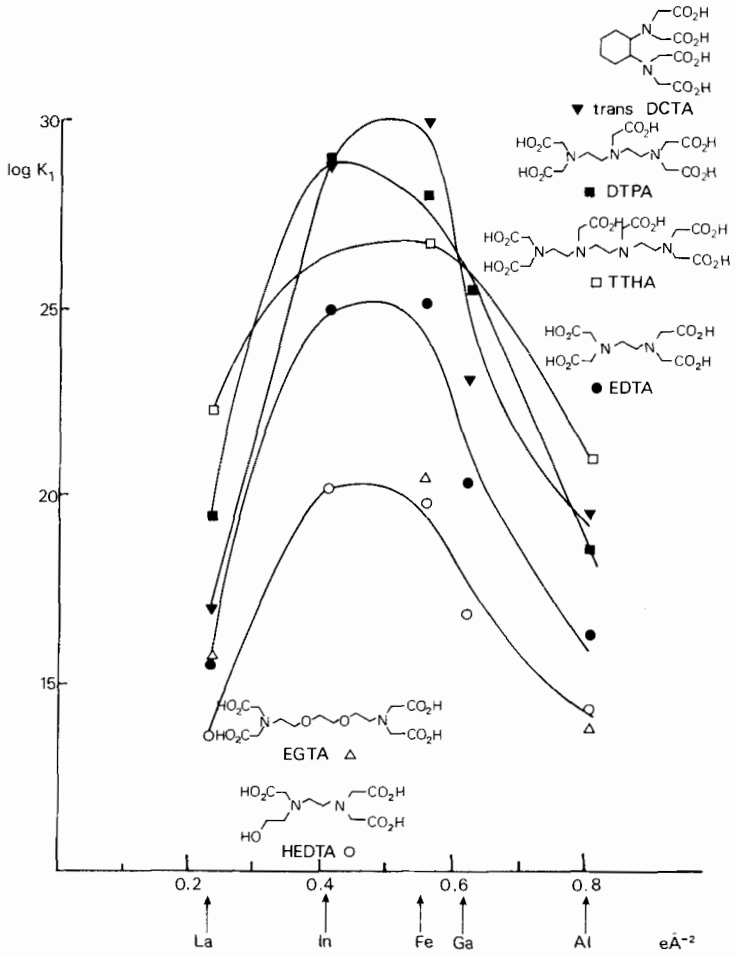
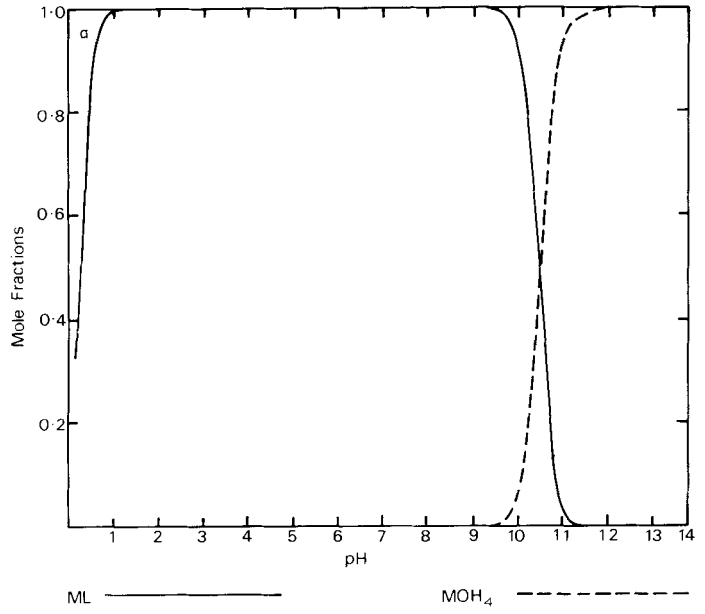


Figure 2.24. Relationship between charge density of M^{3+} cations and log K_1 values of oligodentate aminocarboxylate ligands. From [16–19].

complex possessing a water molecule on a seventh coordination site [120]. The indium and iron EDTA complexes are the most stable of the series and their speciation plots are virtually identical (*Figure 2.25a*). At 10^{-4} M iron or indium, a slight excess of EDTA keeps the metals in solution at pH values below 9.0. However, for gallium, aluminium and lanthanum, the pH range of stable complex formation is markedly reduced; thus for gallium (*Figure 2.25b*), the


a
1·0
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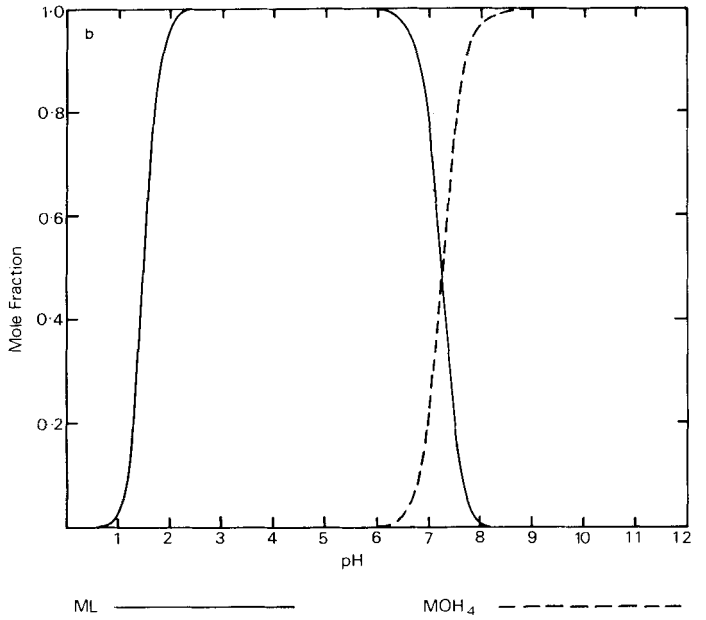
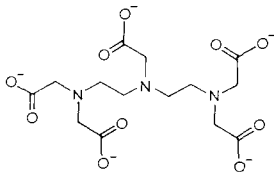
Mole Fractions
1 2 3 4 5 6 7 8 9 10 11 12 13 14
pH
ML
MOH4


b
1·0
0·8
0·6
0·4
0·2
Mole Fraction
1 2 3 4 5 6 7 8 9 10 11 12
pH
ML
MOH4

complex is not stable at pH 7.0 under these conditions. As the carboxylate function possesses a relatively high affinity for zinc(II) and calcium(II), and nitrogen is a good copper(II) ligand, the aminocarboxylate class of chelators are not specific for Group III cations. Thus, both EDTA (25) and DTPA (36) have higher affinities for copper(II) (*Table 2.4*) than for either aluminium(III) or lanthanum(III), and the affinities for gallium(III) are only marginally higher (*Figure 2.24*). The phosphonate analogues of aminocarboxylates have been reported to possess a high affinity for hard cations such as calcium(II) [121]. However, the EDTA analogue, EDTMP (40), has lower affinities for iron(III), copper(II) and calcium(II) than EDTA (*Table 2.4*), and consequently it is improbable that this ligand class will be suitable for the selective chelation of Group III cations. A number of plant and bacterial siderophores are based on hexadentate aminocarboxylate structures, for instance, mugineic acid [122] and avenic acid [123].



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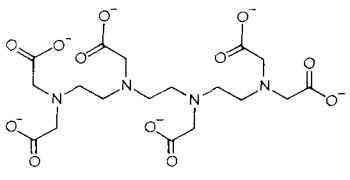

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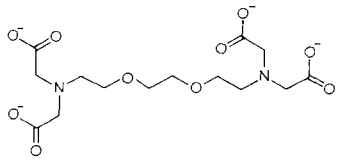

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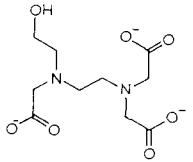

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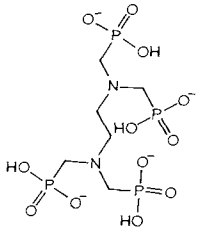


(40)

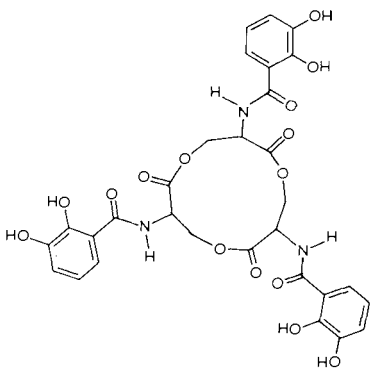
Figure 2.25. (a) Speciation plot of EDTA (25) and iron(III). Concentration of ligand, 4 × 10^{-4} M, concentration of iron(III), 1 × 10^{-4} M. The following parameters were incorporated into the model for this system: log K_{11} *ML = 25.1; p*K_{a1} *= 2.0; p*K_{a2} *= 3.7; p*K_{a3} *= 6.2; p*K_{a4} *= 10.2. Constants for iron-hydroxy complexes, and speciation programme, as* Figure 2.8. *(b) Speciation plot of EDTA (25) and gallium(III). Concentration of ligand = 4 × 10^{-4} M, concentration of iron(III), 1 × 10^{-4} M. The following parameters were incorporated into the model for this system: log* K_{11} *ML = 20.2; p*K_{a1} *= 2.0; p*K_{a2} *= 3.7; p*K_{a3} *= 6.2; p*K_{a4} *= 10.2; log K (MOH/M · OH) = 11.4; log K (M(OH)$_2$/M(OH) · OH) = 10.7; log K (M(OH)$_3$/M(OH)$_2$ · OH) = 9.6; log K ((M(OH)$_4$)/(M(OH)$_3$ · OH) = 7.7.*

Table 2.4 AFFINITY CONSTANTS OF SELECTED HEXADENTATE LIGANDS

	log K_1			
	Fe(III)	*Cu(II)*	*Zn(II)*	*Ca(II)*
DTPA (36)	28.0	21.6	18.4	10.8
TTHA (37)	26.8	20.5	18.1	10.5
EDTA (25)	25.1	18.8	16.5	10.7
HEDTA (39)	19.8	17.6	14.7	8.3
EDTMP (40)	19.6	16.1	–	5.7
MECAMS (31a, $R^1 = SO_3^-$)	41	23	17	–
HBED (44a)	39.7	21.4	18.4	9.3
DFO (33)	30.6	14.1	11.1	2.6

Catechols

As might be predicted from *Figure 2.6*, hexadentate catechols possess an extremely high affinity for Group III cations. Indeed, enterobactin (41) possesses a higher affinity for iron(III) than any other ligand ($K_1 = 10^{52}\ M^{-1}$) [124]. The structure of the molecule is such that the cation can be chelated without inducing strain in the supporting backbone [125]. A wide range of synthetic analogues have been prepared, typical examples being MECAM (31a, $R^1 = H$), MECAMS (31a, $R^1 = SO_3^-$) [118] and spermidine-based analogues, for example (42) [126]. The affinities for iron(III), indium(III) and gallium(III) are similar for this ligand class, the log K_1 values for MECAMS



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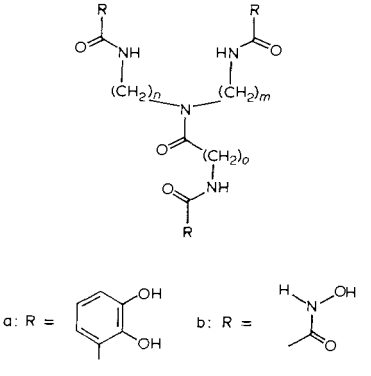


(42)

being 41, 38 and 39, respectively [127]. The catecholates are more selective than the aminocarboxylates (*Table 2.4*) [128], although it has recently been pointed out that sulphonation of ligands, such as MECAM (31a, R^1 = H) to MECAMS (31a, $R^1 = SO_3^-$), with the intent of increasing the water solubility, may in fact lower the selectivity for tripositive cations with respect to a number of biologically important dipositive cations, such as Zn(II) and Ca(II) [31]. This is a direct result of the decrease in basicity of the catecholate oxygen donor atoms resulting from the incorporation of the sulphonate groups [20]. The catechols bind Group III metals more tightly at alkaline pH values than the aminopolycarboxylates, although the complexes are extremely sensitive to pH due to the displacement of 6 protons on complex formation (Eqn. iv), and donate metals such as iron to EDTA under weakly acidic conditions (*Figure 2.26*). Thus, EDTA (25) (log K_1, 25.1) removes iron(III) from the tricatechol (34b) (log K_1, 43.6) at pH 6.0, and from the tricatechol (31c) in the region of pH 6.5.

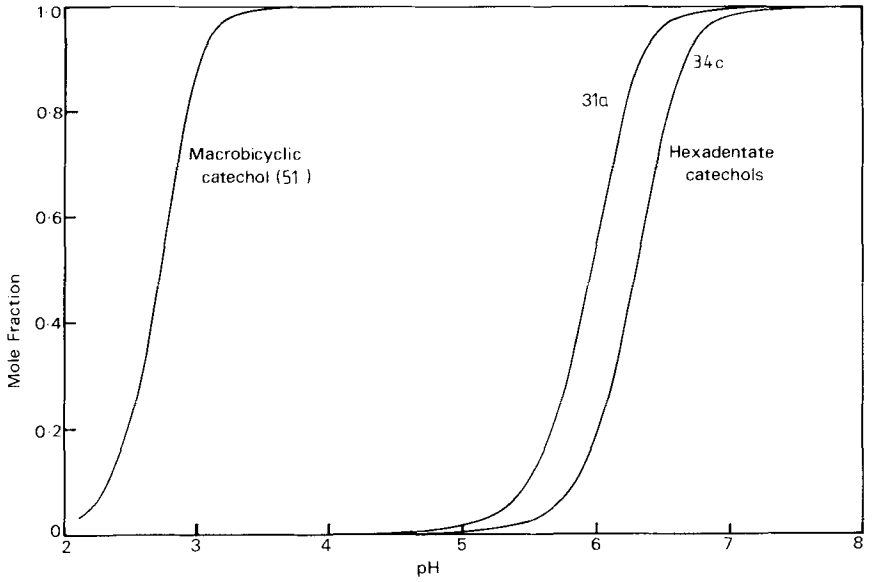
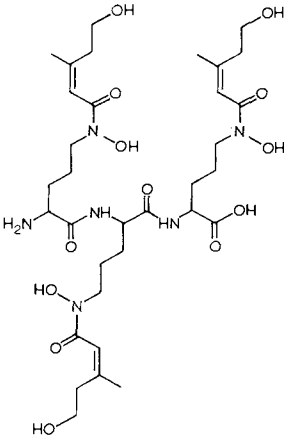


Figure 2.26. Z plots of the hexadentate catechols (31c, 34b) and macrobicyclic catechol (51) vs. EDTA (25) in the presence of iron(III). Concentration of each competing ligand and iron(III) = 1 × 10⁻⁴ M. Data obtained from visible spectra.

Hydroxamates

There is an enormous range of naturally occurring hydroxamate siderophores [32] which posses a high affinity for both iron [(33) log K_1 = 31], and aluminium [(33) log K_1 = 25] [33]. By analogy with other oligodentate ligands, therefore, it can be safely assumed that they will also tightly chelate gallium and indium, but not lanthanum, [(33) log K_1 = 11]. Many hydroxamate siderophores are constructed on extensive peptide backbones, for instance ferrichrome [12] and pyoverdine [129], but others are formed on a simple tripeptide structure (43) [130]. The methodology for oligodentate hydroxamate synthesis is well established [131], and ranges of trihydroxamates based on spermidine (42b) [132,133] and 1,3,5-trisubstituted benzene [134,135] have been prepared. However, none has an affinity for iron(III) greater than that of desferrioxamine-B (33), a widely used iron chelator in clinical medicine (see section on iron).

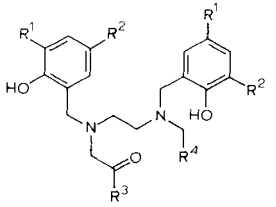


(43)

ortho-*Substituted phenolates*

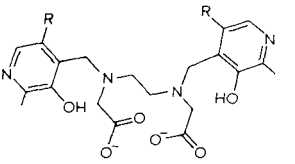
In an attempt to improve the limited selectivity of aminocarboxylates, Martell and co-workers [136] synthesised HBED (44a), in which two of the carboxylate functions of EDTA are replaced by *ortho*-phenolates. The selectivity for Group III metals was markedly enhanced (*Table 2.4*), the affinity for gallium(III) (log K_1 = 39.6) being similar to that of iron(III). Unfortunately, the solubility of HBED metal complexes is low, but can be enhanced by the introduction of

sulphonate functions (44b). Various other analogues have been prepared, but none possesses higher affinity for iron(III) than HBED [136]. Substitution of the phenolic moiety by pyridoxal has also been investigated, the ligand PLED (45a) possessing a high affinity for Group III metals [134]. The advantage of this ligand is its enhanced water solubility when compared to HBED. The closely related HPED (46) possesses a high affinity for iron(III), but is toxic [137].



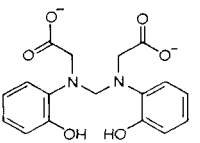
a: R^1 = H ; R^2 = H ; R^3 = OH ; R^4 = COOH
b: R^1 = H ; R^2 = SO_3H; R^3 = OH ; R^4 = COOH
c: R^1 = H R^2 = H ; R^3 = OMe; R^4 = Ac
d: R^1 = Me; R^2 = Me; R^3 = OH; R^4 = COOH
e: R^1 = Me; R^2 = tBu; R^3 = OH; R^4 = COOH
f: R^1 = Me; R^2 = Me; R^3 = OH; R^4 = CH_2OH

(44)



a: R = Me b: R = CH_2OH

(45)

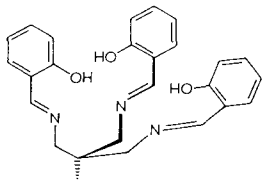


(46)

ortho-Phenolates which lack carboxylate functions, for example 4HBCY (47a) [134] and TX-TACN (48b) [138], also coordinate Group III cations, but again the affinity is lower than that of HBED. These ligands form neutral complexes which possess low water solubility. Similar properties are found with the salicylaldimine derivative $(5MeOSal)_3$ (49) [139].

Hydroxypyridinones

Hexadentate ligands have been prepared from both 3-hydroxy- [62,140] and 1-hydroxypyridin-2-ones [141]. The 3-hydroxypyridin-2-one (34a) forms a neutral 1 : 1 complex with iron(III), indium(III) and aluminium(III). The log K_1 value for iron(III) is 28.8, which is lower than the log β_3 value for the corresponding bidentate analogue (32.3), and consequently there is considerable potential for improving the stereochemistry of the ligand [62]. Nevertheless, this molecule is a powerful scavenger of iron(III) under biological conditions [142]. Unlike the neutral pyridinones (34a), the 1-hydroxypyridinones of the



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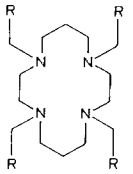
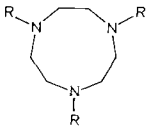
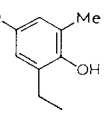


a: R =  b: R = COO^-

(47)



a: R = Ac b: R = 

c: R = CH_2CH_2SH

(48)

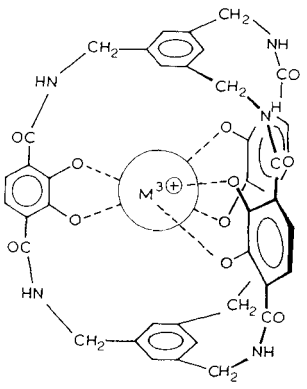
type (31b) [141] are charged at neutral pH values, bearing charges of 2^- or greater. A hexadentate hydroxyquinoline (34c) has been prepared [143]. This molecule and the corresponding Group III metal complexes are not charged at neutral pH values and consequently are extremely hydrophobic.

Macrobicyclic ligands

Macrobicyclic ligands (50) are capable of generating tailor-made coordination sites of considerable kinetic stability [116,144,145]. The first such reagent with a high affinity for Group III metals to be synthesised was the tricatechol (51) [146]. The markedly improved acid stability of these macrocyclic complexes when compared with those of the corresponding acyclic ligand (*Figure 2.26*) results from the much lower pK_a values of the catecholate functions in the macrobicyclic compound [147]. An analogous tricatechol with nitrogen bridge heads has also been prepared [148]. The synthesis of a macrobicyclic trihydroxamate (52) with a high affinity for iron(III) and gallium(III) has also been described [149].

Comparison of hexadentate ligands

The highest affinity ligands are the tricatechol compounds and the acid lability of the simple hexadentate ligands can be overcome by forming macrobicyclic analogues (*Figure 2.26*). These complexes possess a net negative charge of 3^-



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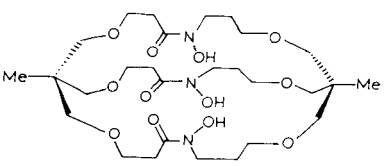

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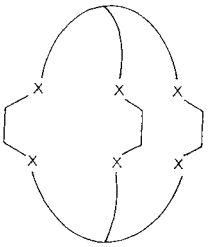

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at neutral pH values. In contrast, hydroxamates, or the substituted phenolates and pyridinones, are capable of forming neutral complexes. All the above classes possess higher affinities and selectivities for Group III metals than the widely used aminocarboxylates (e.g., DTPA (36)).

Hexadentate ligands have some clear advantages over the corresponding bidentate analogues; they are potentially more selective, bind the coordinated metal more tightly at low concentrations ($< 100\ \mu M$) and are kinetically more inert. However, the larger molecular weight of the hexadentate compounds renders it more difficult to achieve efficient oral activity and more difficult for both the free ligand and the complex to penetrate membranes. These latter two properties are not important for many radiopharmaceutical and magnetic resonance imaging applications. Thus, hexadentate ligands are ideal for labelling proteins and for the design of small molecular probes.

SELECTIVITY OF LIGANDS FOR METAL CATIONS

As already discussed, with ligands containing only oxygen donor atoms, the selectivity for metal cations appears to follow a fixed pattern [15]. However, a number of approaches are possible allowing this selectivity order to be modified.

One method is to attach the donor atoms to a rigid molecular framework,

thus enforcing selectivity on the cations which fit the coordination site [31]. This is the reason for the high selectivity of the macrobicyclic ligand (51) [146]. Introduction of alternative donor atoms to oxyanions also allows for changes in selectivity. For instance, incorporation of neutral oxygen donor atoms tends to increase the selectivity of the ligand towards metal ions of larger radius [150]. Another useful donor atom is saturated nitrogen. Comparison of $\log K_1$ [$^-$OH] values with $\log K_1$ [NH_3] values (*Table 2.5*), indicates that changes in selectivity are possible. Thus, incorporation of one or more nitrogen donor atoms into ligands bearing oxyanion donors is predicted to have a much greater effect on Al(III) chelation than on Fe(III), Ga(III) and In(III) chelation [31]. Such properties have to be balanced against the inevitable associated increase in affinity for Cu(II) ions (*Tables 2.2* and *2.5*). A similar argument can be made for the introduction of sulphur donor atoms into ligands (see section on gallium).

Table 2.5 LOG K_1 VALUES FOR SELECTED METALS WITH $^-$OH AND NH_3

	log K_1	
	^-OH	NH_3
Fe(III)	11.8	3.8
Al(III)	9.0	0.8
Ga(III)	11.4	4.1
In(III)	10.0	4.0
La(III)	5.5	0.3
Cu(II)	6.3	4.0
Ca(II)	1.3	−0.2
Zn(II)	5.0	2.2

TRANSFERRIN

Iron is transported in mammalian blood on the glycoprotein transferrin (M_r = 80,000) [151]. Transferrin has two high-affinity iron-binding sites ($K = 10^{20}$ M^{-1} at pH 7.4). These sites are capable of binding all the Group III metals discussed in this review [152]. In the normal healthy person, the concentration of transferrin falls in the range 25–40 μM and it is typically 25–40% saturated with iron. Thus, there is always the possibility of other metals binding to this protein. Exchange of gallium and indium, for instance

between radiopharmaceuticals and transferrin, can limit the utility of many preparations. When designing Group III ligands for clinical use therefore, it is essential to consider how they will interact with transferrin.

Transferrin is a bilobal molecule. Each lobe possesses two domains, the iron binding site being located in the cleft between the domains [153,154]. The iron(III) cation is coordinated by two tyrosines, a histidine, an aspartate and a bicarbonate anion (*Figure 2.27*). The affinity of transferrin for Group III metals is critically dependent on the presence of HCO_3^-, and all *in vitro* studies should be performed in the presence of physiological levels of this anion (25–30 mM) [152]. Iron is transported from the intestine and the liver, via transferrin, to tissues that require iron for normal metabolism. A large proportion of iron is directed to bone marrow, the site of haemoglobin synthesis. Transferrin binds to specific protein receptors on the plasma membrane of iron requiring cells, and is taken into the cell via endocytosis [155]. The endosome population containing the bound transferrin is acidified and as a result, iron dissociates from transferrin. Apotransferrin subsequently dissociates from the receptor and the endosome is recycled, returning apotransferrin to the circulation. It is clear that, should other Group III metals bind to transferrin, they can also gain access to the cytoplasm of cells by the same receptor-mediated process.

The intracellular iron pool is not well characterized, but is probably a small

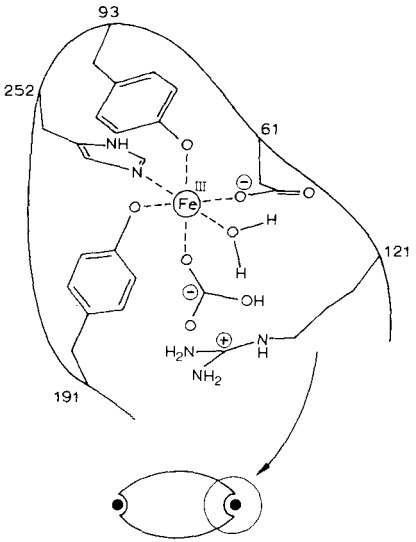


Figure 2.27. Iron-binding site of transferrin (76 kDa) [153,154].

oligomeric iron(III) aggregate ($M_r \approx 1500$) [25,156]. Intracellular 'low molecular weight iron' ($\approx 2\ \mu M$) is used in the biosynthesis of metalloproteins in both the mitochondria and endoplasmic reticulum. Aluminium [156], gallium and indium can enter this cytoplasmic pool. Excess iron is deposited in ferritin [157], a large hollow protein which is capable of storing up to 4,500 iron(III) atoms in a polymeric iron-oxide matrix [158]. Thus, intracellular iron levels are carefully controlled, ensuring that oxygen radical production is kept to a minimum. Other Group III metals may gain access to ferritin; for instance, aluminium has been reported to accumulate in human brain ferritin [159].

Iron–transferrin interactions

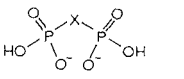
The high affinity of transferrin for iron ensures that the concentration of other forms of iron in the plasma are vanishingly small, thereby exerting a powerful antibacterial and antifungal influence [160]. The closely related lactoferrin, found in milk and other secretory fluids, like transferrin, ensures extremely low iron levels in these fluids. The low level of readily available iron in the blood ensures that oxygen radical production is kept to a minimum.

Iron is bound tightly to transferrin, and consequently any chelator which is capable of competing with transferrin for iron must possess a high affinity for this cation. Hexadentate ligands are generally more likely to be effective than their bidentate analogues, the difference being particularly marked at chelator concentrations likely to be achieved *in vivo* (1–50 μM). However, exchange of iron between two such high-affinity sites will be exceedingly slow unless mixed complex formation is possible, that is, the free ligand must gain access to the transferrin-bound iron, presumably by displacement of the bicarbonate anion (*Figure 2.27*). Thus, the less bulky bidentate ligands are likely to be kinetically more active than their hexadentate counterparts. This observation is largely borne out in practice (*Table 2.6*), where the bidentate ligands are found to reach equilibrium iron distribution with transferrin more rapidly than oligodentate chelators, although 3,4-LICAMS (55b) is a marked exception to this trend. Many *in vitro* competition studies are carried out with a large excess of ligand (up to 5000-fold) whereas under *in vivo* conditions the chelator concentration is likely to be less than or equal to the concentration of transferrin. Under these conditions, the 3-hydroxypyridinones for instance, donate iron to apotransferrin [106]. Thus, in practice donation of iron from a complex to apotransferrin is usually observed.

The two transferrin sites are not equivalent, the N-terminal site possessing a 20-fold higher affinity for iron than the C-terminal site [163,164]. Indeed, different chelator types have different preferences for these two sites [165,166].

Table 2.6 EXCHANGE OF IRON(III) BETWEEN TRANSFERRIN AND SELECTED CHELATORS.

Ligands	$T_{1/2}$*(min)[161]*	*%Fe removed (30 min)[162]*
Bidentate		
Pyrophosphate (53a)	6	–
Acetohydroxamate (2)	14	–
3-Hydroxypyridin-4-one (15)	15	–
Tris and Tetradentate		
NTMP (54)	21	–
NTA (26)	78	
Hexadentate		
3,4-LICAMS (55b)	24	50
EDTA (25)	144	37
MECAM (31a, $R^1 = H$)	–	13
Enterobactin (41)	–	6
Desferrioxamine (33)	–	5



a: X = O b: X = CH_2

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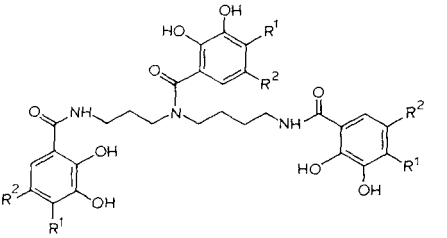
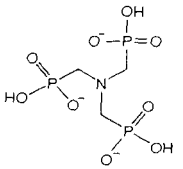


a: R^1 = H; R^2 = H c: R^1 = COOH; R^2 = H
b: R^1 = H; R^2 = SO_3H

(55)



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Group IIIA cation–transferrin interactions

Aluminium binds to the two iron-binding sites on transferrin [167]. The affinity for aluminium, like iron(III), is dependent on the presence of bicarbonate anions [168] and is strongly pH-dependent [169]. As with iron, the affinity of aluminium for the two sites is slightly different [170,171]. Iron readily displaces

transferrin-bound aluminium and the conditional affinity constants for physiological conditions (pH 7.4, $[HCO_3^-] = 20$ mM) are Fe(III), $10^{20}\ M^{-1}$; and Al(III) $2.5 \times 10^{15}\ M^{-1}$ [167]. However, since transferrin is rarely saturated with iron, a large proportion of serum aluminium is associated with transferrin, this being particularly well documented for kidney dialysis patients [172,173]. The observation that aluminium is released from transferrin at pH 4.5 [169] indicates that aluminium can gain access to the cytoplasm of cells via the transferrin receptor. In contrast to the iron-transferrin complex, the aluminium complex is colourless, and some commercial sources of apotransferrin may be contaminated with aluminium [167].

Gallium binds tightly to the two specific iron(III) binding sites on transferrin [174], although the affinity is lower than that of iron(III) by a factor of 400. The N-terminal site has a marginally higher affinity than the C-terminal site [174], and the affinity of both sites is enhanced by the presence of bicarbonate anions. Citrate rapidly donates gallium to transferrin [175]. Gallium bound to transferrin gains access to the cytoplasm of cells [176], and particularly those with high transferrin receptor densities [177,178]. Under physiological conditions, transferrin and EDTA possess about the same affinity for gallium. However, DTPA (36), HBED (44a) and the catechol ligands MECAMS (31a, $R^1 = SO_3^-$) and 3,4-LICAMS (55b) all possess higher affinities for gallium than transferrin, and consequently are unlikely to donate gallium to this protein under physiological conditions [174].

As with gallium, indium binds to transferrin with high affinity. Although no accurate affinity constants are available for this interaction, a number of techniques have been used which give approximate values. Competition studies with iron(III) indicate that indium binds less tightly than iron [179], whilst studies utilising time-integral and time-differential perturbed angular correlation (PAC) with ^{111}In have produced several estimates of the first stability constant of indium-transferrin [180]. These values fall in the range 10^{23}–$10^{25}\ M^{-1}$, with the most accurate value being $6.3 \times 10^{23}\ M^{-1}$ [174]. Based on the general trends of affinity constants with hexadentate ligands (*Figure 2.22*), it seems likely that indium will possess an affinity constant for transferrin similar to that of gallium.

Although indium is undoubtedly transported around the body by transferrin, the metabolism of transferrin-bound iron and indium have been shown to be markedly different [181,182]. For example, indium-transferrin is cleared from the plasma more slowly than iron-transferrin, with very little indium being incorporated into erythrocytes or taken up by bone marrow. The reasons for these differences are unclear at present.

The binding of thallium(III) to transferrin has been reported [183]. The

affinity constant for this interaction is not available, although thallium appears to bind less tightly than iron.

Group IIIB cation–transferrin interactions

There appears to have been very little quantitative work relating to the interaction of Group IIIB cations with transferrin. Some early studies indicated that both scandium and yttrium bind to transferrin [184]. However, no information as to the strength of this interaction is given, and the fact that five scandium atoms and only 0.4 yttrium atoms appear to be bound per transferrin molecule may suggest that relatively non-specific binding was observed. Several studies have investigated the binding of lanthanide elements to transferrin. These largely concentrate on the use of energy transfer from lanthanide cations to provide structural information about the location of the metal binding sites on transferrin (see section on terbium and europium). Whilst the binding of two terbium atoms by transferrin was reported, no details of the affinity of the protein for this metal are available. There are several reports of the binding of gadolinium to transferrin [185,186]. This work indicates that only the C-terminal metal binding site of transferrin is capable of binding gadolinium(III) sufficiently tightly to be characterized. The formation constant for this mono gadolinium-transferrin complex ($7 \times 10^6\ M^{-1}$) is similar to the observed formation constant for the binding of samarium(III) to transferrin ($1.4 \times 10^7\ M^{-1}$) [187]. However, these formation constants are too low to allow any substantial role in the transport of (these) lanthanide cations under conditions of physiological bicarbonate concentration (see section on gadolinium). Both neodymium and praseodymium have been shown to bind at a single site on transferrin [188], although the same studies indicated that four other lanthanide cations, namely terbium, europium, erbium and holmium, all bind with the same stoichiometry as iron.

IRON

There are a number of inherited disease states which are associated with the gradual accumulation of iron, β-thalassaemia and thalassaemia intermedia being particularly well characterized [3]. In some regions of the world, the genes are relatively common; in South East Asia, for instance, approximately 100,000 children are born each year suffering from thalassaemia. The normal total body iron is 4.5 g per adult, whereas some thalassaemic patients may accumulate 50–70 g. This becomes life-threatening, as iron, by virtue of its

facile redox chemistry, is toxic when present in excess, as described earlier in this review. Desferrioxamine (33) has been used for the treatment of iron overload for over 20 years [7], and currently it is the only clinically useful drug available for this purpose. Follow-up of patients on lifelong transfusion programmes has shown that the regular use of the drug prevents the development of iron overload and its pathological consequences [189], whilst patients not taking desferrioxamine succumb to iron toxicity in their late teens or early twenties. However, desferrioxamine suffers from the disadvantage that it is inactive when administered orally, and only causes sufficient iron excretion to keep pace with the transfusion regimes when given either subcutaneously or intravenously over 8–12 h several times per week. For this reason, many patients find it difficult to comply with the treatment, and some even stop taking the drug altogether, subsequently developing the complications of iron overload. There is, therefore, no doubt that an orally active chelating agent is needed to treat patients on lifelong transfusion programmes, such as those with thalassaemia major. The development of an oral iron chelator might also allow the extension of the therapeutic use of red-cell transfusions in sickle-cell anaemia.

In addition to the above applications, chelators may also be of use in chronic inflammatory diseases such as rheumatoid arthritis [190], through their action as scavengers of iron, which can accelerate free radical formation and lipid peroxidation at sites of inflammation [28]. Other potential uses for iron chelators are: reducing post-ischaemic perfusion injury [191], ameliorating bleomycin [192] and paraquat toxicity [193], reducing rejection following organ transplantation [194] and graft versus host disease [195], as anticancer agents through their effect on cell proliferation [196], as well as mobilizing iron from the reticuloendothelial cells in the anaemia of chronic disease [197].

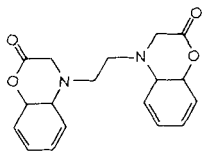
DESIGN OF CHELATING AGENTS FOR THE SELECTIVE REMOVAL OF IRON FROM MAN

The obvious method of choice is to model novel structures on natural hydroxamate and catechol siderophores, for example (32), (33) and (41), which possess extremely high affinities for iron(III) [32]. Hydroxamates possess many advantages for iron(III) chelation, as was outlined in the section on bidentate ligands. However, they tend to possess a low oral activity. Nevertheless, a number have been investigated, including rhodotorulic acid [198], cholylhydroxamic acid [199], synthetic hexadentate [134] and polymeric hydroxamates [200]. None has proved superior to desferrioxamine B (33). In an attempt to overcome the difficulty of poor oral activity, Ciba-Geigy have synthesized a range of desferrioxamine prodrugs by esterifying the labile

hydroxamate functions [109]. Although some of these derivatives are active orally and would be of clinical interest, none has been identified which has comparable activity to desferrioxamine when given to animals intraperitoneally. Catechol, like the hydroxamate moiety, suffers from many disadvantages as a clinical chelating agent, as outlined in the section on bidentate ligands. Despite these limitations, the potential of a number of catechol derivatives has been investigated. The simple 2,3-dihydroxybenzoic acid has been extensively studied but it is ineffective via the oral route [201,202], and many analogues of enterobactin (41), for instance, (31a, 31b, 34b) also lack oral activity. Furthermore, some of these hexadentate catechols are capable of donating iron to pathogenic microorganisms and have been associated with the development of septicaemia [203]. Thus, at the present time, no hydroxamate (with the marked exception of desferrioxamine) or catechol has found clinical application.

Some aminocarboxylates have been investigated for iron chelation; indeed, DTPA (36) is used with patients who develop toxic side-effects with desferrioxamine [204]. DTPA is not orally active and due to its relative lack of selectivity for iron(III) (*Table 2.4*), leads to zinc depletion. As indicated in the section on hexadentate ligands, HBED (44a) was designed to enhance the selectivity for iron(III) [136], but this molecule is not efficiently absorbed via the oral route, and retains a high affinity for zinc (*Table 2.4*). In contrast, the dimethyl ester of HBED (44c) is orally active [137], and this molecule has been highlighted as being of particular interest, pending formal toxicity studies [7]. The subtle prodrug formulation (56) of HPED (46) is also orally active [137].

A completely different line of approach to the selective chelation of iron has been developed by Ponka *et al.* [205] and Hershko *et al.* [206,207]. These workers have synthesized an extensive range of pyridoxal hydrazones, a widely studied compound being PIH (57). Although this compound is reported to be orally active, the Schiff-base link renders it susceptible to hydrolysis in the gastrointestinal tract. PIH is effective in animal studies [208], and has been the subject of a limited clinical trial [209]. PIH is not the most active compound



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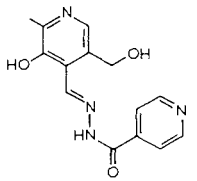


(57)

in the now extensive series of analogues [210], and more toxicological studies will be necessary before other analogues are entered for clinical trial. More recently, the hexadentate pyridoxal-containing chelator, PLED (45a), has been demonstrated to be equieffective with desferrioxamine when given intraperitoneally to iron-overloaded animals [134]. The siderophore, deferriferrithiocin (28), has also been investigated as an orally active chelator [109]; however, it possesses neurological toxicity and is unlikely to reach clinical trial [7].

As indicated in the section on bidentate ligands, 3-hydroxypyridin-4-ones (15,22) are excellent chelators for iron(III), possessing a high selectivity for this metal (*Table 2.2*). They are neutral and can be synthesized with a wide range of K_{part} values [62,77] such that they can penetrate biological membranes either rapidly or not at all [106]. A large proportion of iron present in iron-overloaded animals (including man) is present in the liver, and consequently, hepatocytes have been developed as an *in vitro* assay for the identification of clinically useful chelators [211,212]. Both the bidentate [91], and hexadentate pyridinones [62], are efficient at removing iron from these cells. The ability of these compounds to remove iron correlates well with the K_{part} values of the free ligands [91]. Desferrioxamine (33) is also active in this test, but not as efficient as many bidentate pyridinones [91]. The bidentate pyridinones are orally active in mice [213], and are currently undergoing chronic toxicity studies. They enhance iron removal without increasing the excretion of copper, zinc, calcium or magnesium. Preliminary clinical studies have been promising [214], although some members of the group possess undesirable side-effects [215]. The molecular weight of ligands influences their ability to penetrate membranes [216], and consequently the rate of absorption of oligodentate ligands from the gastrointestinal tract is much lower than that of the smaller bidentate molecules. However, rapid penetration of all cell types can be a disadvantage, as ligand penetration of the blood-brain barrier can cause serious toxicity problems [217]. An important, presently unsolved problem, is to identify the effective molecular weight cutoff for the penetration of both the intestine and the blood-brain barrier. For the brain, this value lies in the region 400–500 [218], but for the intestine the value has not been established, and may be critically dependent on compound type.

Anti-inflammatory activity of iron(III) chelators

Disturbance of iron metabolism is a prominent feature of rheumatoid disease [219,220], many rheumatoid arthritis patients having raised levels of synovial fluid ferritin and iron deposits within their synovial tissue [221–223]. Iron

deposition in early rheumatoid arthritis is associated with a poor prognosis [224], due to the ability of iron to catalyze the production of highly reactive oxygen free radicals (*Scheme 2.3*). Strong iron(III) chelators will remove reactive iron and therefore limit radical production and the concomitant tissue damage [225,226]. Reducing iron levels has also been reported to influence both acute and chronic animal models of inflammation [190,227], iron removal being achieved by chelation or nutritional iron deficiency. When undergoing a respiratory burst, eosinophils, monocytes, macrophages and neutrophils produce superoxide free radicals and hydrogen peroxide [228]. It is thought that these species play an important role in the phagocyte-mediated tissue injury associated with inflammation [229]. In the presence of suitable transition metal catalysts, such as iron, superoxide and hydrogen peroxide react to produce the hydroxyl radical. Iron in a form able to catalyze this reaction has been demonstrated to be present in human synovial fluid [230].

An improvement in both acute and chronic inflammation was noted on administration of desferrioxamine (33) to animal models [190]. However, when given to rheumatoid patients, desferrioxamine caused reversible ocular abnormalities, and co-administration with phenathiazines led to loss of consciousness for up to 72 h [217]. In contrast, lower doses of desferrioxamine administered to anaemic rheumatoid patients have been reported to suppress both inflammation and anaemia [231]. The hydrophilic members of the 3-hydroxypyridin-4-one family, e.g. (22, $R^1 = R^2 = Me$), possess anti-inflammatory ac-

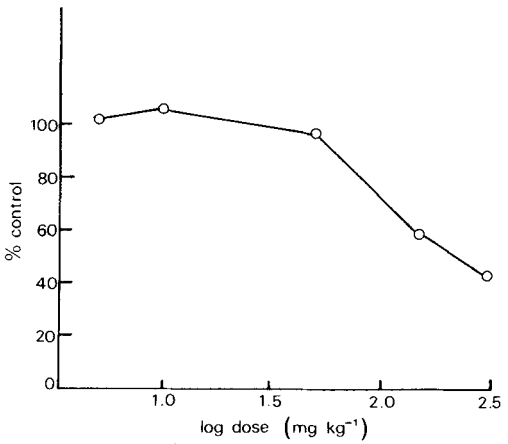


Figure 2.28. Anti-inflammatory activity of the 3-hydroxypyridin-4-one (22) in a carregeenan pleurisy model [219]. Dose response of the reduction of exudate volume with increasing chelator concentration.

tivities in the acute carregeenan-pleurisy model (*Figure 2.28*) [232]. This compound is more active than desferrioxamine in this system.

Although iron chelators undoubtedly possess anti-inflammatory properties, selective direction of these molecules to the site of inflammation is a major problem at the present time.

Prevention of post-ischaemic injury by iron(III) chelators

Tissue injury observed following ischaemic anoxia probably results from oxidative processes [233]. This oxidative damage does not occur during the period of ischaemia, but during the period of reperfusion of the tissues. During ischaemia, mitochondrial oxidative phosphorylation ceases, cellular ATP levels drop and the concentration of the low molecular iron pool increases [233,234]. On reperfusion, the rapid increase in oxygen tension results in the production of a large pulse of hydroxyl radicals due to the elevated levels of the low-molecular-weight iron pool. This pulse, and its consequential damage, can be reduced by chelation of the iron pool by desferrioxamine.

The use of this chelator has reduced brain damage following cardiac arrest [234,235]. Thus, desferrioxamine was able to reduce the intracellular low-molecular-weight iron pool of dog brain to 65% of that of the ischaemic level (*Table 2.7*), but not down to that of the non-ischaemic controls. Simultaneously, a marked reduction of both malondialdehyde and conjugated diene levels (monitors of tissue damage) was observed. In principle, a chelator designed to efficiently permeate the blood-brain barrier would be predicted to be more efficient, and in principle the 3-hydroxypyridin-4-ones would appear to be good candidates. Significantly, 1,2-dimethyl-3-hydroxypyridin-4-one (22, $R^1 = R^2 = Me$) has been reported to prevent post-ischaemic cardiac injury in the rat [236].

Similar problems associated with ischaemia can occur during tissue trans-

Table 2.7 IRON AND LIPID PEROXIDATION FOLLOWING CARDIAC ARREST

	Low-molecular-weight iron	*Malondialdehyde*	*Conjugated dienes*
Nonischaemic controls	9.05 ± 4.04	7.32 ± 1.67	0.604 ± 0.121
Standard intensive care	37.04 ± 4.58	12.2 ± 1.90	1.243 ± 0.608
Standard intensive care + desferrioxamine	24.3 ± 3.40	9.4 ± 0.08	0.642 ± 0.521

plantation, and desferrioxamine has been demonstrated to facilitate the viability of rabbit kidneys subjected to ischaemia and reperfusion [237].

Treatment of anaemia with iron complexes

The comparison of iron(II) and iron(III) uptake by mammalian intestine has recently been the focus of intense research effort. In a study of 14 different iron preparations in man, Dietzfelbinger showed that the iron(III) preparations, without exception, had a lower bioavailability than iron(II) sulphate and were therefore of dubious therapeutic efficacy [238]. Similar conclusions have been reached by others [239]. Unfortunately, orally administered iron(II) sulphate generates hydroxyl radicals in the gastrointestinal tract of mammals [240]. This property, together with the associated acidity of iron(II) sulphate, may cause irritation and damage to the mucosa. A wide range of side-effects have been reported for iron(II) sulphate [241]. Thus, should an efficiently absorbed iron(III) complex be identified [242], it would be of therapeutic benefit.

As discussed in the section on bidentate ligands, the non-toxic hydroxypyrones (3,23) bind iron(III) forming water-soluble complexes. In the pH range 4–7, they possess a lower affinity for iron(III) than EDTA, and by virtue of the kinetic lability of such complexes, are able to donate iron to high-affinity binding sites, while minimizing nonselective binding to foodstuffs. Thus, iron presented as a maltol complex is relatively well absorbed [101,102]. In contrast, the presence of EDTA reduces iron absorption by the intestine. Ferric maltol is the only simple iron(III) preparation which compares favourably with iron(II) sulphate [103], and in contrast to iron(II) sulphate, there are few, if any, side-effects associated with the oral administration of ferric maltol. Consequently, patient compliance is likely to be superior with this iron preparation.

GROUP IIIA METALS

ALUMINIUM

Aluminium, the most abundant metal in the Earth's crust, is not essential to life. It has the smallest radius of the Group III metals (*Table 2.1*), and is nonredox active. Its chemistry is such that it can mimic iron(III), calcium(II) and magnesium(II), and consequently is toxic. However, from the time of its discovery in the first half of the nineteenth century until the early 1970's, it was generally regarded as non-toxic. This view has been modified by the studies of Berlyne [243], Thurston [244], Clarkson [245], Altrey [246] and their

co-workers, who established that aluminium accumulation can be associated with renal failure, retarded growth and encephalopathy. It is now generally accepted that aluminium, if absorbed, can be extremely toxic [247]. Studies on aluminium distribution are severely hampered by the lack of a convenient radionuclide, the quantification of the element being limited to atomic absorption and ICP analytical methods. There is a tendency to use ^{67}Ga as a marker for aluminium, but such practice is of limited use due to the rather different chemistries of the two cations (*Figures 2.3, 2.23, 2.24*). Aluminium, by virtue of its size, is frequently satisfied with tetrahedal coordination, possesses a 'harder' characteristic (*Figure 2.4*) and is kinetically less labile (*Figure 2.5*).

Gastrointestinal absorption of aluminium

The main site of absorption is the gastrointestinal tract, and although it is tempting to assume that aluminium is accumulated by mucosal cells via the iron uptake system, there is considerable evidence to the contrary [248]. Indeed, uptake is more likely to be achieved via citrate complexes [249]. Aluminium owes its presence in the diet to a number of factors, including water, plant materials and cooking utensils. Some domestic tap-water contains aluminium in relatively high concentration, due to the use of aluminium sulphate as a flocculent in the purification process. Aluminium cations coordinate and remove coloured organic materials present in surface waters. Aluminium levels in water are critically dependent on pH. At pH values above 6, aluminium hydroxide tends to polymerise, thus reducing its bioavailability. The aluminium content of some plant material is high, for instance tea [250,251], asparagus [252] and lentils [253], and may be rendered bioavailable by cooking. The use of aluminium utensils can also increase the aluminium content of the diet quite dramatically [254]. The average adult ingests between 5 and 40 mg per day [253,255], but typically $< 0.5\%$ is absorbed. Furthermore, in complete contrast to iron, aluminium is efficiently excreted by the kidneys, and consequently there is low retention in healthy adults. However, patients with impaired renal function, especially children, are susceptible towards aluminium toxicity. Infants can accumulate aluminium from cow's milk formulations [256,257], and premature babies from parenteral feeding [258]. Patients undergoing dialysis are also susceptible to aluminium accumulation either via the dialysate water [259], or oral phosphate binders [260]. Many dialysis procedures are unable to remove phosphate efficiently, leading to hyperphosphataemia, and the most commonly used phosphate binders are aluminium hydroxide preparations.

Biochemical and pathological effects of aluminium

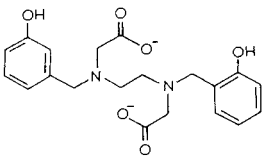
In principle, Al^{3+} can substitute for other hard metal cations such as Mg^{2+} and Fe^{3+}. Mg^{2+} acts as coenzyme for numerous enzymes, facilitating the binding of substrate to the enzyme. Al^{3+} can substitute for Mg^{2+}, and because of its reduced kinetic lability, would severely inhibit the activity of the enzyme [261]. Mg^{2+} is also essential for microtubule assembly and tubulin polymerisation, both of which are influenced by the presence of trace quantities of aluminium [249,262]. Such effects could induce serious disturbances to the normal performance of the cytoplasm of cells. Iron is essential for the synthesis of haemoglobin, and many iron-containing enzymes. Again, in principle, aluminium can substitute for iron and generate inactive enzymes. Aluminium possesses a high affinity for both phosphate and silicate functions [263,264], and depending on the pH of the environment and the levels of other competing cations, can lead to highly localized precipitations of aluminium complexes. Such precipitates could disturb normal cellular function. Elevated levels of aluminium cause microcytic anaemia [265,266] by inhibiting haemoglobin biosynthesis [267]. It is not established whether this effect is due to competition with Mg^{2+} or Fe^{3+}. Accumulation of aluminium in bone can occur in uraemic patients, especially in those undergoing chronic haemodialysis, and can induce osteomalacia. Many patients suffer from symptomatic bone disease with bone pain and/or bone fractures [268]. Aluminium binds to the calcification front [269], where it appears to inhibit mineralisation of osteoid, and because skeletal uptake of calcium is blocked, there is a tendency to hypercalcaemia [270]. Aluminium is also neurotoxic, intracerebral injection in animals leading to neurofibrillary degeneration [271,272]. Dialysis encephalopathy is probably related to this observation as the symptoms, namely, speech disorder, dementia and convulsions, appear after 3 to 7 years dialysis treatment. Increased levels of brain aluminium have been associated with this disease [246]. Alzheimer's disease has also been associated with elevated levels of aluminium [273]. Indeed, the high content of aluminium in the amyloid core of the plaque has added weight to this suggestion [274]. However, there are probably a number of endogenous and exogenous predisposing factors leading to senile dementia, of which elevated levels of aluminium may just be one factor.

The application of chelators to remove excess aluminium

Desferrioxamine was first introduced by Ackrill *et al.* [275] for the removal of aluminium in a patient suffering from dialysis encephalopathy. The improvement in the patient was quite marked, and the removal of aluminium was far

more efficient than that achieved by dialysis methods [276]. The procedure was extended to other patients, with improvement in both bone disease and anaemia [277]. These findings have been confirmed by other groups [278–280]. The affinity constant for the interaction of aluminium with desferrioxamine is 10^{22} M^{-1}, which although large, is smaller than the corresponding figure for iron(III), 10^{31} M^{-1}, and therefore, care must be taken in these non-iron-overloaded patients not to remove iron. The serum aluminium levels rise (between 2- and 10-times normal level) after desferrioxamine infusion, reaching a maximum after 24–48 h. (The aluminium complex of desferrioxamine is not cleared due to either ineffective or absent kidneys.) However, this complex is rapidly removed on dialysis. There are side-effects with the use of desferrioxamine [277], and it cannot be administered orally, but nevertheless it is a remarkably efficient pharmaceutical for the treatment of the adverse effects associated with acute aluminium accumulation. Desferrioxamine has also been shown to have a beneficial effect on aluminium-induced toxicity in rabbits [281].

A series of carboxylate chelators have been investigated by Yokel for their ability to remove aluminium from aluminium-overloaded rabbits [282]. 2,3-Dihydroxybenzoic acid, citrate and NTA (26) were found to be ineffective. Of the hexadentate ligands, including EDTA (25), DTPA (36), EDDHA (58) and HBED (44a), only EDDHA (58) was found to be effective when given orally. HBED and EDDHA had previously been considered for the treatment of aluminium toxicity [283], but HBED was found to be toxic [282]. As indicated in *Figure 2.29*, EDDHA (58) was much less effective than desferroxamine (33) when the latter was given parenterally. EDDHA (58), in contrast to DFO (33), also possesses appreciable affinities for Ca^{2+} and Zn^{2+}, and consequently, would be expected to facilitate the simultaneous removal of these metals. Although studies have indicated that the trismaltol-aluminium complex is extremely neurotoxic in rabbits [96], the 3-hydroxypyridin-4-ones have potential for removal of aluminium [84,86,96,97,284,285].



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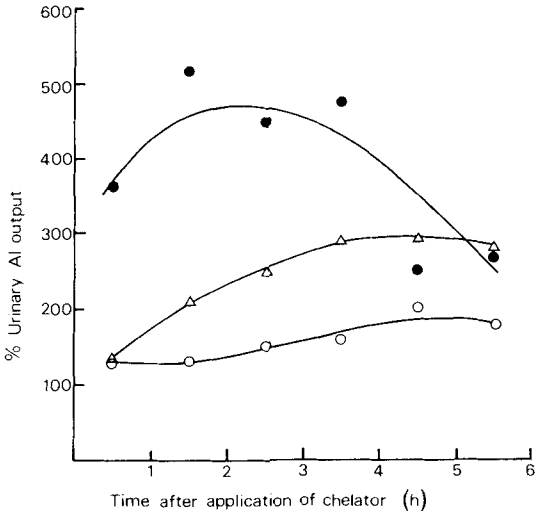


Figure 2.29. Chelator-induced aliminium excretion in the urine of aluminium-overloaded rabbits [281]. ●, *desferrioxamine, 150 μmol kg⁻¹;* ○, *EDDHA, 150 μmol kg⁻¹;* △, *EDDHA, 1500 μmol kg⁻¹.*

GALLIUM

Gallium has no known role in mammalian biochemistry, although its physical properties are such as to allow its extensive use in a wide range of radiopharmaceutical applications.

The three major gallium radionuclides which have found use in investigative nuclear medicine procedures are ^{67}Ga, ^{68}Ga and ^{72}Ga, although ^{72}Ga is rarely used today. ^{67}Ga was first produced for *in vivo* use in 1953 [286]. It is an accelerator-produced radionuclide, made by bombarding either a zinc target with protons [Zn (p,n) Ga], or a copper target with alpha particles [Cu (α,n) Ga] [287–289]. The resultant ^{67}Ga, which may be contaminated with ^{66}Ga, is purified by either ion-exchange or solvent extraction [290,291]. ^{67}Ga decays with a physical half-life of 78 h by electron capture, with no emission of beta particles, producing four gamma photons with energies of 92 keV (42%), 184 keV (24%), 296 keV (22%) and 388 keV (7%) [292]. Although all four photons are in an energy range suitable for detection by most commercial gamma camera systems, the poor abundance relative to ^{111}In (see section on indium) (only 93 photons per 100 disintegrations) means that 3-times as much ^{67}Ga is needed as ^{111}In in order to obtain equal count rates at a fixed detector

geometry [292]. Despite these disadvantages, ^{67}Ga has been used much more extensively than ^{111}In in clinical applications.

In contrast to ^{67}Ga, both ^{66}Ga and ^{68}Ga are positron-emitting radionuclides. The use of positron imaging (or positron emission tomography (PET) as it is more commonly known) in nuclear medicine has several advantages over single photon scintigraphic imaging (often referred to as single photon emission computed tomography – SPECT), particularly in investigations of organs located deep within the body. The physical properties of PET allows the accurate selection of different focal planes, quantitative activity determination and calculation of absorbed radiation doses, as well as advantages in terms of sensitivity and spatial resolution [293]. Due to the technical advances made in high resolution, multiwire proportional chamber positron camera systems since their development [294], the use of ^{68}Ga-labelled radiopharmaceuticals for dynamic or static imaging is likely to receive increasing attention, as spatial resolutions of a few millimetres appear possible. These abilities arise from the emission of positrons (β^+ particles), which have a very short mean free path, producing two high energy gamma photons on interaction with electrons. These two gamma photons (emitted at 180° to each other) are detected by dual-head detector systems, enabling the position of the radionuclide to be accurately determined in three dimensions. The development of increasingly sophisticated positron imaging devices has increased the need for radiopharmaceuticals labelled with positron emitters. Much work is carried out with radionuclides such as ^{11}C, ^{13}N, ^{15}O and ^{18}F, all of which are positron emitters which can be readily incorporated into biochemical moieties [295], allowing *in vivo* metabolic studies to be performed in real time. However, the short half-life of these nuclides limits their use to centres with access to a cyclotron. ^{68}Ga, in contrast, is available from a number of generator systems [296], and hence is likely to find application in a wider range of centres. ^{68}Ga, with its half-life of 68.3 min and β^+ energy of 1.9 MeV, is obtainable as a generator product from its parent nuclide, ^{68}Ge [297]. Generator systems are available which produce ^{68}Ga in either an ionic form or as a chelate complex. Until fairly recently, the only commercial ^{68}Ge-^{68}Ga generator was of the latter type, being eluted with an EDTA solution, to yield ^{68}Ga-EDTA [298]. This was suitable for brain and renal imaging studies, but for other applications it was necessary to remove the EDTA. Due to the high stability of the EDTA-gallium complex, procedures for removal of the EDTA were time-consuming and not totally effective, resulting in loss of usable radioactivity [299]. Coupled with this, the long working life of the generator of about 1 year (resulting from the long physical half-life of the ^{68}Ge parent of 275 days), presented problems with maintenance of generator sterility, due to elution at neutral pH [300]. These problems have since been

overcome by the introduction of a ^{68}Ge-^{68}Ga generator producing gallium in an ionic form. The generator consists of a tin dioxide column carrying ^{68}Ge, which is eluted with 1 M HCl, to yield ionic gallium chloride. High yields of ^{68}Ga are obtainable from such generators (typically 75%), and this, together with the extremely low levels of ^{68}Ge (a major radiation hazard to the patient due to its long half-life) present in the eluate, ensure its future in nuclear medicine.

Although the 68 min half-life of ^{68}Ga allows the use of this radionuclide in some procedures not accessible with shorter-lived positron emitters, its use is limited in situations where the labelling procedure is time-consuming, or where delays between radiopharmaceutical preparation and administration are unavoidable [301]. In these circumstances, the longer lived positron-emitting radionuclide of gallium, ^{66}Ga, may have advantages. ^{66}Ga is produced in a cyclotron by bombarding a copper target with α particles. It decays to ^{66}Zn by both positron emission (56.5% – 4.2 MeV) and electron capture, with a physical half-life of 9.5 h [302]. However, its use in diagnostic applications is somewhat limited by both the high positron energy and the presence of high-energy γ emissions (1.039 MeV – 37% and 2.753 MeV – 22.7%).

^{72}Ga, a reactor produced radionuclide, is rarely used today in clinical investigations. This is due mainly to its short physical half-life of 14.1 h, which obviously precludes its use in studies carried out over periods of more than a few hours, as well as the low specific activities typically attainable.

Clinical use

Initial studies on the biodistribution of gallium began in 1949, when ^{72}Ga lactate was administered to rats and rabbits [303,304]. Due to the non-availability of high-efficiency detection systems at this time, detection of the gallium was performed chemically, the results indicating that the majority of the gallium was deposited in bone. The same studies indicated that gallium is transported in the blood by plasma proteins, with most being removed from the plasma within 24 h of administration. Some activity is cleared through the kidneys, whilst the only other significant site of deposition, apart from bone tissue, is the liver. The first application of gallium radiopharmaceuticals to human disease appears to have been in 1951, when ^{72}Ga lactate was administered to 12 patients suffering from metastatic bone disease [305,306]. This confirmed the results of earlier animal work, indicating that the amount of gallium deposited in bone lesions was up to 20-times greater than that found in normal bone. In 1953, the disadvantages of ^{72}Ga prompted the replacement of this nuclide by the newly available ^{67}Ga, and a number of studies were conducted

using ^{67}Ga citrate [307], which was easier to prepare than gallium lactate [308]. Initially, these studies indicated that the amount of gallium deposited in bone tissue was less than had been observed with gallium lactate, although it soon became apparent that this was a function of the amount of gallium carrier present in the preparation. In 1969, the effect of gallium carrier on the efficacy of gallium citrate as a bone scanning agent was examined [309], indicating that gallium uptake in the bone was only enhanced when the plasma was 'saturated' with gallium. When 'carrier-free' gallium citrate was administered, the gallium was preferentially bound by plasma proteins, this binding being dependent on both the pH and citrate concentration. Despite the fact that this potentially toxic, non-specific deposition of gallium in the plasma suggested the abandonment of this agent for bone scanning, the same study highlighted another application for gallium citrate. It was observed that when 'carrier-free' gallium citrate was administered, the gallium was found to concentrate in soft tissue tumours [310]. This application now accounts for many of the clinical procedures involving gallium radionuclides.

Use of gallium citrate in tumour detection

Gallium, when administered as gallium citrate, has been shown to accumulate in a range of primary and metastatic tumours, although the mechanism of accumulation is unclear. Following intravenous administration, the majority of the gallium is bound to various plasma proteins, including transferrin, haptoglobin and albumin, whilst most uptake by blood cells is into leukocytes [311]. Within the first 12 h of administration, approximately 12% of the administered dose is cleared by the kidneys. After this stage, the major route of excretion is faecal. In normal patients, at 48–72 h after administration, most gallium is located in the liver, spleen and bone tissue [312].

The uptake of gallium into tumour tissue depends on several factors, including metabolic activity and rate of growth of the tumour, its size and histology, the relative extent of vascular and necrotic tissue within the tumour, as well as patient-related factors, such as the state of the immune system and the types of prior treatment received [313]. There is strong evidence from clinical studies that the site and histology of the tumour have a profound effect on the extent of uptake of the ^{67}Ga. This limits the use of gallium citrate as a diagnostic aid to only a limited range of tumour types, discussed briefly below.

Tumour types usefully imaged with gallium citrate

Gallium citrate has proven useful in determining the extent of Hodgkin's disease, particularly in detection of tumours of the neck and thorax [314]

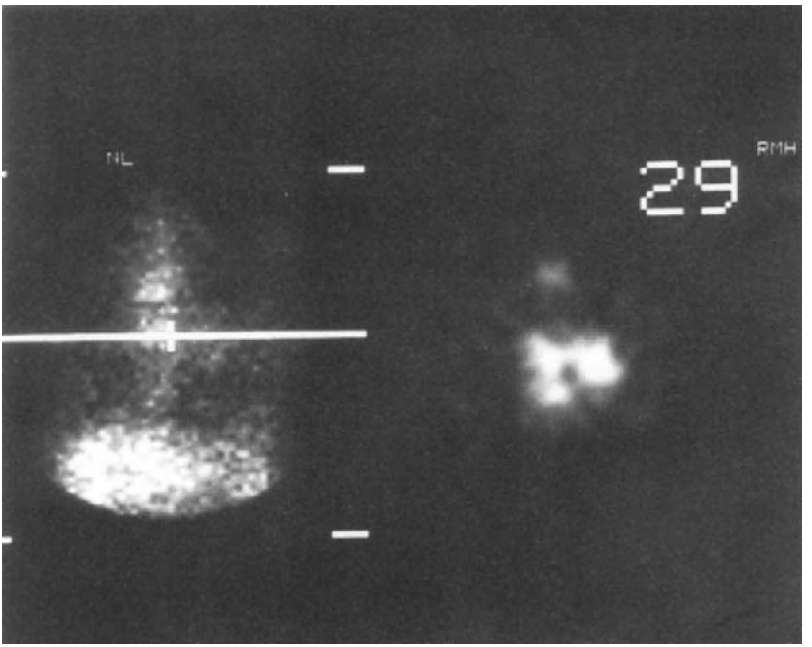


Figure 2.30. Scintigraphic images showing left planar view, right transaxial section of ^{67}Ga citrate uptake in a patient with Hodgkin's lymphoma. The image shows high uptake at the tumour site, as well as increased uptake of gallium by the sternum and spleen.

(*Figure 2.30*). The overall detection efficiency, as monitored by surgical procedures, is between 70 and 80%, with a very low incidence of false-positive results. The detection of tumours found below the diaphragm is, however, less efficient, being of the order of 50%, due to the high background levels in the abdomen arising from the high uptake of gallium by the liver. Although the primary use of gallium citrate in this condition is in lymph node scanning, it also plays a vital role in the detection of extralymphatic tumours. Bronchogenic carcinoma is detected with high efficiency using gallium citrate [315]. Studies have indicated that up to 85% of lesions in patients suffering from such carcinomas have been detected. In addition to the detection of primary disease, other uses include the evaluation of therapeutic procedures, as well as the detection of recurrent disease. Initial studies on the use of ^{67}Ga in melanoma did not produce encouraging results [316]. The specificity of detection was extremely high (up to 98%), whilst the sensitivity was disappointing at 50% or less, the remaining being false-negative results. Nevertheless, the presence of

sites of uptake which cannot be correlated with areas of inflammation is almost diagnostic of sites of metastatic disease. The usefulness of this procedure in the detection of metastatic melanoma is not diminished by its inability to produce clear images of metastases in the liver or lungs, as alternative means can be used in these specific instances. ^{67}Ga imaging is also used in the detection of hepatomas [317]. Whilst most hepatomas exhibited uptake of gallium equal to or greater than normal tissue, the major use has been in the differentiation of true hepatoma from cirrhotic pseudotumours. These pseudotumours do not demonstrate gallium uptake to any significant extent, resulting in 90% detection efficiencies. However, gallium uptake cannot be used to differentiate hepatoma from either abscesses or metastases, and in these instances, the diagnosis must be made on the basis of both the gallium citrate and a technetium sulphur colloid scan. Although gallium citrate has been used only rarely in investigations of leukaemic patients, it has proved to be of benefit in the detection of focal leukaemic masses, in monitoring response to various therapeutic regimens, as well as in the identification of concurrent inflammatory disease [318]. Other uses of gallium citrate include applications in detection of Burkitt's lymphoma [319], non-Hodgkin's lymphoma [320,321], in addition to a fairly small scale use in detection of brain tumours [322]. In this last instance, gallium citrate can be used as an adjunct to ^{99}Tc^{m} pertechnetate scanning, which does not specifically detect tumour, but rather areas of vascular lesions resulting from such tumours.

Tumour types poorly visualized with gallium citrate

In general, tumours of the head and neck are poorly visualized using ^{67}Ga. This is mainly due to increased uptake of gallium in the nasopharynx, which tends to mask tumours located in this region [323]. Carcinoma of the thyroid is visualized in only approximately 40% of cases, whilst benign thyroid lesions may also exhibit increased gallium uptake, rendering gallium citrate so non-specific as to be inadequate for routine use in evaluation of thyroid disease [324]. With the single exception of the liver, the sensitivity of gallium for the detection of malignant diseases of the gastrointestinal tract and related organs is unusably low, often being of the order of 15% [325]. The same is true for lesions throughout the majority of the genitourinary tract. Breast tumours are inadequately visualized with the use of gallium citrate, due to the relatively high uptake of gallium by normal breast tissue [326]. This is probably due to the increased levels of lactoferrin present.

Mechanism of gallium localization in tumours

A number of hypotheses have been proposed to account for the mechanism by which gallium localizes in tumour cells, although in general, these are highly speculative and controversial. One early explanation suggested passive diffusion of the gallium through the cell membrane [327,328]. Indeed, the highly permeable walls of tumour blood vessels, together with the large volume of extracellular fluid associated with many tumours, may play a part in the initial localization of gallium at the tumour site. Transferrin is likely to play a major role in gallium transport, as it has been demonstrated to possess 14 binding sites for colloidal gallium in addition to the two 'true' binding sites for ferric ions, to which gallium(III) will also bind [329] (see section on transferrin). However, the extent to which transferrin is intimately involved in entry of gallium into tumour cells is unclear. It has been suggested that free, 'ionic' gallium in the plasma becomes incorporated into the tumour rather than transferrin-bound gallium [330]. Support for this hypothesis comes from studies which indicate that the uptake of gallium into tumour cells increases as the available transferrin binding sites are saturated with other trivalent metal ions, such as scandium or iron [330–332]. It should be noted, however, that under physiological conditions, gallium will not exist in an ionic form as Ga^{3+}, but rather will be present as a mixture of the hydroxy complexes $Ga(OH)_3$ and $Ga(OH)_4^-$, together with some oligomeric anions [333].

Contradictory evidence relating to the involvement of transferrin in the localization mechanism has shown that the uptake of gallium by tumours is dependent on the transferrin concentration, initially increasing as the plasma transferrin concentration increases. Above a certain transferrin concentration, gallium uptake decreases, possibly due to saturation of the membrane bound transferrin receptors [315,331]. The subcellular localization of ^{67}Ga in tumour cells also supports the involvement of transferrin. Once taken up into the cell, the majority of the gallium is located in the cytoplasm, within lysosomes, in both normal liver and tumour cells [334,335]. Gallium-transferrin, if exposed to such an environment, would dissociate, leaving the gallium to be distributed to other intracellular sites, such as ferritin, which has been shown to be responsible for the storage of small amounts of ^{67}Ga [336]. In addition, lactoferrin, an iron transport protein related to transferrin, has also been shown to bind high levels of ^{67}Ga *in vivo* [337]. It has been suggested that lactoferrin may play a role in gallium accumulation in certain specific tumour types, such as Hodgkin's disease, Burkitt's lymphoma and melanoma [338], as well as being responsible for gallium accumulation in certain normal tissues, such as breast, and biological fluids such as milk, tears, seminal fluid and mucus [339].

Recent studies on the effect of both carrier gallium and citrate concentration on the uptake of ^{67}Ga by tumours indicated that the rate of entry of 'carrier-free' ^{67}Ga citrate into the tumour followed the simple physical diffusion of citrate into the cell [340]. This diffusion is inhibited by the presence of carrier gallium, which also significantly reduces the extent of radiogallium uptake. Such results appear to support the hypothesis that increased membrane permeability of tumour cells is the principal mechanism for gallium accumulation. Concanavalin A (a membrane-active agent which modifies membrane permeability as a result of structural changes caused by its binding to membrane glycoproteins), has also been shown to increase tumour cell uptake of gallium [341], providing further support for this hypothesis.

Alternative mechanisms, although apparently incompatible with the membrane permeability hypothesis, are difficult to ignore. For example, it has been demonstrated that over 50% of the gallium associated with tumour cells is bound to a protein having a molecular weight of approximately 40,000 [342]. This protein appears only to be produced by tumour cells, or is produced by normal cells in quantities too small to be detectable. Other workers have demonstrated in virally transformed tumours the presence of siderophore-like growth factors which have a high affinity for tripositive metal ions, such as iron and gallium [343], although the widespread importance of such molecules is at present unclear.

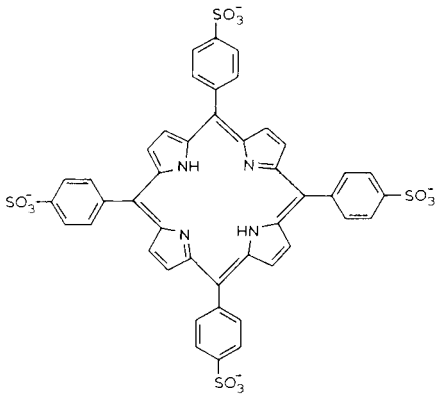
In view of the mass of evidence supporting most of these apparently contradictory mechanisms, it is probable that there is no single mechanism responsible for gallium accumulation in all tumour types, and that there are several different mechanisms involved. Indeed, the uptake mechanism(s) may not be gallium-specific. Most tumour cells contain raised levels of iron-dependent enzymes, such as ribonucleotide reductase, and the observed similarities between the kinetics of iron and gallium within the tumour may indicate that the gallium is simply riding on a pathway designed to increase the uptake of iron by the cell. If this were indeed the truth of the matter, it is perhaps somewhat surprising that other tripositive metals similar to iron, such as indium, do not show such a marked accumulation in tumours.

Alternatives to gallium citrate have been investigated, in an attempt to increase the amount of ^{67}Ga which can be delivered to tumours. In the main, these rely on the use of liposomes to prolong the serum half-life of the gallium complex. Two major approaches have been adopted, namely either the straightforward incorporation of gallium into liposomes, or the labelling of preformed liposomes with bifunctional chelates. A recent report of liposome labelling with gallium uses NTA (26) as the complexing agent [344]. When incorporated into small unilamellar vesicles, more gallium could be delivered

to the tumour than with gallium citrate. This was due to uptake of the liposomes by the tumour, although as much as 40% of the accumulated activity arose from direct uptake of gallium-NTA, after leakage from the liposome. This leakage of the gallium complex from the liposome poses a potential problem in that whilst gallium in the plasma is available for tumour uptake, it is also open to excretion, either via the kidneys or bile. This was a major problem with liposomes labelled with gallium oxine [345]. The success of oxine as a cell-labelling agent arises from its ability to readily permeate biological membranes (see section on indium), although this also results in the rapid loss of gallium oxine from liposomes. In an attempt to overcome this problem, DTPA (36) has been covalently attached to a long hydrocarbon chain, such as that in octadecylamine, which is subsequently incorporated into liposomes during their preparation [346]. Liposomes prepared in this way may be labelled with ^{67}Ga at virtually 100% efficiency by standard methods, the labelled product showing considerably greater *in vivo* stability than gallium-oxine labelled liposomes. Increased tumour uptake is obtained as a direct result of liposome uptake by the tumour [345].

There have been few reports of the labelling of proteins with gallium radionuclides. Studies have examined the carbodiimide coupling of DFO (33) to human serum albumin [347], although there are few indications that this approach will be extended to other proteins.

The uptake of porphyrin derivatives by tumours has attracted much attention following the observation that administration of haematoporphyrin to tumour bearing animals led to intense fluorescence in the tumour (see section on photosensitizing agents). This uptake was not tumour-specific, but occurred in all rapidly dividing tissues, with greater uptake in kidneys, liver and spleen than in tumour [348]. In an attempt to increase tumour uptake, a synthetic, water-soluble porphyrin was prepared, tetraphenylporphyrin sulphonate (TPPS) (59), which was shown to accumulate in tumours in rats [349,350]. However, labelling of TPPS with either ^{57}Co or ^{111}In resulted in the loss of a large proportion of its tumour affinity [351], probably due to a change in the net charge on the molecule [352]. The tumour uptake of tetrasulphophthalocyanine (TSPC) (60), and its γ-emitting metal complexes has also been investigated [353]. On administration of ^{67}Ga-TSPC to rats, the majority of the activity was found to pass through the hepatobiliary system, with activity residing in liver, kidneys and spleen. Despite the fact that overall uptake of activity by the tumour was not as high as found with gallium citrate, the tumour-to-blood and tumour-to-blood and tumour-to-muscle ratios were greater for Ga-TSPC than for Ga citrate [353]. This is due primarily to an increased rate of clearance of Ga-TSPC from the blood stream, when compared with gallium citrate.



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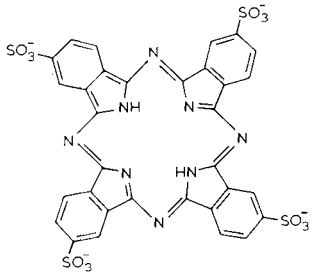

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Other clinical applications of gallium-67

Although the early clinical use of ^{67}Ga was centred on tumour imaging, its potential use in the detection of occult inflammatory lesions was also investigated [354]. Indeed, the current use of radiogallium (as the citrate complex) for diagnosis of inflammatory disease probably exceeds that for the evaluation of malignancies. This technique is of most use when applied to specific anatomical regions, as detailed below.

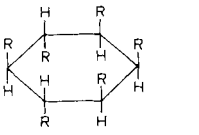
Detection of inflammatory lesions of the lung has been particularly successful with gallium [355], localization being observed in virtually all pulmonary lesions in which inflammation is present as either a primary or secondary phenomenon. Disease states typically involved are pneumonia [356], tubercular or mycotic granulomatous lesions [357], pneumoconiosis [358], sarcoidosis [359], abscesses and idiopathic fibrosis [360]. In contrast, gallium uptake is not observed in cases of uncomplicated pulmonary infarction [361]. From a clinical viewpoint, the most useful applications of non-tumour related gallium scans of the lungs are related to the early detection of opportunistic infection, in staging idiopathic pulmonary fibrosis, the evaluation of various sarcoidosis treatments, and differentiation of areas of pulmonary infection from areas of infarction. Although the use of gallium in the detection of other pulmonary lesions has been reported, the clinical significance is doubtful. For instance, despite reports of the use of gallium scanning as a means of differentiating active and inactive tuberculosis [362], very few clinical studies have been performed.

A large number of gallium studies have been performed on the detection of

intra-abdominal infection [363–367]. Even though a wide range of protocols have been used, the sensitivity and specificity of detection both appear to exceed 90%. The gallium scan can be used to detect both abscesses and peritonitis [368,369], with advantages over the use of ^{111}In-labelled neutrophils in that no significant difference has been found in the extent of gallium accumulation in abscesses of varying ages, rendering the detection of old abscesses more reliable. In an attempt to improve the abscess to soft-tissue distribution ratio of gallium, some synthetic tricatecholamide siderophore analogues of enterobactin (41), such as LICAM-C (55c), have been investigated [370]. These have potential clinical applications, due to their ability to decrease gallium levels in all tissues apart from liver and spleen, these latter organs being labelled with colloidal gallium hydroxides, which cannot be sequestered by the siderophore. Although the gallium levels in abscesses are slightly reduced by LICAM-C, the overall increase in distribution ratio more than compensates. An additional advantage of this approach, is that the gallium mobilized from the tissues is rapidly excreted via the kidneys, thus reducing the radiation burden to the patient.

The use of gallium in the detection of inflammation at other sites, such as joints and the kidneys, has been reported [371,372], although such uses currently account for only a small percentage of the use. As with gallium uptake by tumours, the mechanism by which gallium localizes at sites of infection is also unclear, although three possible explanations have been proposed. These include leukocyte labelling, lactoferrin localization at the site of infection, and direct bacterial uptake. ^{67}Ga has been demonstrated to be incorporated into leukocytes [373], which in turn localize at the site of inflammation. Indeed, the absence of leukocytes is associated with diminished gallium uptake [374], although it is doubtful that this fully explains the degree of localization observed. Lactoferrin (see section on transferrin) may be involved via its presence in high concentrations in leukocytes [375]. When leukocytes localize at inflammatory sites, they not only ingest bacteria, but also secrete vesicular contents, including lactoferrin [376]. This released lactoferrin tends to remain at the site, attached to receptor sites on tissue macrophages. Any gallium entering this region, possibly via leakage through permeable blood vessels often associated with inflammatory sites, will be retained by complexation with the apolactoferrin. Direct uptake of gallium by bacteria is assumed to involve siderophores [377,378], produced by most pathogenic bacteria to enable them to survive in the low (free) iron environment of the body. As in the case of gallium uptake by tumours, it is believed that a combination of mechanisms is responsible for uptake into inflammatory sites.

^{67}Ga, as the gallium-phytate complex, has also been used for the imaging of



$R = PO_4^-$

(61)

lymph nodes [379]. Phytate (1,2,3,4,5,6-cyclohexanehexaphosphate) (61) is a multidentate ligand often used for the removal of trace quantities of heavy metals. Upon intravenous administration of the phytate complex, colloidal particles are formed by interaction with calcium ions in plasma. This application, despite producing useful clinical results, appears not to have been followed up.

Gallium-67 citrate is also used to aid diagnosis of primary amyloid nephrotic syndrome [380,381]. However, this technique provides no additional information over that obtainable from conventional total urine protein measurements. The mechanism of gallium accumulation within amyloid deposits is unknown, although reports that gallium is mimicking the calcium uptake by glycoprotein amyloid P [381], should be treated with caution.

Although currently of little clinical importance, the complexation of gallium by 3-hydroxypyridin-4-ones (15) has been reported [382], indicating that the complex distributes significantly differently from gallium citrate, with most of the 3-hydroxypyridin-4-one complex being cleared via the kidneys within 20 min, as compared with 24 h for similar clearance of the citrate complex. This difference is attributed to the high stability of the hydroxypyridinone complex ($\beta_3 \approx 38$ for gallium) compared with citrate, resulting in little loss of gallium to transferrin.

Clinical applications of gallium-68

Many of the remaining uses of gallium radionuclides utilize ^{68}Ga, a positron emitting nuclide. Complexes of ^{68}Ga have been used for brain tumour scanning [383] (*Figure 2.31*), bone scanning [384], measurement of glomerular filtration rate [385] and for blood cell labelling [386], although arguably one of the most important potential applications is in the measurement of myocardial and cerebral blood flow [295,387,388]. A variety of techniques have been described for the measurement of cerebral blood flow, of which the use of labelled albumin microspheres remains the standard. Whilst microspheres are the standard with which other tracers are compared, the measurement of cerebral blood flow in

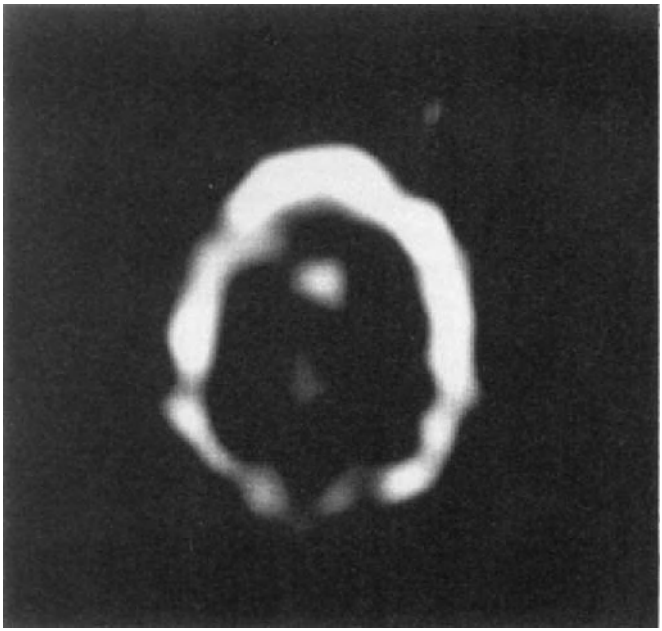


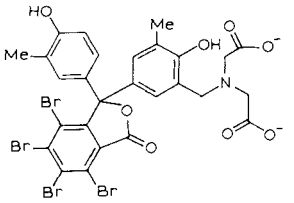
Figure 2.31. Positron emission tomographic image obtained from a 6 mm thick transaxial section through the brain of a patient with a cerebral lymphoma, using ^{68}Ga-EDTA. The image shows breakdown of the blood-brain barrier at the site of the tumour.

patients is no longer performed clinically with arterially administered microspheres, due to the high risk of potential embolization. Several methods have been described for the labelling of microspheres with positron-emitting nuclides such as ^{11}C and ^{68}Ga, which in general use either a direct labelling [389–391] or bifunctional chelate approach [387,392]. The use of ^{68}Ga allows the quantitative measurement of regional blood flow, assuming that the label is sufficiently stable *in vivo*. Typical of the bifunctional chelate approach, is the use of DTPA, the mixed anhydride of which can be coupled to human serum albumin [387]. This derivatized protein may then be incorporated directly into albumin microspheres, and subsequently labelled with ^{68}Ga. Despite the fact that many of the literature labelling methodologies give very high labelling efficiencies (typically $>90\%$), the *in vivo* stability of many of these complexes is not acceptable. For instance, 1 h after intravenous administration of the labelled microspheres, the percentage of gallium lost ranges from 2% (for the bifunctional DTPA label) up to 15%, the majority of this gallium being bound

to plasma transferrin [387,392]. In order to obtain accurate blood flow measurements, increased *in vivo* stability is required, and this has been achieved by the use of procedures involving direct labelling of the protein with gallium (via the amino-acid side-chains), without the use of a gallium chelate. Despite the very low labelling efficiencies typically attainable when using such methods, less than 0.2% of the administered activity was removed from the microspheres during a 2 h distribution study in animals [388].

Other uses of ^{68}Ga labelled radiopharmaceuticals include imaging of the reticuloendothelial system in the liver and the spleen with ^{68}Ga-labelled dihydroxyanthraquinones, such as alizarin and alizarin red [393]. In contrast to other ^{68}Ga-colloids proposed for liver scanning, the preparation of ^{68}Ga-alizarin requires no inorganic carriers such as ferric hydroxide, stabilizers such as gelatin, or time-consuming heating procedures. Reproducibility of the colloid size, coupled with the ease of preparation commend this agent for routine use. This complex is also extremely stable, evidence suggesting that it possesses a higher affinity constant than the gallium-EDTA complex [394].

An alternative agent for assessment of hepatobiliary function is an iminodiacetate derivative of tetrabromo-*o*-cresylphthalein (BP-IDA) (62) [395], which is known to be selectively removed by the hepatobiliary system. This agent has been found to bind gallium extremely tightly. The ability to determine absolute ^{68}Ga activity in the liver by tomographic imaging, the high level of liver uptake (> 60%), rapid biliary clearance (> 75% within 1 h), low urinary clearance (< 1%) and weak competition for gallium from bilirubin, all suggest that this agent has promise for the quantitative study of hepatobiliary function.



(62)

The major drawback to the use of cationic positron emitting radionuclides for brain imaging is their inability to cross the blood-brain barrier, either due to their hydrophilicity or to the high molecular weight of the complex [218,396]. A number of low-molecular-weight ligands, based on TACN (48, R = H),

which have a high affinity for gallium and indium, have been reported [138,397]. The amino-thiol ligand, TS-TACN (48c), is of particular interest, as it possesses a low molecular weight for a hexadentate ligand, and yet appears to present a sufficiently stable coordination environment to both gallium and indium, that the complexes are stable *in vivo*. There is little or no loss of the metal ion to transferrin [397,398], with the complexes being cleared rapidly via the liver. The complex is not particularly hydrophobic (log K_{part} (octanol/water) = 0.06), presumably due to its compact size, and consequent exposure of the N and S donor atoms to solvent. However, macrocyclic derivatives are being investigated, which are predicted to be more hydrophobic [397].

INDIUM

As with gallium, indium has no known role in mammalian biochemistry. However, three indium radionuclides, namely ^{111}In, ^{113}In^{m} and ^{114}In^{m}, are used in nuclear medicine, although the current use of ^{113}In^{m} is not extensive.

^{111}In, which has ideal physical characteristics to allow scintigraphic images to be obtained up to 1 week after administration of the radiopharmaceutical, is produced in a cyclotron by bombarding either a cadmium target with protons or a silver target with alpha particles [399–401]. ^{111}In decays by electron capture to stable ^{111}Cd with a 67.5 h physical half-life, emitting two gamma rays in cascade in very high abundance (173 keV – 89%, and 247 keV – 94%), in addition to internal conversion electrons, X-rays and Auger electrons [402]. Both photons, which are produced in high yield (183 photons per 100 disintegrations), are in the desirable energy range for use with commercially available gamma-camera systems [403]. A prerequisite for several clinical imaging procedures is a delay of between 1 and 5 days following radiopharmaceutical administration and subsequent imaging [404]. This allows time both for maximum accumulation in the target tissue, and clearance of reasonable amounts of background activity. The physical half-life of ^{111}In is ideally suited to such studies.

^{113}In^{m} is commercially available in the form of a ^{113}Sn-^{113}In^{m} generator. The parent ^{113}Sn decays by electron capture with a half-life of 118 days to ^{113}In^{m}, which in turn decays by isomeric transition with a half-life of 1.66 h to stable ^{113}In [405]. The 118 d half-life of the parent results in this type of generator having an effective life of approximately 6 months [406]. The gamma ray emitted during the decay of ^{113}In^{m} has an energy of 390 keV. This low radiation dose to the patient permits the use of millicurie amounts of the radionuclide, providing a high photon flux, although some limitations are presented by the high internal conversion ratio [407].

Table 2.8 CLINICAL APPLICATIONS OF TRIPOSITIVE METALS

Application/organ system	*Metal*	*Ligand or carrier*
Abscess detection	^{67}Ga	citrate
	^{111}In	leukocytes
Antigenic tissue imaging	^{67}Ga, ^{111}In	bifunctional chelate labelled antibodies
Blood – plasma protein volume	^{68}Ga	transferrin, DTPA-albumin
– red blood cell volume	^{68}Ga	erythrocytes
Bone marrow imaging	^{67}Ga, ^{111}In	Cl^-, transferrin
	^{157}Dy	HEDTA
Bone scanning	^{68}Ga	EDTMP, polyphosphate
	^{72}Ga	lactate
	^{111}In, $^{113}In^m$	EDTMP, HMDTP, DTPMP
	^{153}Sm	EDTMP
	^{157}Dy, ^{167}Tm	HEDTA
Brain – cerebral perfusion	^{68}Ga	EDTA, albumin microspheres
– detection of blood-brain barrier defects	^{68}Ga	EDTA
	^{111}In	DTPA
	^{169}Yb	EDTA, DTPA
	Gd	DTPA
Cerebro-spinal fluid – cisternography	^{111}In	DTPA, HSA, transferrin
Heart – cardiac perfusion	^{68}Ga	albumin microspheres
Hepatobiliary function	^{68}Ga	alizarin
		BP-IDA
		3,4-DiP-LICAM
		Br-EHPG
		Br-HBED

Kidney – renal function	^{68}Ga	PLED, EDTA, polymetaphosphate
	^{111}In	DTPA
	^{169}Yb	EDTA, DTPA
Liver/spleen/reticulo-endothelial system imaging	^{68}Ga	alizarin, Ga/Fe hydroxide colloid
	^{111}In	erythrocytes
	Fe	ferrite particles
Lung – pulmonary perfusion	^{68}Ga	albumin microspheres
– scintigraphy	^{111}In, $^{113}In^{m}$	In/Fe hydroxide colloid
	$^{113}In^{m}$	albumin microspheres
Lymph node imaging	^{67}Ga	phytic acid
	^{111}In	lymphocytes, In/Fe hydroxide colloid
Thrombus localization	^{68}Ga	platelets
	^{111}In	platelets, fibrinogen
		anti-myosin monoclonal antibody
Tumour – imaging	^{67}Ga	citrate, TSPC
	^{111}In	Cl^{-}, bleomycin
	^{111}In, $^{113}In^{m}$	EDTA, DTPA
	^{169}Yb	citrate
	Gd	DTPA
Tumour – therapy	^{90}Y	bifunctional chelate labelled monoclonal antibody
	Fe, Al, Ga	ClAlPc, ClAlSPc

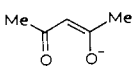
Indium was introduced into routine use in nuclear medicine in 1966, when $^{113}In^{m}$ was first used to image lungs and liver [408–410]. Since then, however, its use has become much more widespread, and indium radiopharmaceuticals are currently used in a range of imaging procedures (see *Table 2.8*).

CLINICAL USES OF INDIUM

Cell labelling

Although blood-cell labelling was used prior to the introduction of indium chelates in the mid-1970s, the radionuclides used (^{51}Cr, ^{67}Ga and $^{99}Tc^{m}$) were not as suitable for this type of study as ^{111}In [411]. For instance, the physical half-life of ^{51}Cr (27.7 days) is far longer than the imaging period required, resulting in an unnecessarily high radiation dose to the patient. In addition to this, although the emitted gamma photon has a suitable energy for imaging (320 keV), it is only produced in 10% of the disintegrations. In comparison, $^{99}Tc^{m}$ has ideal physical characteristics for imaging, but the cell labelling efficiency using technetium (as pertechnetate) is low, presumably due to the charged nature of the species [412].

Ionic indium, with a charge of 3+, will not penetrate cell membranes. However, indium can be coordinated by a range of bidentate chelating agents to yield an electrically neutral tris complex (see section on coordination chemistry). Depending on the nature of the bidentate ligand used, the properties of the final complex can be manipulated to produce a species which is sufficiently lipophilic to permeate membranes. Currently, a range of ligands are available for cell labelling – (6), (7), (19), (63), (64). Of these, the most widely used is 8-hydroxyquinoline (oxine) (6). This was first used to label cells with indium in 1976 [413]. Since then, a range of alternative ligands have been developed and have, in some centres, replaced oxine as the labelling agent of choice [414]. Of the other ligands available, acetoacetonate (63) gives clinical results closely comparable with oxine [415], but both suffer from the major drawback that cell labelling cannot be performed in the presence of plasma. Of the alternative chelating agents, both tropolone (7) and 2-mercaptopyridine *N*-oxide (19) have been shown to label cells in plasma [416,417], although to date, insufficient



(63)

(64)

clinical data are available for 2-mercaptopyridine *N*-oxide (19) and the alkyldithiocarbamates (64) [418] to suggest whether they will eventually replace oxine and acetoacetonate. However, this must be considered a possibility, in view of the cellular toxicity of both oxine and tropolone [419,420].

All of the ligands currently used for cell labelling form neutral, lipophilic complexes with indium, and with the exception of indium oxine, all are readily soluble in water [411,416,417,421]. The mechanism by which cells are labelled using such complexes is thought to involve the passive diffusion of the complex across the plasma membrane into the cell [422,423]. Here, the complex dissociates, allowing the free ligand to diffuse back out of the cell. The indium subsequently becomes bound to a macromolecular species within the cell, as evidenced by the presence of protein-bound indium found on cell lysis [422,424,425]. This ensures that on reinjection of the labelled cells into the circulation, only very small amounts of indium are released, typically 2–10% of the total [425–427].

The inability to label blood cells in the presence of plasma using indium complexes formed with ligands such as 8-hydroxyquinoline (6) and acetoacetonate (63), is due to the preferential binding of indium to plasma proteins such as transferrin, and to a much lesser extent, the α_2-globulins [428]. In contrast, the newer ligands such as tropolone (7), 2-mercaptopyridine *N*-oxide (19) and the alkyl dithiocarbamates (64) do label cells in the presence of plasma, although the amount of indium incorporated into the cells is less in the presence of plasma than in its absence [416]. This is due to the interaction with plasma globulins being reduced rather than eliminated completely. This lower labelling efficiency attained in plasma is preferable, however, as certain classes of blood cells, including platelets and leukocytes, do not behave normally when removed from plasma [429,430]. Unlike other radiopharmaceuticals used for cell labelling, such as $^{99}Tc^{m}$ sulphur colloid and [^{3}H]thymidine, none of the indium complexes are selective for particular classes of blood cell. Thus, labelling in whole blood leads to the non-specific incorporation of indium into all cell types. In order to obtain the clinical information required, it may be necessary to isolate a particular cell sub-type prior to labelling, using procedures described in detail elsewhere [411].

Indium-labelled blood cells are currently used for a wide variety of diagnostic and cell kinetic studies [411]. The majority of these techniques use ^{111}In, as the kinetics of uptake of the labelled cells are such that the half-life of $^{113}In^{m}$ is too short for many applications. Labelled lymphocytes have been used to visualise the lymph nodes in patients suffering from Hodgkin's disease [427], sites of chronic inflammation associated with joint disease [431], as well as the measurement of blood clearance rates from patients suffering from lymphomas

and leukaemias [431,432]. ^{111}In-labelled platelets have been shown, in a series of normal volunteers, to behave in the same way as unlabelled platelets, and have been used in patients with thromboses or atherosclerosis to visualize the sites of the lesions [433]. The International Committee for Standardization in Haematology (ICSH) has recently published guidelines for the recommended method for platelet survival studies, recognizing the advantages of using ^{111}In to label platelets in preference to ^{51}Cr [434]. Granulocytes localize in sites of inflammation and infection, and have been used to aid in the diagnosis of abscesses [435], lesions in inflammatory bowel disease, including Crohn's disease and ulcerative colitis [436], and osteomyelitis [437]. Indium labelled erythrocytes are not widely used clinically, the more established methods of ^{99}Tc^{m} labelling still finding favour, despite evidence that ^{113}In^{m}-acetoacetonate labelling provides better results in the measurement of red-cell volume or splenic red-cell pool [426].

The use of indium complexes as cell labelling agents does, however, have limitations. For the results obtained from such studies to have clinical relevance, the labelled cells must behave identically to normal cells. However, the process of labelling can damage cells in one of three ways. The use of indium-oxine or indium-acetoacetonate requires the cells to be labelled in a plasma-free environment. Mechanical damage to the cells can then arise either from changes caused by the absence of plasma, or as a result of the *in vitro* manipulations necessary to isolate the particular cell class to be labelled. Chemical damage can be caused by some of the ligands used to form the indium complexes, 8-hydroxyquinoline (6) and tropolone (7) being particularly toxic to cells [419,420]. Lastly, radiation damage to the cells can, potentially, present very serious problems. Lymphocytes, in contrast to other types of blood cells, are long-lived cells capable of division, making them radiation-sensitive. Radiation damage, incurred as a result of labelling with indium complexes, is capable of inducing a mutagenic change which, on reinjection of the cells, could result in the formation of a tumour [438,439]. Indeed, the radiotoxicity of ^{111}In-oxine has been reported to be far greater than the chemotoxicity of oxine [420], being related to the accumulation of indium in the cell nucleus [431]. This finding may have serious implications in view of the large amounts of indium reported to localize in the nuclei of labelled cells undergoing replication ($\approx 30\%$ in lymphocyte nuclei with indium-tropolone and $\approx 80\%$ with indium-oxine) [420].

Cisternography

Cisternography is the study of the flow of cerebro-spinal fluid (CSF), typical studies requiring an imaging period of between 48 and 72 h [440], for which

^{111}In is ideally suited. Radiopharmaceuticals labelled with ^{111}In were first used to study CSF in the early 1970s [441], in response to the problems associated with ^{131}I-human serum albumin (HSA), the agent of choice at that time. Up to 41% of patients suffered a febrile response to the radiopharmaceutical, due to the presence of contaminating pyrogens in the HSA [442]. Alternative radiopharmaceuticals investigated were indium complexes of EDTA (25), DTPA (36) and transferrin [443,444], as well as other EDTA and DTPA chelates [445]. Simultaneous studies with ^{131}I-HSA and ^{111}In-transferrin showed that the distribution of the two complexes in the CSF was identical [443]. The indium-EDTA and -DTPA complexes, administered intrathecally, also showed similar distribution, although differences were apparent in the rate of urinary clearance. Approximately 65% of both ^{111}In-chelates appeared in the urine within 24 h, increasing to 85% at 3 days, in contrast to ^{111}In-transferrin, which showed less than 5% urinary excretion of indium at 7 days [443]. This high excretion rate with the carboxylate ligands was not, however, disadvantageous to imaging. Although the amount of activity present in the CSF was lower at imaging with the ^{111}In-EDTA and -DTPA, the clearance of the background activity via the kidneys improved the image contrast. The counting rates at times later than 4–6 h are lower with the chelates than with transferrin, but the counts obtainable at 24 and 48 h (using only the 173 keV photons) compare favourably with ^{131}I-HSA, whilst the patients receive a lower radiation dose. The advantages of ^{111}In-transferrin are offset by the time-consuming preparation and quality control procedures, a problem which does not arise with the commercial availability of ^{111}In-DTPA.

Brain and renal studies

DTPA complexes with ^{111}In and ^{113}In^{m} have been used for both brain imaging, and studies of renal structure and function [446,447]. Indium-EDTA and -DTPA chelates tend to accumulate in cerebral tumours and in some types of brain lesion [446,447], although there is currently considerable interest in the development of small, neutral ligands capable of chelating indium, which could be used as tissue perfusion agents. Of particular interest are those ligands with lipophilicities and molecular weights enabling them to permeate the blood-brain barrier (see sections on iron and gallium). A range of ethylenediamine-based ligands (HBED (44a), SHBED (44b), deoxy-PLED (45b), tetramethyl-HBED (44d), and *t*-butyl-HBED (44e)) have been investigated [448]. Although these ligands form stable complexes with indium, they are unable to permeate the blood-brain barrier, presumably by virtue of their high molecular weights (>400). Recently, two ligands based on cyclic ethylenediamines, TA-TACN

(48a) and TX-TACN (48b) were studied, but again no brain uptake was found with either complex [449].

DTPA and its chelates are mainly excreted by glomerular filtration, as evidenced by measured $^{113}In^{m}$-DTPA/[^{14}C]inulin clearance ratios of 0.99 [450]. Indium-DTPA is used for renal scanning, renography and rapid estimation of the glomerular filtration rate.

Reticulo-endothelial system studies

Imaging of the reticulo-endothelial system (RES) is performed with a variety of colloids labelled with suitable radionuclides. The uptake mechanism is phagocytosis of the colloid by the RES, the distribution of colloid being determined by the particle sizes present in the colloid preparation. Phagocytic cells in the liver remove particles between 50 and 100 nm in diameter, whereas particles smaller than this tend to be removed by the bone marrow [407]. There is also uptake in the spleen of small (3–6 nm) particles. Several indium colloids have been used, of which the simplest is indium hydroxide produced by the addition of indium chloride to aqueous solution at pH 4 or above. The formation of the colloidal particles results from the extreme insolubility of indium (as evidenced by the solubility product of 10^{-33} M for $In(OH)_3$) [451,452]. The use of this material is somewhat surprising, in view of the fact that indium is one of the most toxic metals known, comparable in toxicity with mercury [307], with the hydroxide exhibiting particular toxicity. This accounts for the fact that 'carrier added' indium is rarely used in nuclear medicine. A number of studies have indicated that indium is essentially non-toxic to animals when administered orally [453], although subcutaneous, intravenous or intraperitoneal administration can produce extensive tissue damage [454,455]. Large differences are seen in the toxicity of 'ionic indium' ($InCl_3$) and hydrated (colloidal) indium oxide in animals, the LD_{50} value being 12 mg/kg and 0.3 mg/kg respectively, in mice [456,457]. Intraperitoneal administration of indium chloride produces rapid changes in haem metabolism in the liver [458], whilst intravenous administration of indium hydroxide produces drastic effects on the RES, in addition to extensive necrotic damage to the liver and kidneys [455].

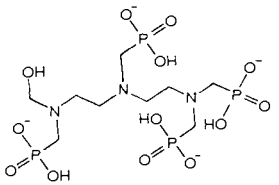
Most uses of this colloidal preparation require the addition of a stabilizer such as gelatin, mannitol or PVP and sodium bicarbonate [459–461]. Various methods have been devised to allow preferential fixation of the colloid in either the liver or the spleen, most using ferric chloride as a carrier [462]. Other colloidal preparations may be prepared with stannous chloride, albumin microaggregates or indium sulphide [463,464]. The complex of $^{113}In^{m}$ with

acetoacetonate (63) has also been suggested as a radiopharmaceutical suitable for imaging of the liver [407]. Alternatives to many of the potentially toxic colloidal indium preparations have become available since the introduction of $^{99}Tc^{m}$ radiopharmaceuticals with similar distribution properties.

Specific imaging of the spleen can be accomplished much more successfully using damaged, labelled erythrocytes than with colloidal preparations [465]. Red blood cells are labelled with indium using the indium complexes described earlier, and deliberately damaged, usually by heat, prior to reinjection. Such damaged cells are removed from the circulation by splenic cell sequestration, enabling visualisation of the spleen whilst minimizing uptake by the liver.

Bone imaging studies

The ligands used most widely for the imaging of bone tissue are phosphate derivatives, the majority of clinical investigations being performed with $^{99}Tc^{m}$ complexes of methylene diphosphonate (MDP) (53b) [412]. The corresponding ligands for indium are derivatives of NTA (26), EDTA (25) and DTPA (36), modified by substituting methylene phosphoric acid for acetic acid [466]. These compounds retain their ability to chelate indium, but due to the introduction of the phosphate groups, they have increased affinity for bone tissue over the parent carboxylic acids. Excellent scintigraphic images of skeletal tissue are obtainable with ^{111}In-labelled EDTMP (40), HMDTP (65) and DTPMP (66), whilst the tetradentate ligand NTMP (54) exhibits rather poorer results, usually attributed to the lower thermodynamic stability of the indium complex [466]. $^{113}In^{m}$ complexes of EDTMP and DTPMP have also been investigated for their potential in detecting myocardial infarcts [467].



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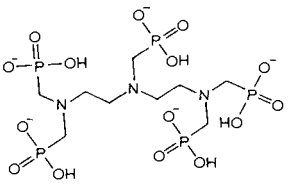

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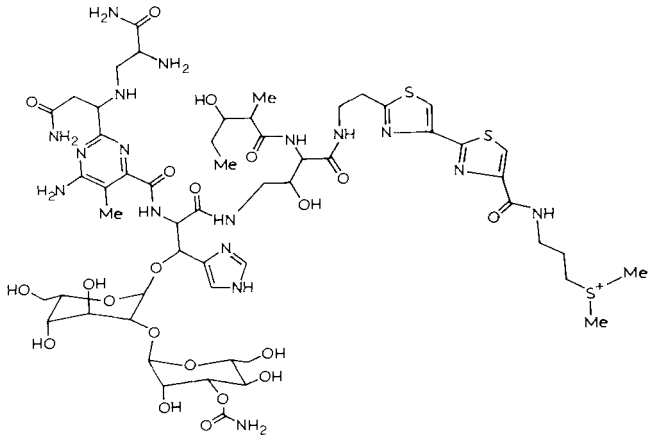
As mentioned above, imaging of the bone marrow can be performed with the use of labelled colloids. These have been used successfully to diagnose a range of haematological diseases, as well as various types of tumour affecting the bone marrow. However, this technique depends on the distribution of reticulo-

endothelial cells within the marrow being the same as that of the haemopoietic marrow (HPM). This assumption may be valid in normal subjects, but evidence suggests that the distribution may change in response to disease states [468]. To overcome this potential problem, imaging of bone marrow is usually performed with ^{111}In-transferrin, for which it was found that 24–72 h after administration, the majority of unremoved activity was deposited in the bone marrow, where it remained complexed [441]. Evidence from comparative studies on the distribution of indium and iron suggests that the indium activity is probably associated with the HPM, rather than with RE cells. However, metabolic differences in the utilization of iron and indium have been observed in animal models, notably the partial block on the incorporation of indium into erythrocytes following its deposition in the bone marrow [469]. Human studies provide evidence that ^{111}In-transferrin distribution represents the HPM rather than the RE cells, as uptake of this material in leukaemic patients was decreased, whilst uptake of labelled colloid was normal [470]. Indium transferrin is, therefore, more likely to provide accurate diagnostic information about the bone marrow than indium-labelled colloids, whilst also avoiding competing uptake of activity into the spleen.

Tumour imaging

If 'carrier-free' indium chloride is injected intravenously at pH 1.5–3.0, transferrin binds the In^{3+} ion quantitatively. Initial studies in animals suggested that indium chloride had potential as a tumour-imaging agent, with some selective uptake of indium into the tumour [471]. Human studies utilizing ^{111}In-transferrin indicated a plasma clearance half-time of approximately 10 h, associated with increasing deposition in the bone marrow at periods up to 48 h [472]. Although it proved possible to image certain tumours utilizing indium-transferrin, the high background activity, particularly in areas adjacent to, or overlying, the bone marrow, was a major problem. The clearance of background activity over the imaging period was minimal, no indium being excreted via the GI tract, and only 1–2% being excreted via the kidneys, precluding further development of this approach. The biodistribution of a range of indium complexes has been investigated, utilizing ligands such as citrate, lactate, acetate, HEDTA (39), tetraphenylporphyrin (TPP), bleomycin (67), 8-hydroxyquinoline (6) and 8-mercaptoquinoline [473,474]. Of these ligands, citrate, lactate and acetate form such weak complexes, that the distribution of indium following administration is virtually the same as that of indium-transferrin. The indium-HEDTA complex appeared promising due to both its high thermodynamic stability, and its rapid clearance from blood, enabling the achievement of very low



(67)

background levels, although it was finally abandoned due to its lack of tumour-specific uptake. The indium complexes of TPP, 8-hydroxyquinoline and 8-mercaptoquinoline were considered because of their lipophilicity, enabling them to readily permeate cell membranes. Tumour uptake of these complexes is, however, poor with tumour to blood distribution being as low as 0.1. In the case of ^{111}In-oxine, this was possibly due to particulate material remaining in the blood, or being deposited in the lungs, whilst ^{111}In-TPP and ^{111}In-mercaptoquinoline accumulate in the liver.

Bleomycin (67), a mixture of closely related antibiotics (the structure of one member of which is presented), has been shown to have cytostatic properties towards malignant cells, in addition to therapeutic effects on artificially induced tumours in animals [475]. It is rapidly cleared from the plasma following intravenous administration, 85% being excreted in the urine within 24 h. The major structural feature, shared by all bleomycins, contains several nitrogen-atom-chelating moieties, which are capable of binding divalent cations, such as Cu^{2+}. Bleomycin was also shown to be capable of forming a 1 : 1 complex with indium in yields of more than 99%, suggesting that ^{111}In-bleomycin may be suitable for tumour-imaging studies [476]. Indeed, animal work showed high tumour-to-blood radioactivity ratios, and patient studies indicated that bleomycin was rapidly concentrated in some, but not all, tumours. In those tumours which did show specific uptake, most of the activity was accumulated within the first hour, and the tumour retained this activity for at least 48 h, whilst the bone marrow, the liver and the kidneys showed increasing uptake between 24

and 48 h [441]. Despite the encouraging results, ^{111}In-bleomycin has not been widely used. The clearance of ^{111}In from the blood was slower in humans than in animals, and the persistent accumulation of ^{111}In in the liver and bone marrow prevented the detection of small lesions [477]. This may be related to the finding that, although 95% of the indium is still associated with bleomycin 5–10 min after injection, within 4 h this level has fallen to 5%, the remaining indium apparently being bound to transferrin. The future of bleomycin in tumour imaging now appears to lie in other directions. The biodistribution of four different radiometals, ^{111}In, ^{67}Ga, ^{57}Co and ^{59}Fe, has been studied when administered as a bleomycin complex, and has been found to be significantly different [478]. With equivalent doses of bleomycin, less ^{67}Ga was found to accumulate within the tumour than ^{111}In, whilst in one particular patient study, ^{57}Co-bleomycin managed to localize 80% of known tumour sites compared with a 20% success rate for ^{111}In-bleomycin. This latter finding is thought to be due to a combination of both an increased excretion rate of ^{57}Co-bleomycin compared with ^{111}In-bleomycin, leading to a higher tumour-to-background ratio, and perhaps more importantly to the observation that, whilst bleomycin will bind a range of metal ions, only the kinetically inert Co(III) complex remains associated long enough for successful use as an *in vivo* diagnostic imaging agent. This is as predicted from the earlier section on chelation chemistry, where certain cobalt complexes have been shown to possess extraordinary kinetic stability. Whilst ^{57}Co-bleomycin is currently used in diagnostic imaging, the long physical half-life of ^{57}Co (270 days) does present serious problems to patients in terms of absorbed radiation dose. It is likely that the use of bleomycin labelled with ^{58}Co (a positron-emitting radionuclide with a physical half-life of 71 d, which therefore exposes the patient to a lower radiation dose) will become more widespread as the accessibility to positron imaging systems increases.

An alternative approach being adopted to the problem of low tumour-to-background distribution ratios of ^{111}In, is the development of chelators with the ability to scavenge the metal from non-tumour tissue [479,480]. Such molecules are designed with the intention of removing the indium from sites responsible for high background activity, such as liver, spleen and in particular, from transferrin in plasma, whilst not significantly affecting the uptake in tumour. These chelators, MECAM (31a, R^1 = H), LICAM (55a) and their sulphonated analogues (31b, R^1 = SO_3^-; 55b), are catecholamide analogues of enterobactin (41), and were originally developed for use as ferric ion sequestering agents (see section on iron) [481,482]. Detailed studies on these chelators indicate that they are both thermodynamically and kinetically capable of removing indium from transferrin (see section on transferrin), and animal

studies have demonstrated that they will rapidly clear indium to the kidneys, although subsequent clearance to the bladder is slow. *N*-Alkylated catecholamide chelators show particular promise in this area because of their rapid clearance from the liver [479].

The applications of bifunctional indium chelates

The ideal approach to the targeting of radiopharmaceuticals to specific tissues is to label biomolecules possessing natural specificity for such sites (cf. the labelled bleomycin approach). Despite the fact that many proteins and other molecules of biological significance may be labelled with radioisotopes of iodine, the end-products of these procedures are often either unstable *in vivo*, or exhibit reduced receptor binding [483–488]. A further important factor is the lack of a radionuclide of iodine possessing both a suitable physical half-life for this type of imaging procedure (ideally between 2 and 3 days), and a gamma photon of suitable energy for imaging. In contrast, labelling with radionuclides by chelation can offer the advantage of highly stable products with unchanged distribution profiles. However, due to the paucity of suitable chelation sites, relatively few of these biologically important molecules can be successfully labelled in their native state. In an effort to overcome this problem, the so called 'bifunctional chelate' approach has been adopted, involving the covalent attachment of chelating groups to biomolecules of interest [489–493]. Bifunctional chelates are compounds containing both a chelating moiety and a functional group capable of forming a covalent bond with macromolecules. Utilizing this approach, it is possible to target the radionuclide to a particular site utilizing the specificity of the particular macromolecule chosen. The majority of chelating groups utilized in this approach are derivatives of the aminopolycarboxylates, such as EDTA (25) and DTPA (36), although the coupling methods used, and the molecules to which the chelates are attached vary widely [494]. The choice of chelate is of particular importance, and is considered below.

The first attempts at protein labelling with chelates used simple systems, such as protein acylation with DTPA derivatives carrying activated carbonyl groups. Although widely used, this approach has now largely been superseded by alternative methods giving more specificity in determining the site at which the protein is labelled. Bifunctional chelates are available with a variety of reactive groups, ranging from simple carboxylic acid or amine groups [347], to anhydrides [491,495,496], isothiocyanates (both of which couple via amines) [492,493], haloacetamides (for coupling via sulphydryl groups) [492] and azides (which are photoaffinity reagents) [489,497]. Perhaps the most widely

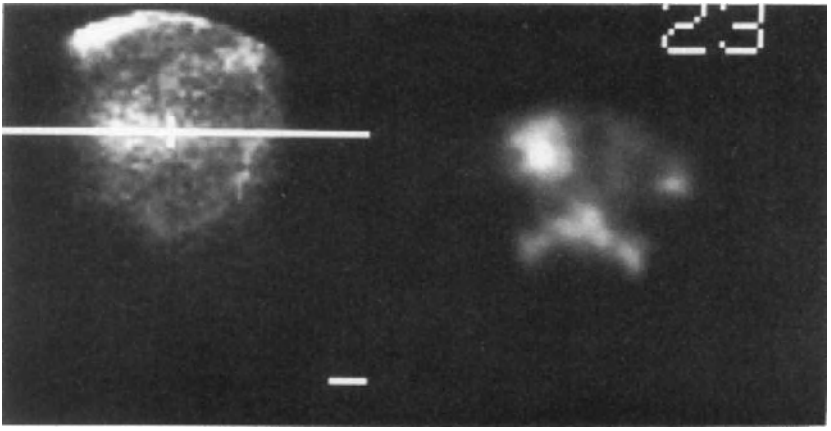


Figure 2.32. ^{111}*In-DTPA antibody scintigram (left planar view, right transaxial section). Uptake of the anti-EMA antibody is seen in tumour in the abdomen, as well as uptake of indium in the bone of the sacro-iliac joints.*

used bifunctional chelates are the various anhydride derivatives of DTPA [495,496] (*Figure 2.32*). These suffer from the major disadvantage that following covalent attachment to the protein, one of the carboxylate moieties is involved in the amide bond to the protein, resulting in the formation of the diethylenetriaminetetraacetate (DTTA) structure. Due to the non-symmetrical nature of its chelating centre, this is predicted to have a lower affinity for metal ions than DTPA itself, and this has been confirmed from *in vivo* measurements of the rate of indium loss from DTTA ligands compared with DTPA [498]. The importance of using true bifunctional chelates (which do not utilize any of the chelating moiety in the formation of the covalent bond with the protein) is now understood. A range of such chelates has been reported, all based on either EDTA or DTPA, but with an additional substituent on one of the carbon atoms in the ethylene or diethylene chain, via which the chelate is coupled to the protein [493]. Such substituents have been shown to have a striking effect on the rate at which a chelated metal ion dissociates from the complex. One study has shown that whilst transferrin removes about 20% of the indium from In-EDTA, and about 35% of the indium from In-DTPA over a period of approximately 300 h (under physiological conditions), when there is a substituent present on one of the ethylene carbons, transferrin is able to remove only 1–2% of the indium over the same period [499,500].

Many of the clinical applications of ^{111}In-labelled bifunctional chelates have

been associated with the development of tumour-imaging agents, intended to have improved properties over those described in the previous section. The progress of this work is undoubtedly related to the advances in hybridoma technology, and in particular, the ability to readily generate monoclonal antibodies to a wide range of human carcinomas. Such antibodies present ideal opportunities for the detection of tumours, assuming that the problems of stable attachment of suitable radionuclides can be overcome. The original reports relating to the labelling of antibodies date back to 1974, but these early studies suffered from significant reductions in the immunoreactivity of the antibody, due to the harsh labelling conditions employed [489,497]. It has since been amply demonstrated that the chemistry utilized in the antibody labelling procedure is a primary factor in determining the extent of tumour localization. However, at such early stages of development, the factor of primary concern was the desire for a stable antibody-^{111}In complex, to reduce background uptake. In an attempt to achieve this, alternatives to the EDTA-DTPA systems have been investigated.

A widely used substitute has been DFO (33), which in addition to its avidity for iron(III), has also been found to have a high affinity for indium(III). The approach used in the conjugation of DFO to antibodies is slightly different from those already described. Instead of forming the bifunctional chelate and subsequently reacting this with the protein, the entire process is performed in a single step. Glutaraldehyde is used as the coupling agent, one end of which reacts with primary amine groups on the protein, the other end reacting with the terminal amine in DFO [347]. However, the labelling efficiency for this reaction is low, with very little DFO being bound in this way. The major problem appears to be from the competing cross-linking reactions, which can result in either the coupling of two DFO molecules, or even the coupling of two antibodies, although this latter possibility is less likely due to steric considerations. Desferrioxamine does appear to have disadvantages when compared with the simpler aminopolycarboxylates, not only in terms of the chemistry involved in the antibody coupling, but also in the less well-characterized complexation of indium by the ligand [494].

With the exception of the EDTA, DTPA and DFO derivatives already mentioned, there have been very few reports of alternative bifunctional chelates. The only one worthy of note, although its use is limited, is the aminopolycarboxylate derivative, TTHA (37) [501]. This has been investigated principally because it carries six carboxylic acid groups, and it may therefore be expected that its metal binding ability would be less adversely affected on antibody conjugation than DTPA. One potential advantage of TTHA is that it has been possible to label antibodies with higher specific activities of ^{111}In, even though

the antibody/bifunctional chelate ratio is lower than is usual with DTPA. However, despite this, the tumour to background activity ratios were consistently higher with DTPA-labelled antibodies than with TTHA-labelled antibodies. In addition, the thermodynamic stability of the indium-TTHA complex is such as to allow transferrin to sequester the metal at a higher rate than from the DTPA complex [477]. This may be due to the stereochemistry of TTHA, which affords a larger potential site for chelation of the metal ion, resulting in lower affinity for metals when compared to DTPA. In view of this, DTPA remains the chelate used most widely for the labelling of antibodies.

Bifunctional chelates have also been used to derivatise a range of other molecules, including bleomycin. An EDTA derivative has been coupled to the (non-radioactive) cobalt-bleomycin complex [502], and studies of tumour uptake of this material in humans have been promising. The most significant aspect is that a bifunctional chelate ($M_r \approx 400$), can be attached to a relatively small molecule such as bleomycin ($M_r \approx 1500$), and not significantly change, at least in this particular case, its biological properties or distribution. The variety of other biomolecules labelled using the bifunctional chelate approach are limited in number and, almost without exception, use DTPA derivatives as the chelating agent. Examples are labelled fibrinogen for the detection of thromboses [441], the labelling of low-density lipoprotein (LDL) for the determination of early atherosclerotic disease and lipoprotein biodistribution [503], and the labelling of 'bifunctional haptens'. A hapten is a pair of compounds which possess a high binding affinity for each other, one example being avidin and biotin [504–506]. For example, biotin may be conjugated to a biomolecule, such as an antibody, whilst avidin is labelled with a chelate, such as indium-DTPA. The biomolecule and chelate are subsequently administered separately, the biotinylated antibody being given first and allowed to accumulate in the tumour, over a period of days if necessary. On administration of the radiolabelled avidin, rapid accumulation is seen as a result of its high affinity for the tumour-bound biotin. This enables imaging to be performed within a few hours, opening up possibilities for imaging with short half-life radionuclides. Whilst the most common hapten system used is avidin-biotin, a number of others have been reported [507–509]. The radiolabelled hapten approach was developed in an attempt to improve tumour-to-background distribution ratios, by enabling the rapid clearance through the kidneys of any non-tumour-bound radiolabel. Indeed, biotinylated antibodies are found to be cleared rapidly from the blood on infusion of avidin [510], suggesting that this approach will find increasing use in the future.

PHOTOSENSITIZERS BASED ON GROUP IIIA CATIONS

Photodynamic therapy (PDT) involves the use of selected light-absorbing molecules, called photosensitizers, to irreparably damage, or kill, particular types of cell. This technique is finding particular application in the treatment of a range of solid tumours in humans, in which photosensitizers have been found to accumulate. These photosensitizers are, almost without exception, porphyrin (59) or phthalocyanin derivatives (60), and are often used as complexes with Group III metals including iron, aluminium and gallium. Unlike the majority of ligands discussed in this review, these compounds are planar tetradentate ligands, which utilise nitrogen as co-ordinating atoms. The cavity provided by these structures is such that it can accommodate both the small Group IIIA cations and iron (III). The planar tetradentate structure permits ready access by molecular oxygen, an essential feature for this class of photosensitizers.

The basic requirements of a photosensitizer suitable for clinical photodynamic therapy are that it should possess minimal chemical toxicity, its light-absorbing properties should be maximal in the wavelength range available for therapeutic use, and that it should display enhanced uptake into the lesion over normal tissue [511]. In combination with visible (red) light from a laser, a photodynamic effect is generated, which causes tissue disruption. This arises from the excitation of the photosensitizer into a triplet state, which may then either react directly with tissue components, or with molecular oxygen, producing toxic species such as singlet oxygen. $^1O_2$ and free radicals [512]. In common with other oxygen radical species, singlet oxygen is very reactive, having a mean diffusion distance of 0.1–0.2 μm [513]. This may suggest that the cell membrane may be the principal target, although cytoplasmic components adjacent to the membrane may also be affected [514]. The production of singlet oxygen depends on photons being absorbed by the photosensitizer molecule, which should, therefore, possess intense absorption bands at wavelengths as high as possible in the 600–800 nm region of the visible spectrum. It is at these wavelengths that tissue penetration by the light is greatest, with penetration of 780 nm radiation being typically twice that of 630 nm radiation [515].

Currently, the most widely used photosensitizer is haematoporphyrin derivative (HpD) [516], or a mixture of its likely active components, Photofrin II [517]. This preparation, however, suffers from a number of disadvantages [518], not least that the clinical effect is unpredictable, due to the presence of a large number of porphyrin species. In addition, the absorption band of such porphyrins is not only fairly weak (molar extinction coefficients typically less

than 5000 l mol^{-1} cm^{-1}), but also occurs at wavelengths around 630 nm, which are too short for optimal tissue penetration by the activating laser light. Side-effects may also be produced as a result of the tendency of HpD to accumulate in the skin, from where it is subsequently cleared only slowly. If patients do not avoid exposure to strong sunlight for periods up to 4 weeks following administration of HpD, dramatic effects may be produced in the exposed tissue [519]. In an attempt to overcome these disadvantages, a wide range of potential photosensitizers has been studied, including phthalocyanins. These have improved absorption properties over the porphyrins, having much more intense absorption bands (molar extinction coefficients are typically > 100,000 l mol^{-1} cm^{-1}), whilst the absorption maximum is also higher than the porphyrins, at approximately 670 nm [520–522]. Indeed, the naphthalocyanins may prove to be even more suitable for PDT, since they also possess strong absorption bands, but at rather higher wavelengths (about 780 nm) than the phthalocyanins themselves [523]. Of the phthalocyanins investigated to date, the leading contenders are chloroaluminium phthalocyanin, ClAlPc [524], or its sulphonated counterpart, ClAlSPc [525]. These can be synthesized easily, are chemically stable and possess suitable absorption characteristics (λ_{max} 675 nm). The triplet quantum yield is acceptably high at 0.4, with a long-lived associated triplet state (510 $\pm$ 50 μs), enabling energy transfer for the production of singlet oxygen to be accomplished [526]. Sulphonation of the phthalocyanin simply serves to enhance the water solubility of the complex, with virtually no effect being seen on the cytotoxicity [527]. Indeed, the non-sulphonated aluminium complex produced total cytotoxicity within 3 days of exposure to red light [527]. The major difference between the two forms arises from the sensitivity to ambient light. Cells treated with the sulphonated form were sensitive to red light and insensitive to normal room light, whereas room light produces measurable cytotoxicity in ClAlPc treated cells. Phthalocyanin complexes with other metals including magnesium, zinc and zirconium are all cytotoxic under ambient lighting conditions [527].

It should be noted that the effects of phthalocyanin sulphonation are not equivalent for all metal complexes, as evidenced by studies on a series of sulphonated chlorogallium phthalocyanins [528, 529]. This has indicated that the mono- and disulphonated derivatives possess the highest light-induced cytotoxicity, whereas the tri- and tetrasulphonated analogues show no photoactivity [529]. This has been shown to be due not to a change in the kinetics of singlet oxygen production, but rather to the effect of sulphonation on the ability of the phthalocyanin to permeate biological membranes [530]. Whilst lower degrees of sulphonation tend to promote complex dimerization, or higher aggregation, with associated reductions in photochemical activity [529], this is

offset in the biological system by the inability of the highly charged tri- and tetrasulphonated derivatives to penetrate membranes. This is supported by the observation that whilst the tetrasulphonated gallium phthalocyanin induces little damage on intact cells, it is as effective as the monosulphonated gallium phthalocyanin when exposed to permeabilized cells [531]. Observed differences between the phototoxicities of the aluminium and gallium complexes may arise from the effect of the metal ion on the quantum yield and lifetime of the triplet state, although differences in photosensitizing properties resulting from altered extents of sulphonation cannot be entirely ruled out.

GROUP IIIB METALS

YTTRIUM

The Y^{3+} cation has a radius of 0.90 Å, and consequently has a charge density between that of La^{3+} and In^{3+} (Table 2.1). Its co-ordination chemistry is, therefore, predicted to be similar to these two metals, and affinity constants for hexadentate ligands are found to be intermediate between those for the two aforementioned metals (*Figures 2.3, 2.21* and *2.22*).

The clinical interest in yttrium arises from the fact that ^{90}Y is a pure β emitter, with a physical half-life of 64.1 h, and is considered to be one of a very small number of radionuclides suitable for the treatment of cancer by radioimmunotherapy [532]. A further advantage of this radionuclide is its ready availability from a $^{90}Sr/^{90}Y$ generator system. Yttrium is normally eluted from such a generator in the form of the acetate, although small amounts of citrate often have to be added to mobilize the yttrium from the resin bed into the acetate carrier solution [533]. The purity of the yttrium obtainable from early examples of such generators was normally in the order of 99.5%. The remaining 0.5% was the strontium parent nuclide, and as such presented a considerable potential radiation hazard to the patient. One method of reducing the risks from this hazard was to expose the generator eluate to a chelator with a high specificity for yttrium. Such a chelator is DTPA, the difference in the K_1 values for yttrium and strontium being greater than 10^{12} (log K_1 for yttrium = 22.28, log K_1 Sr = 9.57). Such a high affinity ensures that all the yttrium is converted from the acetate complex to the DTPA complex, and under normal circumstances, no strontium contamination in the final yttrium-DTPA solution was detectable. Generators currently available are much improved, with elution efficiencies being greater than 95%, and levels of strontium in the eluate being less than

0.003%. At such levels, the quantities of strontium administered to patients in typical clinical situations is below the detectable level.

Most of the clinical studies performed with ^{90}Y have involved the labelling of monoclonal antibodies with yttrium, via the bifunctional chelate approach described earlier. Owing to the high stability and selectivity of DTPA for yttrium, this is the chelate most often chosen for such work [534, 535]. One drawback to the use of yttrium is that, as a pure β emitter, it is not possible to follow the biodistribution of the labelled antibody by external imaging techniques. Studies with antibodies labelled with other radionuclides, either direct labelling with ^{125}I, or bifunctional chelate labelling with ^{111}In, are of limited use, as the biodistribution of each antibody is different [533, 534]. In order to overcome this lack of knowledge, the biodistribution of a ^{90}Y labelled anti-ovarian antibody was determined in a number of patients by biopsy [536]. The urinary excretion of activity was low, at 7% of total injected dose over a 2-day period. Levels of yttrium found in other organs were 8% in serum, 10% in liver, 7% in bone marrow, 19% in bone, with the tumour/normal tissue yttrium distribution varying between 3 and 25 [511]. The accumulation of yttrium in the liver following injection of the labelled antibody may arise from two possible routes, either the dissociation of the metal from the DTPA-coupled antibody, or as the result of antibody clearance into the RES in the liver, followed by release of the yttrium. Removal of the intact yttrium-DTPA complex from the antibody would tend to lead to the yttrium being cleared from the body via the kidneys.

If lanthanides, including yttrium, are injected into the bloodstream as the free metal, substantial amounts are accumulated in the liver. This is probably due to clearance of a colloidal form of the metal into the RES. Studies implicating iron transport proteins such as transferrin [537], should be considered with some scepticism, following the demonstration that yttrium-labelled DTPA-coupled antibody was stable in serum, with no evidence of transcomplexation to transferrin [534]. In a chelated form, such metal ions are either excreted by the kidneys or are taken up into the bone tissue.

At the current time, the potential for yttrium-labelled antibodies to be used in therapy is good, although progress should be made with restraint. For instance, it is suggested [536] that intraperitoneal administration may be more advantageous than intravenous administration, in terms of reducing the radiation exposure to the bone marrow. Alternative approaches, including the co-administration of soluble chelates, which have been shown to reduce the liver uptake of lanthanides [533], may also be adopted.

THE LANTHANIDES

Many lanthanide ions possess unpaired electrons and consequently are coloured and are paramagnetic [538]. In contrast to the *d*-block transition elements, the *4f* electrons are inner electrons, shielded from external influences by the overlying $5s^2$-, $5p^6$- and $6s^2$-electron shells. Ligand field effects are thus relatively weak, which has the dual effect of producing narrow absorption bands resulting from transitions between different *f*-orbitals, and preventing the magnetic properties of the ions being affected by their chemical environment. It is a characteristic of the M^{3+} lanthanides that coordination numbers exceeding six are very common. Indeed, very few six coordinate species are known, but coordination numbers of 7, 8 and 9 are more important. The ionic radii of the entire series of 15 lanthanide tribasic cations falls in the range 0.86–1.03 Å, with lanthanum(III) being the largest. The radii of the clinically important cations are: samarium(III), 0.96 Å; gadolinium(III), 0.94 Å; europium(III), 0.95 Å and terbium(III), 0.92 Å. Thus, the coordination chemistry of these cations, like yttrium, falls between indium(III) and lanthanum(III) (*Figures 2.3, 2.21* and *2.22*).

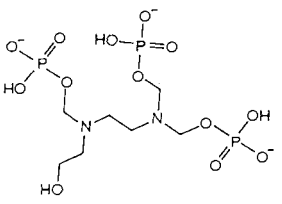
SAMARIUM

The clinical usefulness of samarium results from applications of the radioactive forms of samarium in nuclear medicine. ^{152}Sm finds little applications clinically, as it is an expensive radionuclide to produce, and possesses a high thermal neutron capture cross-section. ^{153}Sm, however, has emissions of use in clinical procedures, namely 640 (30%), 710 (50%) and 810 (20%) keV beta particle emissions, a 103 keV gamma-ray emission (28%) and 55 keV internal conversion electrons (41%) [538]. The physical half-life of ^{153}Sm is also suited to a range of applications, being 46.8 h. The energies of the β^- emissions are suitable for use in therapeutic regimes, the short half-life allowing for the application of the dose in several discrete stages, reducing the radiation burden to the patient at the outset of the treatment. The 103 keV gamma-ray is also usable, allowing the biodistribution of the radionuclide to be quantified using conventional gamma camera scintigraphy. Owing to the fact that samarium has been shown to form stable chelates with phosphonate ligands [539], the suitability of these complexes for the treatment of bone tissue tumours has been investigated [540].

Bone metastases occur very commonly in patients suffering from a range of carcinomas (including carcinoma of the lung, prostate and breast), with up to 50% of such patients predicted to develop such metastases. The major

symptom associated with such metastases is bone pain, which has conventionally been treated by a combination of various analgesics and external beam radiotherapy. More recently, there has been a move towards the use of radiopharmaceuticals which accumulate in bone tissue, to treat this pain. Examples of such compounds include strontium-89, and a range of phosphorus-32 labelled compounds [541, 542]. However, in a large number of cases these have been shown to cause suppression of the bone marrow.

The biodistribution of samarium complexes of a number of multidentate aminophosphonate ligands (NTMP (54), EDTMP (40), DTPMP (66), HEEDTMP (68) and NBTP (69)), as well as NTA (26), EDTA (25) and DTPA (36) for comparative purposes, were determined in animal models to assess the suitability of such compounds for clinical use [540]. Lanthanide complexes of small phosphonates such as MDP (53b), which is routinely used (as its $^{99}Tc^{m}$ complex) for bone imaging, were unsuitable for use as therapeutic bone agents due to their high uptake in the RES. This result is interpreted as an indication of colloid formation due to low *in vivo* stability, a finding confirmed by earlier work with relatively unstable lanthanide complexes, which also exhibited high RES uptake [543]. Similar results obtained with the diphosphonate (70), which localized in the RES to a smaller but still unacceptably high degree, suggest that, in general, all small ^{153}Sm-diphosphonate complexes are likely to be unsuitable for use as bone agents. The samarium complexes of NTMP (54) and NBTP (69) behave in a similar fashion to NTA (26) and EDTA (25), and also accumulate in the RES despite showing a high degree of bone uptake. In contrast, Sm-DTPMP and Sm-DTPA show low RES and soft tissue uptake, but are also unsuitable due to the low skeletal uptake of these complexes. Samarium-EDTMP does, however, accumulate to a large extent in skeletal tissue, showing no significant clearance from these sites over a 3-day period, whilst clearing rapidly from soft tissue. Indeed, the bone/blood and bone/muscle ratios for this complex appear to be superior to commercially available $^{99}Tc^{m}$-MDP kits. The uptake of this complex is also significantly



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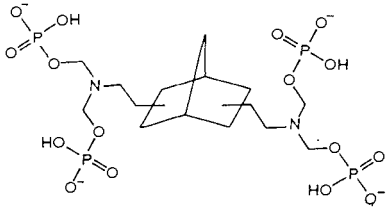

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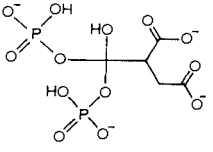

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higher in bone lesions than in normal bone tissue, with a typical distribution ratio being 17/1 [540].

GADOLINIUM

Clinical interest in gadolinium arises from the fact that paramagnetic ion complexes and stable free radicals are currently the subject of intense scrutiny for use as magnetic resonance imaging (MRI) contrast agents. Magnetic resonance imaging is a highly promising diagnostic technique, especially in the areas of neurology and neurosurgery, as images of the brain, spinal cord and pelvic organs, in particular, are not hindered by the presence of bone tissue. MRI of the abdominal area tends to be much less reliable, due to unavoidable motion artifacts. The technique is non-invasive, and does not rely on the use of ionizing radiation, and since there is little evidence that the radiofrequencies and magnetic fields typically used cause any tissue damage, possible harmful effects to patients are minimized. In common with other imaging techniques such as PET and SPECT, MRI can create images in a series of different planes, allowing very accurate determination of the extent and size of various types of lesion. For instance, under ideal circumstances, MRI is capable of differentiating areas of oedema from tumours, and areas of haemorrhage from oedema. It is also possible to detect brain lesions resulting from multiple sclerosis, allowing an accurate diagnosis to be made at an early stage in the disease process [544,545]. Indeed, in diagnosis of various demyelinating diseases, brain stem infarctions and tumours of the posterior fossa, MRI has been shown to be clinically superior to computed tomography [546]. However, despite the wide variety of MRI pulse procedures available, consistent differentiation between areas of infarction and tumour, and between areas of oedema and tumour is difficult. Similar problems in computed tomography can be overcome by the use of iodinated contrast media [547]. The non-availability of corresponding MRI contrast media, in addition to the high capital cost of imaging equipment, have been the major reasons preventing widespread clinical application of the technique.

The chelates of paramagnetic ions are, however, suitable for use as MRI contrast agents. The usefulness of paramagnetic transition elements as contrast agents lies in the fact that they tend to shorten both the spin-lattice (T_1) and the spin-spin (T_2) proton relaxation times, and hence change the NMR signal strength. This change in signal strength results in enhanced tissue contrast in both T_1- and T_2-weighted MRI [548]. The gadolinium (Gd^{3+}) ion has attracted most interest due to its high magnetic moment [549]. The variation in T_1 relaxation induced by gadolinium as a function of applied frequency

indicates a maximum in relaxivity for frequencies in the range 20 to 60 MHz. The form of the chelate is relatively unimportant with respect to the efficacy of the complex as an MRI contrast agent, the major influence of the ligand being to contain the paramagnetic metal ion in a non-toxic form to the body. However, the ligand must allow access of at least one exchangeable water molecule to the metal. It is the exchange of such an inner sphere water molecule with solvent which produces the observed relaxation effects [550]. Lanthanides have no known physiological functions and no specific transport systems for the free metal ion are known to exist *in vivo*. Lanthanides are known to be toxic to mammals, with gadolinium having an LD_{50} value in rats of approximately 80 mg/kg. Chelation of the gadolinium to DTPA neutralizes the toxicity of the metal ion, and it is this metal complex which has been used most widely in the clinical situation. Gadolinium-DTPA is rapidly becoming accepted as the first general-purpose MRI contrast agent. In combination with fast imaging, transit and clearance studies may be performed in addition to the more usual 'structural' studies. The time-scale resolution currently achievable is of the order of 2–3 s, although advanced techniques currently being tested on commercial instruments seem capable of reducing this to less than 50 ms.

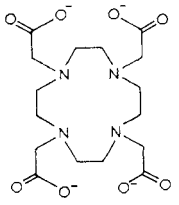
There have been very few reports of side-effects associated with the use of Gd-DTPA, although in some instances, raised bilirubin and serum iron levels have been measured [551]. Possible causes of this are slight alterations in the permeability of the erythrocyte membrane, or more likely, changes to the iron-saturation of transferrin. This is supported by the observation that gadolinium will bind to human serum transferrin, although the binding appears to be weak enough such that at physiological carbonate concentrations, the formation of non-binding carbonate complexes of Gd(III) preclude transferrin assuming a major role in the transport of gadolinium *in vivo* [186].

On intravenous injection, Gd-DTPA is distributed within the vascular system, and is excreted unchanged (by glomerular filtration) with a half-life of approx. 90 min in man [552]. Gd-DTPA is an extracellularly distributed ion, and with the exception of the kidneys, no uptake of Gd-DTPA by an intact organ has been reported [553]. Gd-DTPA is believed to rapidly diffuse into the entire interstitial (extravascular) space, and hence tends to cause image enhancement in tissues containing a high proportion of interstitial fluid [554]. The contrast enhancement obtained in MRI after a bolus administration of Gd-DTPA will, therefore, initially be related to the amount of Gd-DTPA located in that organ [555]. The contrast is thus dependent on organ blood flow. With time, this contrast will change depending on the total extracellular space in the organ, and the rate of Gd-DTPA excretion via the kidneys. The mechanism of MRI contrast agent uptake in brain lesions is attributed mainly

to destruction of the blood-brain barrier [556]. Changes in the structure of tumour capillaries have been shown to facilitate the passage of higher-molecular-weight species from intravascular space to interstitial space [557], and this mechanism of uptake has also been demonstrated to occur with X-ray contrast agents of similar molecular weights to gadolinium-DTPA [558].

Studies on MRI of brain tissue have shown that usable contrasts can be obtained when gadolinium is administered intravenously at a concentration of about 20 mg/kg body weight [557]. This should be compared with studies on MRI of liver tumours, where contrast was obtained at gadolinium concentrations as low as 1.5 mg/kg [551], although for this purpose, a typical Gd concentration used in an imaging protocol is nearer 10 mg/kg. Potential problems may arise if high doses of Gd-DTPA are administered, as, with some spin-echo image procedures, the T_2 relaxation time can be shortened markedly, leading to a dramatic reduction in signal intensity.

A number of other polyaminocarboxylic acids capable of coordinating gadolinium are currently being investigated with regard to their potential as clinical MRI contrast agents [559–561]. These ligands are based on cyclic polyaza ligands with a number of pendant carboxylate groups, and include 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) (71) and 1,4,8,11-tetraazatetradecane-1,4,8,11-tetraacetic acid (TETA) (47b). These two chelators display a number of properties not possessed by similar, but non-cyclic ligands, such as EDTA and DTPA. The lanthanide complexes of both DOTA and TETA are very rigid, highly symmetrical and appear to possess the same geometry in both solution and the solid state [561]. The possession of rigid geometry is of prime importance in the application of such compounds as MRI contrast agents. A lack of symmetry allows the possibility of different relaxation effects being brought to bear, depending on the conformation of the complex, making quantification of the process difficult. In addition to these advantages, the lanthanide complexes of DOTA are remarkably stable, a property not generally exhibited by lanthanide complexes. This has been attributed to the



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imposition of precise steric criteria by the polyaza macrocycles. The stability constants of a number of DOTA-lanthanide complexes indicate that they are significantly more stable than either of the open-chain analogues, EDTA and DTPA, with the DOTA complex being approximately 10^7-times more stable than the corresponding DTPA complex, and approximately 10^{10}-times more stable than the EDTA complex [560,562]. This dramatic increase in affinity does not come from a 'macrocyclic effect', as seen with the crown ethers, for example. The internal cavities possessed by both DOTA and TETA are too small to accommodate a lanthanide M^{3+} ion, hence the polyaza rings simply appear to be acting as templates to hold the carboxylate groups and the ring nitrogen atoms in a sphere of the correct geometry to complex the metal ion.

The usefulness of these MRI agents would be greatly enhanced if they could be coupled to macromolecules capable of targetting specific tissues or lesions [563]. One example is the labelling of monoclonal antibodies for the NMR detection of tumours [564]. Labelling with gadolinium DTPA appears not to adversely affect the antibody biodistribution when compared with indium- or gallium-labelled antibodies. The only alternative to DTPA so far suggested for protein labelling is DOTA [559]. This would have significant advantages over DTPA in preventing transchelation of the gadolinium to transferrin, resulting in lower toxicity [565]. In addition, lower patient doses of gadolinium may be used, typically 1 mg/kg Gd as opposed to approximately 10 mg/kg Gd for non-protein bound gadolinium.

Metalloporphyrins have also been investigated as MRI contrast agents, in mice bearing subcutaneous human colon carcinoma xenografts [566]. Porphyrins are known to accumulate in tumours, and hence may have advantages over materials such as Gd-DTPA, which is distributed evenly throughout the extracellular space. The Mn(III), Fe(III) and Gd(III) complexes of TPPS (59) were examined, and of these, the manganese and gadolinium complexes caused image enhancement, whilst the iron complex suffered from decreasing relaxivity above pH 6, due to the formation of oxy-dimers. The Mn-TPPS complex appeared to have somewhat higher stability in human plasma than Gd-TPPS, remaining stable even after incubation in plasma for nine days. Studies in the animal models showed enhanced relaxation of tissue water in several tissues including kidney, liver and tumour, although this effect in tumour tissue appeared to be reversed at high doses 4 days post administration. At the present time, metalloporphyrin contrast agents are the only paramagnetic materials which appear to concentrate selectively in tumours. The usefulness of such agents in MRI will depend on a number of factors still to be evaluated, including toxicity, selectivity of tumour uptake and stability *in vivo*.

Virtually all of the other MRI contrast agents proposed as alternatives to the

gadolinium complexes are iron(III) complexes [563]. Although iron(III) is toxic when free (LD_{50} value = 7 mg/kg in rabbits), it is an essential nutrient, and hence a number of intricate systems have evolved for its transport and storage, as already described. Thus, despite the fact that iron(III) is only about half as effective as gadolinium(III) in causing proton relaxation, its complexes may have significant advantages in toxicity terms as MRI contrast agents. No theory has yet been proposed which adequately explains all of the observed relaxation behaviour of iron compounds in the body, be they in the blood or in various sites of iron storage. However, MRI has been used to investigate the distribution of iron in some patients suffering from thalassaemia, on the assumption that Fe(III) ions should substantially alter the contrast in areas of high iron concentration (sites of iron deposition associated with iron overloaded), as a result of their large magnetic moments, arising from the possession of unpaired electrons [567]. Although results were obtained relating to the effect of the iron core in ferritin, this is likely to be of limited use in normal patients not suffering from iron loading diseases. One of the most promising iron-based MRI contrast agents in ferrite particles, intended for MRI of the reticuloendothelial system. Ferrites are crystalline iron oxides possessing superparamagnetic properties, which acquire extremely large magnetic moments in the presence of an externally applied magnetic field. Studies investigating the suitability of magnetite as a contrast agent in animals indicated that within 30 min of injection, large effects in the T_2 relaxation associated with liver and spleen were noted, whilst no effect was seen on the lung, kidney or muscle [568]. This effect persisted for up to 6 months following administration, pointing to the major disadvantage of this material, namely the poor clearance from the RES. Commercial development of such materials is likely once biodegradable alternatives have been developed.

TERBIUM AND EUROPIUM

In common with the other lanthanide elements, terbium and europium have incompletely filled inner electron shells. Absorption of visible light by these two elements in particular can give rise to excited states of relatively low energy, many of which can subsequently relax back to the ground state by emission of photons, a phenomenon known as fluorescence. The lanthanides are unique amongst metallic cations in their ability to fluoresce in aqueous solution at room temperature [569]. Indeed, virtually all metal ions or associated complexes, either occurring naturally or used as biological probes, are not fluorescent in aqueous solution at temperatures above $-196°C$ [570]. Due to the high sensitivity with which fluorescence can be measured, this effect has found wide

application in the probing of biomolecular structure. Indeed, this approach allows structural information relating to solution conformations to be obtained which could previously only have been obtained in the solid state from X-ray crystallographic studies. A further advantage of the lanthanides as fluorescent probes, is that the long fluorescent lifetimes of chelated lanthanide irons ($T_{1/2} \approx 1$ ms) allows the quantification of energy transfer without interference from scattering or the presence of organic fluorophores.

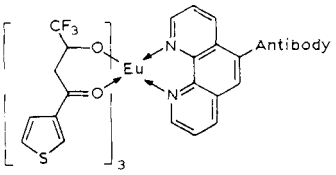
Applications of fluorescent probes in biological systems include the determination of structural information from discrete components in an equilibrium mixture, the magnitude of structural changes brought about by changes in solution conditions, the extent of solvent interaction with biomolecules and information about the binding of ligands and metal ions to biologically important proteins. However, for the lanthanide ion to be suitable for use as such a probe, a number of criteria must be fulfilled. The coordination chemistry of the metal should be complementary to the metal binding site of the molecule under investigation, the binding of the metal to the target should exhibit a degree of site selectivity and, lastly, the fluorescent properties of the metal must be retained when bound to the biomolecule.

Most of the recent interest in europium and terbium as structural probes has been directed towards applications involving their substitution for calcium and magnesium. These latter two metals are extremely abundant in biological systems, yet it is relatively difficult to investigate their transport or protein binding adequately, due to the lack of any suitable spectral characteristics. The biological activity of proteins containing calcium or magnesium is often retained when either of these metals is replaced by a lanthanide. This occurs as a result of the general similarity between the lanthanides and calcium(II); the ionic radii of the trivalent lanthanides range from just below to just above that of calcium(II), whilst both the lanthanides and calcium have a preference for oxygen donor ligands (see section on coordination chemistry). The additional positive charge carried by the lanthanide ions is often of little relevance, as macromolecules can often accommodate the gain of a single charge owing to the presence of multiple potential acceptor groups. Examples of calcium-dependent systems activated by lanthanide ions in the absence of calcium are concanavalin A [571], trypsinogen [572], phospholipase A_2 [573], thermolysin [574] and elastase [575].

The use of terbium and europium is not, however, limited to studies on calcium- or magnesium-containing proteins, provided that the target molecule possesses one or more suitable binding sites for the lanthanide ion. In cases where there are several metal ions bound, energy transfer between the metal ions can occur, allowing measurement of the intermetal distance from the

magnitude of the energy transfer. Two such examples of energy transfer are Eu(III) to Co(II) transfer in galactosyltransferase [576], and Tb(III) to Fe(III) energy transfer in transferrin [577]. Both these studies allowed measurement of the intermetal distance before crystallographic data became available. Related studies on the terbium-transferrin system, involving energy transfer to cobalt complexes in solution, allowed the distance from the protein surface to the two iron binding sites to be measured as approximately 1.7 Å [578]. Such studies are, therefore, capable of providing valuable information on the structure of metal containing proteins.

Europium chelates capable of extended lifetime fluorescence have also been investigated [579]. Such chelates (72) contain a conjugated aromatic system, which is capable of energy transfer from the ring system to the adjacent chelated metal ion. An immunoassay procedure, in which the europium chelate is used to label an antibody, has been developed from this particular compound (72) [580]. Pulsing of a solution containing such a chelate with an intense light source, such as a laser, causes many components in the solution to fluoresce. However, the majority of fluors will have very short fluorescence lifetimes, typical fluorescence half-times being of the order of 10 ns. It is thus possible to allow the fluorescence of all the contaminants in the solution to decay before the fluorescence arising from the europium chelate tagged antibody is measured (*Figure 2.33*). More applications based on this technology are likely, with further advances in the conjugation chemistry necessary to couple these metals to macromolecules.



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SCANDIUM

A number of enterobacterial pathogens produce siderophores which are endogenous growth factors *in vivo*. Of these, enterobactin (41), which is produced by a number of pathogenic organisms, including *Escherichia coli*, *Klebsiella pneumoniae* and *Salmonella typhimurium*, has the highest affinity for iron(III) of any known iron chelator (see section on iron). A number of studies investi-

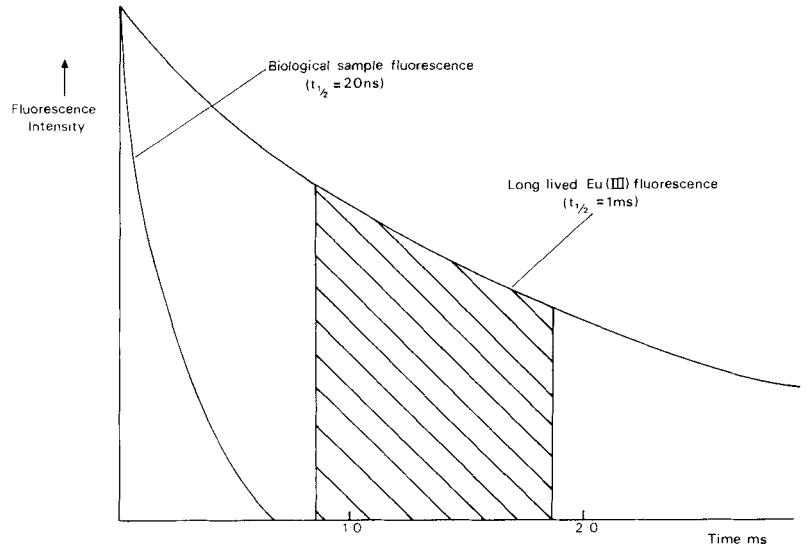

Figure 2.33. Diagram illustrating the extended fluorescent lifetime of europium chelates compared with the natural fluorescence of the biological sample. Observation is performed in the time window between 1 and 2 ms.

gating the use of complexes of enterobactin with metals other than iron, as anti-metabolites, have been reported.

Initial studies examined the antibacterial effects of 19 enterobactin-metal complexes [581], the metals under investigation ranging from dipositive to hexapositive cations, and including the tripositive metals aluminium, gallium, indium, yttrium, lanthanum and scandium. The only complexes found to produce any antibacterial effect were the scandium and indium complexes. In *in vitro* measurements in both horse serum and rabbit plasma, the scandium complex was found to exert a bacteriostatic effect, whilst the indium complex did not produce complete bacteriostasis, but rather a marked increase in the generation time (*Figure 2.34*).

Initially, the mechanism of antibacterial action was unclear, although the inability of additional enterobactin to reverse the effects of scandium-enterobactin suggested that the toxic effect arose from interference in the assimilation of iron via the enterobactin uptake pathway. However, subsequent studies on a number of mutant bacterial strains, with impaired enterobactin uptake mechanisms, indicated that the scandium-enterobactin complex acts as a highly specific anti-metabolite, which interferes with the transport of

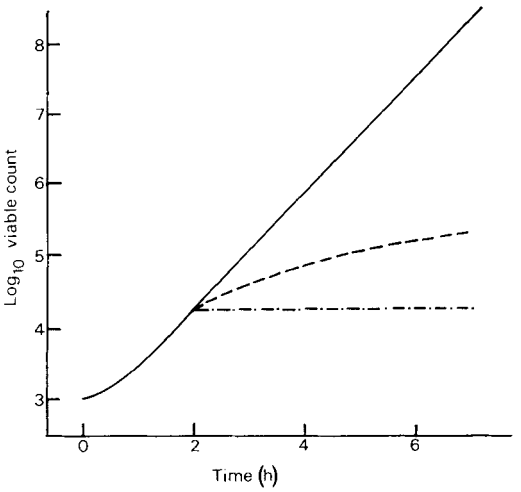

Figure 2.34. Antibacterial effect of complexes of enterobactin added to Klebsiella pneumoniae *growing in rabbit plasma.* ———, *control (no addition);* ------, *indium-enterobactin;* –·–·–·–, *scandium-enterobactin.*

iron-enterobactin [582]. It is likely that, once incorporated into the bacterial cell, the scandium-enterobactin complex is degraded, releasing scandium. This is likely to be responsible, in part, for the observed toxic effects on the cell. Evidence from earlier studies on the bactericidal effect of aqueous solutions of the lanthanides, which indicated that scandium was the most toxic metal investigated, particularly against Gram-negative bacteria such as *K. pneumoniae* [583], supports this view. The overall effect of scandium-enterobactin is thus likely to arise from a combination of both the inherent toxicity of scandium, which is incorporated into the cell by riding on a specific uptake system, and the concomitant reduction in the uptake in iron.

The use of enterobactin-metal complexes as anti-metabolites has been studied in mice with experimental infections of either *K. pneumoniae* or *E. coli*, indicating that indium-enterobactin has only a slight therapeutic effect, whilst the scandium complex exerts a significant therapeutic effect [584].

CLINICAL APPLICATIONS OF OTHER LANTHANIDE ELEMENTS

Of the remaining members of the lanthanide series, only three have found any significant clinical application, these being dysprosium, thulium and ytterbium.

All of these have been used in nuclear medicine, although none of them to any great extent.

Dysprosium-157 (^{157}Dy) has been used for imaging of both bone and bone marrow. It is an accelerator produced radionuclide, the two methods of production being neutron bombardment of terbium-159 [585] and bombardment of gadolinium with alpha particles [586]. ^{157}Dy has a physical half-life of 8.1 h, and decays with emission of a 330 keV gamma photon. The majority of the applications of dysprosium for skeletal imaging, although limited in number, have used HEDTA (39) as the chelating agent [587].

A beta-emitting isotope of dysprosium, ^{165}Dy, has also found limited therapeutic application in radiation synovectomy. This radionuclide is a short-half-life ($T_{1/2}$ = 139 min) beta emitter (1.3 MeV), which is produced at high specific activity by reactor irradiation of natural dysprosium. Radiation synovectomy is a non-invasive alternative to surgical synovectomy in rheumatoid arthritis, in which a beta-emitting nuclide is injected into the synovial sac. Most of these applications have been in the treatment of arthritic knee joints, using a variety of colloidal particles in the range 10–60 nm labelled with radionuclides such as ^{198}Au, ^{90}Y [588] and ^{169}Er [589] in addition to ^{165}Dy. However, this approach suffered largely from leakage of the colloid from the joint, with subsequent accumulation in other organs. This disadvantage was overcome by the use of ferric hydroxide macroaggregates, coprecipitated with therapeutic doses of dysprosium, which have sizes in the micron range [590]. In animal studies, these were found to exhibit lower joint leakage than gold colloid, and would be expected to present lower radiation doses to the patient as a result of both this reduced leakage and the short physical half-life of ^{165}Dy.

Both thulium and ytterbium have been used for positive contrast tumour scintigraphy [591–593]. ^{167}Tm is produced by neutron bombardment of ^{167}Er [594], and decays with a physical half-life of 9.6 days with emission of a 210 keV gamma photon. The use of this radionuclide in bone imaging studies was prompted by reports of other lanthanides such as ^{153}Sm and ^{171}Er (as the HEDTA complexes) being used in bone imaging [595]. Initial studies employing thulium utilized the thulium citrate complex [596]. This exhibited reasonable bone uptake, although other agents such as ^{167}Tm-HEDTA, were subsequently used [597]. These were found to be superior to the widely used ^{85}Sr agents, not only in terms of image quality, but also because of the lowered patient radiation dose arising from the shorter physical half-life of thulium. The use of thulium citrate in tumour scintigraphy has also been investigated. This was prompted by the observation that ^{169}Yb-citrate had an affinity for tumours, although the decay characteristics of ytterbium are not ideal for such applications. Animal studies examining the distribution kinetics of several lanthanides

(^{155}Tb, ^{167}Tm, ^{169}Yb and ^{171}Lu) have shown that thulium has advantages over the other lanthanides in terms of faster urinary excretion and lower liver uptake [592]. Indeed, thulium citrate also exhibits distribution characteristics which are more favourable than those of gallium citrate, in terms of faster blood clearance and lower accumulation in the liver and GI tract when compared to gallium. This rapid clearance allows tumour imaging to be performed within 4–6 h of administration of the radiopharmaceutical, at which time the thulium level in the tumour is up to twice that normally seen with gallium 50 h after injection. The major disadvantage to the routine use of thulium for tumour imaging arises from the enhanced skeletal uptake compared to gallium. Studies on the mechanism of tumour uptake of lanthanide elements have indicated that a protein-mediated mechanism is involved [598,599]. Measurements of the stabilities of lanthanide-serum protein complexes, as well as serum fractionation, indicate that 95% of administered lanthanide appears to be associated with albumin in serum, and less than 0.5% with transferrin [600]. However, since it is known that transferrin is capable of binding lanthanide cations (see section on transferrin), the enhanced tumour uptake is more likely to arise from a transferrin-mediated uptake process, as is the case with gallium, than from an uptake process involving albumin.

Whilst ytterbium citrate has been used for tumour scintigraphy, the majority of the applications of this lanthanide, particularly in Eastern Europe, have been associated with renal and cerebral imaging, as either the EDTA (25) [601,602] or DTPA (36) [603,604] complexes. However, the use of ytterbium in such imaging procedures has now been superseded by the use of ^{99}Tc^{m} DTPA.

LIKELY FUTURE TRENDS IN CHELATION OF GROUP III CATIONS

The clinical relevance of Group III cation chelation may be divided into two major categories; the removal of unwanted trace metals (iron and aluminium) and the use of high stability complexes for imaging (gallium, indium and gadolinium), therapy (yttrium) and immunoassay (europium). The requirements for these two broad classes are rather different, and are summarized below.

LIGANDS DESIGNED FOR THE REMOVAL OF GROUP III CATIONS

A high selectivity for the cation to be removed is essential. This, together with the required ability to permeate membranes, in both the free ligand and com-

plexed forms, severely restricts ligand choice. Carboxylate and nitrogen containing ligands lack sufficient selectivity for Group III cations, whilst catecholate ligands form charged complexes. In contrast, the hydroxypyridinones are highly selective for Group III cations, and under physiological conditions, both the free ligand and complex are uncharged, and can, therefore, penetrate membranes by simple diffusion. Hydroxypyridinones have an advantage over hydroxamates, in that they are generally orally active. In principle, both bidentate and hexadentate molecules can be utilized. However, the higher molecular weight associated with the hexadentate chelators (≥ 400) limits their biodistribution, and depending on their application, this can be considered to be either an advantage (limited permeation of the blood-brain barrier) or a disadvantage (reduced oral availability). Ideally, the newly formed complex should also be sufficiently stable such that the toxic metal is not readily donated to apotransferrin.

The major application of ligands within this class are for the removal of iron (thalassaemia, sickle-cell anaemia), the redistribution of iron (ischaemia and inflammation) and the removal of aluminium (dialysis encephalopathy).

LIGANDS INTENDED FOR IMAGING AND THERAPY

In contrast to the ligands designed for trace metal removal, compounds within this class are administered as the preformed metal complex, which may or may not be covalently attached to proteins. The chelation requirements are, therefore, different from those outlined for the previous group. With the exception of gallium citrate and the indium cell-labelling agents (which are required to donate their chelated metal ion to endogenous ligands to obtain the required biodistribution), the dominant property of these ligands should be high kinetic and thermodynamic stability. This ensures that under physiological conditions there is little or no donation of the chelated probe metal to natural biological species. For this reason, ligands used for these applications are limited to oligodentate (and in particular, hexadentate) classes. Such stability can be achieved using oxygen-rich ligands, for instance, hydroxamates, catechols and hydroxypyridinones, covalently linked in a favourable stereochemical manner to form the required octahedral environment for the metal cation. The kinetic stability of such complexes can be further enhanced by formation of the corresponding macrobicyclic ligand. This emphasis on high complex stability reduces the requirements for the ligand to be highly selective; if the complex remains intact *in vivo*, there is no opportunity for chelation of other metals. Thus, oligodentate ligands involving nitrogen and/or sulphur donor atoms, may

find increasing application in this field, whereas their use for removal of trace levels of Group III metals *in vivo* is unlikely.

The probe metals finding most widespread application are gallium and indium. Currently, the majority of applications utilize the γ-emitting nuclides of these elements, although the increasing availability of the β^+ (positron)-emitting ^{68}Ga is likely to have a profound effect on the radiopharmaceuticals used in diagnostic imaging procedures, for uses such as blood flow measurements and in the staging of AIDS. The major use of indium is for the labelling of a variety of blood cell types, although there are limitations to the use of the currently available complexes, for instance, the inability to label selected cell types.

The use of immunoglobulin-linked chelates for the detection of various tumour types is an expanding activity, presently limited by both the availability of suitable immunoglobulins, and the published methods for chelate conjugation to proteins, which are less than satisfactory. Considerable development in this latter area is required before the clinical use of chelate-linked immunoglobulins becomes widely established. Advances in this field are likely to signal an increased usage of gadolinium (as an immunoglobulin label, for the improved NMR detection of tumours), and for immunotherapy, both with immunoglobulin-linked photosensitizing agents and suitable β^--emitting radionuclides, such as ^{90}Y.

ACKNOWLEDGEMENTS

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3 Safer Total Parenteral Nutrition based on Speciation Analysis

JOHN R. DUFFIELD, Ph.D.[1], SIMON B. HALL, Ph.D.[1], DAVID R. WILLIAMS, D.Sc.[1,*] and MICHAEL I. BARNETT, Ph.D.[2]

[1] School of Chemistry and Applied Chemistry, University of Wales College of Cardiff, P.O. Box 912, Cardiff, CF1 3TB and [2] Welsh School of Pharmacy, University of Wales College of Cardiff, P.O. Box 13, Cardiff CF1 3XF, U.K.

*To whom correspondence should be addressed.

INTRODUCTION

Total parenteral nutrition (TPN) is a means of providing all the nutritional requirements of seriously ill patients using carbohydrates, fats, nitrogen (as amino acids), electrolytes, trace elements and vitamins, so that a positive nutritional status is achieved. In this review, TPN may also be used to denote 'total parenteral nutrition' as in TPN fluid.

Speciation in the chemical sense of a particular chemical entity (for example, an element, an ion, a molecule) in a given system is defined as the identity and abundance of each and every physico-chemical form in which that entity occurs.

Since the first recorded demonstration of intravenous infusion in 1656 by Wren [1], the field has shown steady and marked improvements. The increased usage of TPN and the sophistication of the components therein over the last decade has been of immense benefit to patients suffering from gastrointestinal obstruction and disease, from wasting disease, burns, trauma, prolonged ileus, and cancer. In addition, the approach is now more widely used in the pre-operative support of undernourished patients. Approximately 5% of hospital patients require parenteral nutritional support of some form. The literature contains numerous reports of new uses, new compositions and increasingly venturesome operations totally dependent on TPN support for the patient for periods of months or even years [2–4].

Research reports concerning TPN are widely spread through the medical and pharmaceutical literature and these articles constitute a complex and voluminous body of knowledge. As yet, no amount of appraisal and evaluation of such knowledge has enabled prediction of the precise TPN regimen for each and every occurrence. However, now that computers have become widely available, it is possible to assimilate this knowledge in a systematic manner so that the therapy, in terms of its benefit-to-risk ratio, can be significantly improved. The preferred means of quantifying interactions between the components of TPN and other biological fluids, such as blood plasma, is to consider the chemical speciation involved. This can markedly facilitate decision making and long-term planning of benefits to patients.

In some respects, the clinical uses of TPN are ahead of the scientific theories but, by a concentrated attack upon quantifying the speciation present in TPN fluids and plasma-TPN mixtures, it is possible to narrow this gap and to enhance safety considerations.

The basic concept is that computer simulation of the speciation can be more thorough and give a more complete appraisal of species present in solution than is possible by solely experimental measurements of just one or two of the

concentrations of the species more accessible to modern instrumentation [5]. This review describes progress made to date and discusses future targets in this new area.

Scheme 3.1 shows the six fundamental components for TPN regimes and the subheadings represent examples of some of the items included amongst those components. When these components are brought together, using the formulation procedures [6] indicated by TPN suppliers, a wide range of new chemical bonds are formed and complex interactions occur, thus creating several hundred different chemical species in the solution being intravenously administered. Further interactions will then occur with the biochemicals present in blood plasma.

A study of the compositions of these species, their concentrations and their net charges is known as 'chemical speciation' [5,7]. For example, when metals present as minerals interact with nitrogen present as amino acid anions, a wide range of classical inorganic metal ion-ligand complexes may be formed. Such species are often formed relatively rapidly upon mixing and so TPN fluids tend to approximate to thermodynamic equilibrium. Similarly, blood plasma is in a 'steady state' which is also close to thermodynamic equilibrium. Unfortunately, as each of these fluids involves many hundreds of different species present at concentrations well below those generally analysable by the most sophisticated techniques, computer simulation is necessary in order to extrapolate our knowledge down to these levels. Such computer programs rely heavily upon thermodynamic equilibrium constants and mass balance equations which dictate the concentrations of solution complexes and solids present under steady state conditions.

The physical chemistry of such interactions may be described in terms of

WATER

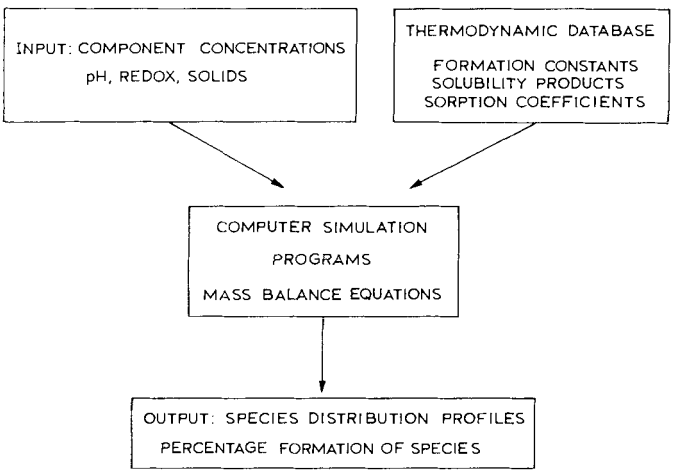
ENERGY	MINERALS
Amino acids	Electrolytes
Glucose	Trace elements
Soya protein	Impurities
Phospholipid	
Glycerol	VITAMINS
NITROGEN	DRUGS
Amino acids	Anticoagulants

Scheme 3.1. TPN components and subcomponents. Considerable expertise already exists in determining the speciation of a selection of these interactions, for example, metal ions interacting with amino acids in blood plasma.

mass balance equations and embodied in computer simulation programs after which thermodynamic databases containing equilibrium constants are compiled and experiments designed to validate the approach. The information from such programs permits an improvement of biochemical awareness and of desirable safety features by avoiding the use of components which lead to highly undesirable side-effects. These include precipitation within a vein (leading to thrombosis), emulsion flocculation, or creaming (a serious problem with TPN fluids containing lipids) and fluid coloration during shelf storage.

Over the last two decades, we have modelled the chemical speciation of trace metals for blood plasma, milk, perspiration, saliva, urine, and other biofluids and used such models to predict the likely changes to the speciation profile when pharmaceuticals, or contaminants, are administered. These may be related to pharmaceutical efficacy, biological response and side-effects [8]. Computer software and hardware have advanced markedly over the last 20 years, as has the fund of thermodynamic data, and it is now possible to calculate equilibrium speciation profiles for a wide range of scenarios. Such profiles are now being applied to TPN therapy and enhanced safety awareness arising from such speciation data.

The simulation approach is to combine total concentrations of metals, amino acids, and ligands for a particular fluid with the appropriate formation constants and solubility products (*Scheme 3.2*). In practice, this compilation is referred to as a database. Large computer programs use this input to calculate



Scheme 3.2. Chemical speciation modelling.

the concentrations of all species present at equilibrium – the complexes, the free ligand or metal ion concentrations and any solids which precipitate.

In order to prepare a solution for intravenous administration, clinical pharmacists choose from a wide range of suppliers' packs and 'compound' such solution packs into the total TPN fluid. It is feasible for computers to hold details of each pack and to examine regimens for incompatibilities, and the related problems of storage and ageing (that is, stability) of such solutions, the influence of impurities present in stock solutions, order of mixing characteristics, and plasma interactions (that is, the local interaction when the TPN fluid meets the plasma in the vicinity of the catheter tip, and also the far more widespread interactions which occur when the TPN fluid is systematically circulated throughout the patient's bloodstream). A competent predictive model should be capable of suggesting features to be monitored during the therapy. For example, the amount of zinc passing out in the urine depends upon amino acid administration and may be related to the concentration of charged zinc complexes present in plasma. Supplementing the TPN fluid with additional zinc may then represent a practicable approach to solving this problem.

The number of patients receiving TPN is increasing and new clinical techniques are being developed. It is thus important to alert scientists, physicians, surgeons, pharmacists, nurses and technicians who design, prepare or administer TPN fluids to the incompatibilities that can arise and also to the help which is available in terms of chemical speciation knowledge. Finally, we indicate areas for future development.

Problems under consideration include:

- the solubility of additives and precipitation propensity in TPN fluids;
- the long-term stability of such fluids in the presence of metal complexes;
- the side-effects caused by impurities present in the stock solutions;
- the 'knock-on' effects of co-administering drugs with the TPN fluids;
- the kinetics of slow precipitation reactions;
- selection of a fluid so that the free metal-ion concentrations are buffered at similar values to those present in normal blood plasma so that the disturbance caused by administration is minimized.

TPN fluids often contain lipid emulsions as a source of energy and these emulsions are often unstable in the presence of electrolytes. A related problem is that when TPN components are being compounded, it is possible to momentarily exceed solubility products or to produce flocculation and creaming of the emulsion because of inadequate stirring; such processes are often irreversible. Complex regimens prepared for patients have a limited stability which can range from hours to several months depending upon a number of factors including the storage temperature, the presence of minerals, electrolytes and the prevail-

ing pH. If, for example, the solubility product of calcium phosphate is exceeded, a solid may precipitate. Visual inspection of such TPN fluids gives an insufficient safety margin. Thus, a quantified underpinning of the safety of such fluids is necessary, and this review aims to extend the use of computer-assisted chemical speciation in compounding.

The review considers each of the fundamental components of TPN fluids, namely, water, nitrogen and energy sources, minerals, vitamins and administered drugs. The equations governing the physical chemistry are indicated and overlapping interactions are suggested.

SPECIATION OF TPN COMPONENTS

WATER

Some 70–75% of human body weight is water. This represents about 50 litres per adult. Shenkin and Wretlind have pointed out that, in a newborn suckling child, up to 20% of its two litres of water is replaced daily [9]. Adults under normal circumstances require 1.5–2 litres per day to be absorbed from the gastrointestinal (GI) tract. The presence of nutrients and especially electrolytes in water raises the osmotic pressure and also modifies the ability of the fluid to pass through cell membranes. Indeed, the properties of a cell membrane which permit two different aqueous phases to be completely separated, with the exception of certain chosen lipophilic compounds which can pass between these aqueous layers, contributes markedly to the patient's ability to survive.

The figures given above emphasize the following points: –

(a) Bypassing the GI tract by using TPN therapy places a great responsibility upon the pharmacist and clinician to ensure that the correct volume of water is admitted directly into a vein.

(b) In order to administer sufficient energy and protein by TPN, it is sometimes necessary to raise this volume of water to almost 4 litres per day for an active adult.

(c) One natural safety barrier – the intestine – is not always active in TPN patients and, in addition, injured persons often accumulate sizeable volumes of fluid. This situation can be exacerbated by poor kidney function. Thus, the matching of TPN components to the water volume in which they are prepared requires careful consideration. Fortunately, equations relating the solubilities of TPN components in water are well established.

(d) In addition to patient water-balance data, water formed by the metabolism

of carbohydrates, fats and proteins should be taken into account in assessing water balance.

Chemical speciation is critical to the health of the patient in many respects. Government legislators are now realizing that to limit an element in the enteral diet in terms of its total concentration is insufficient and requires data pertaining to valence state and complexation to be specified. This is important in TPN where contaminating metals must also be taken into account.

When considering the toxicity of metal ions, whether contaminating or essential, it is important to appreciate that this may well be a function of oxidation state and/or the presence of complexing agents. For example, arsenic(III) and its compounds are highly toxic, whereas As(V) species are much less toxic; chromium(III) is essential to humans but Cr(VI) is toxic. The presence of such species in the TPN bag, or subsequently in blood plasma, may be assessed by using computer simulation programs.

The computer simulation techniques used in the chemical speciation of biofluids are based, in part, upon the same equilibrium concepts as are used by pharmacy students, for example, in calculating the bioavailability of aspirin from its pK value and the pH of the stomach. The net neutral $[\text{aspirinate}^- \cdot \text{H}^+]^0$ species is formed at stomach pH and uptake occurs whereas the small intestine at pH 6.5 contains $[\text{aspirinate}]^-$ and so the analgesic is not absorbed.

Similar physico-chemical constants can be measured and combined in large computer-based speciation models which can describe the total speciation occurring in the wide range of biofluids mentioned earlier. These models are organized as shown in *Scheme 3.3* and require well-validated and verified computer programs which are now readily available. In addition, two types of database are necessary:

(a) a compilation of all of the physico-chemical constants relating to equilibrium conditions at the biofluid temperature and ionic strength; and
(b) an analysis of the total components of each metal and ligand component moiety present in the biofluid.

A knowledge of the speciation occurring under normal biochemistry is the first output from the model. Next, the cumulative effects of adding a new ingredient such as a pharmaceutical or a polluting component to this biochemical equilibrium may be predicted and indicate a requirement to monitor for possible side-effects. Of late, many newly introduced pharmaceuticals have been screened by such speciation analysis. In addition, it is possible to select drugs which are reasonably specific for a particular contaminant metal ion and thus to facilitate removal of the metal from the body by chelation therapy. Thus, a combination of two drugs may be chosen so that the first mobilizes the

offending metal from tissue deposits into blood plasma and then the second drug moves the metal complex from blood plasma out through the kidneys. This approach which utilizes the physico-chemical properties of two drugs is known as synergistic chelation therapy.

The enhanced efficacy of pharmaceutical administration and the minimization of side-effects can now be combined using computer simulation models. The field has recently been reviewed in several papers and is described in a later section [5,7,8,10,11].

NITROGEN

Normally, a regulatory control mechanism exists between the polymerization of amino acids into peptides and proteins and the breakdown of such proteins within the body. It is only for those amino acids which are deaminated or otherwise metabolized that it is necessary to introduce additional sources of nitrogen on a daily basis. The sole source of this nitrogen in TPN therapy is a mixture of amino acids. However, the liver is able to act as a buffer that absorbs excessive amounts of amino acids present in the bloodstream and can even replace, to a limited degree, such amino acids in plasma. Just as proteins are able to buffer the free metal ion concentration in a biofluid (see later), so too the liver is able to buffer the amino acid concentrations therein.

There have been numerous viewpoints concerning the ideal amino acid levels for patients under normal and under trauma conditions. Recommended dietary allowances have been published in different countries and *Table 3.1* indicates a typical listing.

In general, two sources of amino acid preparations have been used. Early preparations employed protein hydrolysates and these have since been replaced by mixtures of the purified and crystalline amino acids.

Table 3.1. AN EXAMPLE OF A COMMERCIALLY AVAILABLE AMINO ACID MIXTURE (g or mg per dm^{-3}) [12]

L-Alanine	160.0 g	L-Lysine	9.0 g
L-Arginine	11.3 g	L-Methionine	5.6 g
L-Aspartic acid	3.4 g	L-Phenylalanine	7.9 g
L-Cysteine/cystine	560.0 mg	L-Proline	6.8 g
L-Glutamic acid	5.6 g	L-Serine	4.5 g
Glycine	7.9 g	L-Threonine	5.6 g
L-Histidine	6.8 g	L-Tryptophan	1.9 g
L-Isoleucine	5.6 g	L-Tyrosine	230.0 mg
L-Leucine	7.9 g	L-Valine	7.3 g

Clearly, it is necessary to have a well-balanced mixture of all of the essential amino acids as well as a wide spectrum of the non-essential ones. The optimal utilization of such amino acids given intravenously requires a constant source of energy. Thus, carbohydrates or lipids are co-administered (and discussed in the next section).

At the pH values prevailing in blood, such amino acids are zwitterionic and are able to influence a wide range of TPN speciation so that, for example, the anions can complex essential metal ions present.

ENERGY

Energy is normally provided in TPN fluids in the form of carbohydrates, alcohols, or fats and oils. If the patient's sole energy requirement is given in the form of glucose, about 700–800 g per day are necessary. This would usually require extra insulin to be added in order to permit all of this energy to be utilized. Other carbohydrates, such as fructose or maltose, have been used, as have alcohols (even ethanol!) [13]. The intravenous administration of such a large amount of material requires the use of a large central vein rather than a peripheral vein.

Smaller volumes of material can be employed when lipids are used as an energy source. Although they suffer the disadvantage of not being water-soluble, they may be readily emulsified. Current high-energy TPN diets tend to contain a fat emulsion of either soya bean oil or cotton-seed oil, with glycerol to provide isotonicity. An emulsion is a type of colloid whereby both the continuous phase and the dispersed phase are liquids, the dispersion being stabilized by an emulsifier or surfactant.

It is now possible to prepare emulsions which are stable for several years. Surfactants commonly used in preparing intravenous emulsions include phospholipids having an organic chain which becomes attached to the oil droplets and a charged phosphate end which protrudes into the aqueous solution and becomes highly solvated.

The use of a central vein and infusion of a mixture of fat emulsions and carbohydrates is now the preferred means of treatment. The main advantage is that large amounts of energy can be given in a relatively small volume of a fluid which is isotonic with blood plasma. This is not possible with diets in which carbohydrates alone provide energy. Typically, lipids for intravenous infusion are administered as 10% or 20% isotonic emulsions through a peripheral vein or in a TPN mixture through a central vein.

MINERALS

As a great deal of knowledge has yet to be acquired concerning the intake, uptake, and bioavailability of minerals in our enteral diet, it is not surprising that much progress remains to be made in the analysis and assessment of the mineral requirements for TPN support.

It is necessary to differentiate between adequate requirements for a healthy person and for those who have an extra requirement arising from a diseased state. The safety of administering these minerals must be assessed because their presence as ions and complexes can markedly influence the speciation of other biochemical components and also the integrity of lipid emulsions.

Furthermore, whereas amino acid and other *organic* biochemical concentrations tend to be fairly constant for healthy humans, there seems to be a wider variation in *mineral* concentrations which are, in turn, related to the concentrations of chemically similar elements. The well-known phenomena of antagonism and stimulation between different elements will undoubtedly influence TPN therapy [15]. *Table 3.2* lists the main electrolytes supplied in TPN

Table 3.2. THE MAIN ELECTROLYTES INVOLVED IN TPN ADMINISTRATION
The ranges shown cover basal amounts through to high supply figures [9]. Figures are per kilogram of body weight.

Ingredient, unit	*Adult*	*Neonates and infants*
Water, dm^{-3}	0.030–0.150	0.100–0.150
Energy, kJ	130–250	380–630
Amino acids, g	0.09–0.50	0.3–0.5
Glucose, g	2–7	12–30
Fat, g	2–4	4–6
Sodium, mmol	1–4	1–5
Potassium, mmol	0.7–4	2–5
Calcium, μmol	110–200	500–2000
Magnesium, μmol	40–400	150–1000
Iron, μmol	0.25–1	2–4
Manganese, μmol	0.1–0.6	0.3–1
Zinc, μmol	0.7–3	0.6–1.5
Copper, μmol	0.07–1	0.3
Chromium, μmol	0.015	0.01
Selenium, μmol	0.006	0.04
Molybdenum, μmol	0.003	
Chlorine, mmol	1.3–4	2–6
Phosphorus, mmol	0.15–1.0	0.4–3
Fluorine, μmol	0.7–1.5	3
Iodine, μmol	0.015	0.04–0.1

therapy. Ranges are given depending upon whether the patient requires moderate amounts or high concentrations because of severe catabolic trauma as in, for example, burn victims. Under ideal conditions, monitoring of the losses through different routes should be carried out so that the input at least satisfies, or even exceeds, the losses. However, this is an exceedingly difficult target to achieve.

Because of the uncertainties listed above, the exact choice of input, patient for patient, is a matter of good and experienced judgement; Shenkin and Wretlind have surveyed different element concentrations for different conditions [9]. In the computer programs (mentioned later) for optimizing safety and minimizing side-effects, the above-mentioned wide variation in concentrations can easily be accommodated, since the specific choices of the clinicians and pharmacists may be used as input data. In reality, not all degrees of freedom, with respect to TPN component concentrations, are readily available to these specialists. This is because certain groups of concentrations of trace elements are often unavoidable and depend upon the multiple element packs supplied by the manufacturers of TPN fluids.

In addition to those elements added intentionally to the packs as supplied by the manufacturers, it should be noted that a number of other elements may be present as impurities. Such trace metal impurities may give rise to beneficial and/or toxic effects depending upon their physico-chemical form(s) and on whether or not they are essential to man. Elements involved in such 'beneficial impurity' versus 'contamination' discussions include aluminium, antimony, arsenic, barium, boron, cadmium, lead, nickel, rubidium, strontium, tin and even vanadium. At one time or another, each of these trace elements has been implicated as being essential to animal life. No doubt the future will reveal the true importance of these suggestions.

With the technical improvements which now make it possible for TPN to be administered for several months, there has been increasing demand for adequate mineral supplies and sometimes with raised levels of elements such as zinc necessary to promote healing. A range of mixtures of such trace elements has been suggested in the literature and recently reviewed [16].

Analysis of minerals present as trace elements is an on-going challenge. Dietary intake is measured in terms of milligrams, whereas metals present in tissues rarely exceed 50 μg per g of tissue. When TPN is used as a substitute for enteral feeding, it is prudent to assume that there has been inadequate intake of trace elements over some time, that is, tissue levels and reserves are low. Increased trace element losses arising from disease states *per se* is a well-established phenomenon. In addition, a secondary requirement for trace elements may be necessary because of renal or diabetic problems.

The assessment of a patient's trace element status can be extremely difficult. Total plasma concentrations can be measured, but only with the more predominant elements such as calcium, sodium and selenium can normal plasma concentrations be correlated with balance. Normally, there is an indirect relationship between plasma concentrations and tissue concentrations. Urine losses may be measured, but whole-body balance studies are far safer, especially when the elements being considered are those excreted through the bile duct rather than through the kidneys. However, the manifold difficulties of collecting faeces, aspirations, fistula losses, and all of the other specimens necessary for a whole-body balance study are self-evident. Tissues can be analysed but this is a painful and unpleasant task from the viewpoint of the patient. Some researchers have tried to correlate trace element status with biochemical reactions which are governed by those trace elements; for example, alkaline phosphatase is dependent upon zinc status and xanthine oxidase depends upon the molybdenum concentration. Similarly, element-dependent physiological functions such as muscle-power measurements have been tried.

Clearly, the experience of clinicians and pharmacists is worth far more than an arbitrarily fixed diet of trace element concentrations to be used under all circumstances. When their experience suggests a set of recommended daily intakes, then speciation analysis can take over and assess safety margins and likely side-effects.

Shenkin and Wretlind [9] have assessed those elements which are a matter of serious concern and those that are chosen for rather academic reasons. Zinc, copper, selenium, chromium, iron, molybdenum, manganese and cobalt fall into the 'elements for concern' at present. *Table 3.3* shows a typically required elemental package but, as has been discussed earlier, there will be unspecified elemental impurities such as aluminium present. In response to this latter problem, Hewitt *et al.* [17] have analysed the aluminium levels in infant milk formulations and in intravenous fluids. Intravenous feeding bypasses the GI tract, so that aluminium, which would normally be excluded by the GI tract, is able to gain direct access to the blood plasma and, hence, the body tissues. This can present a serious problem in children fed intravenously who have poorly-developed or damaged kidneys. Paradoxically, the fact that the environment is exceedingly rich in aluminium [18], and yet tissue and fluid levels of this element are extremely low, means that there are special problems in calibrating equipment such as atomic absorption spectrophotometers used for this work.

Sometimes, the essential trace elements or the impurity elements are subject to localized precipitation near the tip of in-dwelling catheters. Stennet *et al.* have carefully analyzed such precipitates and found them to contain both

Table 3.3. RECOMMENDED DAILY INTAKES OF MICRONUTRIENTS BY THE PARENTERAL ROUTE (KABI NUTRITION, STOCKHOLM)

Inorganic micronutrients			*Inorganic micronutrients*		
Iron	(μmol)	20	Fluorine	(μmol)	50
	(mg)	1.1		(mg)	0.95
Zinc	(μmol)	38–100	Chromium	(μmol)	0.2–0.3
	(mg)	2.5–6.4		(mg)	0.01–0.015
Copper	(μmol)	8–24	Selenium	(μmol)	0.4
	(mg)	0.5–1.5		(mg)	0.03
Iodine	(μmol)	1.0	Molybdenum	(μmol)	0.2
	(mg)	0.127		(mg)	0.02
Manganese	(μmol)	3–15			
	(mg)	0.15–0.8			

essential and non-essential elements in the form of calcium, phosphorus, silicon and some organic residues [19]. These precipitates, which could lead to thrombosis, are to be avoided.

Ideally, trace elements should be provided to the patient at the same levels as would be received from a normal balanced diet via a healthy and well-functioning GI tract. In practice, this is a very difficult goal to realize because of the different speciation occurring in the stomach, gastrointestinal tract, blood plasma, and the TPN fluid. However, suggestions later in this review go some way towards achieving this end.

VITAMINS

Vitamin supplementation is an essential part of nutritional support via TPN. As most patients requiring intravenous nutrition are usually in a rather poor condition with vastly depleted vitamin stores, vitamin supplies far in excess of those normally required are usually desirable; doubling the vitamin input is not unusual.

In general, the vitamins fall into the two classical groups of water-soluble and fat-soluble vitamins. The water-soluble vitamins include thiamine, riboflavin, niacin, pyridoxine, folic acid, vitamin B-12, pantothenic acid, biotin, ascorbic acid and choline [9]. The fat-soluble vitamins include A, D and K.

As the half-lives of vitamins in the human body are the order of a week or two, vitamin deficiency can be detected 9 or 10 days following some TPN administrations. Thus, vitamin supplementation as part of the therapy *ab initio*

is highly desirable. There are satisfactory water-soluble vitamin supplements such as are given in *Table 3.4*, but the method of supplementation of fat-soluble vitamins is much less clear [12]. In addition, some of the vitamins such as vitamin K, vitamin B-12 and folic acid, are unstable in aqueous solutions, thus presenting storage and compounding problems.

Table 3.4. EXAMPLE OF AN ADDITIVE PACKAGE PRODUCED BY ONE COMPANY (KABI NUTRITION, STOCKHOLM)

Thiamine mononitrate (B-1)	3.1 mg
Sodium riboflavine phosphate (B-2)	4.9 mg
Nicotinamide	40 mg
Pyridoxine hydrochloride (B-6)	4.9 mg
Sodium pantothenate	16.5 mg
Biotin	60 μg
Folic acid	0.4 mg
Cyanocobalamin (B-12)	5.0 μg
Sodium ascorbate	113 mg
Glycine	100 mg
Sodium edetate	0.5 mg
Methyl 4-hydroxybenzoate	0.5 mg

DRUGS

When the oral route for drug administration is excluded, for the same reasons that make TPN a requirement, it is necessary to add drugs intraveneously. The TPN fluid bags and associated lines and tubes termed 'giving sets' are an alternative which obviates the need to locate another vein. The number of drugs that can be injected is increasing daily [20] and already reports are appearing in the literature (for example, Ref. 21) of such drugs perturbing the chemistry of the TPN fluid by changing its chemical speciation.

As both the usage of TPN and the number of weeks of therapy increases, the administration of both drugs, vitamins and trace elements by this route becomes an increasingly important aspect for future researches.

CHEMICAL SPECIATION MODELLING AND ITS COMPUTATION

The term speciation has been defined earlier as a knowledge of the individual chemical forms in which an element exists and the abundance of each of these forms. Usually, such species are composed of a number of ligands and metal

ions which collectively produce a net overall charge [5]. The density of this charge or, conversely, the electroneutrality of the complex formed, determines whether or not the species will be hydrated and remain in aqueous fluids such as blood plasma and/or will be excreted through the kidneys. If of low or zero charge density, the species is probably lipid-soluble and able to pass from biofluids into tissues, or it may precipitate, as occurs during the formation of bone and tooth enamel. Thus, knowledge of speciation can assist in formulating hypotheses concerning how best to encourage a metal to move around the body's biochemistry and play its desired roles.

Analytical problems prevail, since the equilibria present are sometimes extremely delicate and easily disturbed and also the concentrations present are well below those analysable by established, reliable laboratory techniques. This situation, however, lends itself to speciation modelling techniques using computers which can extrapolate data down to extremely low concentrations based upon well-determined physico-chemical constants, that is, the chemical laws that govern nature (*Scheme 3.2*). If such 'analyses' from the computer are reliably validated by specifically designed experiments, it is then possible to challenge the model system to predict the influence of impurities, trace element imbalances, disturbing the system by administering a pharmaceutical or other intrusion.

History has been kind to chemical speciation modellers in that the thermodynamic, physico-chemical data necessary for many of these reactions have been determined over the last few decades and are already available from the literature. Data for the formation of mixed ligand complexes (called ternary complexes) are more difficult to obtain but may still be calculated mathematically by using a scale of stabilization factors. Indeed, it is often these ternary complexes which are the most important trace element biochemicals, since they act as intermediaries for placing a metal in a protein or for bridging between one moiety to another. Free trace metal ions rarely, if ever, exist in meaningful concentrations *in vivo*.

A useful approximation is to regard each trace metal ion as being present in one of four distinguishable forms (see, for example, *Table 3.5*) [22,23].

(a) The first is that of the metal being bound to so-called inert metalloprotein (inert in the sense that the metal cannot be extracted by treatment with a chelating agent although, of course, it can be released when the whole protein is catabolised in the liver or spleen).
(b) There is labile protein which permits the metal ion to come and go from its active site in balanced equilibrium with
(c) low molecular mass complexes of the metal ion which, in turn, are in equilibrium with

Table 3.5. INERT AND LABILE PROTEINS IN VIVO

The equilibrium arrows indicate rapid exchange under the correct speciation conditions whereas insertion and extraction of metal ions to/from the inert species requires a biochemical mechanism which usually involves destruction or recomposition of the protein [22, 23]. lmm, low-molecular-mass.

Inert and/or thermodynamically non-reversible	*Labile and thermodynamically reversible*		
(a)	*(b)*	*(c)*	*(d)*
Iron Haemoglobin Myoglobin (Ferritin)	transferrin $\rightleftharpoons$	lmm Fe^{3+} complexes	$\rightleftharpoons [Fe(H_2O)_6]^{3+}$
Copper Ceruloplasmin (Metallo-thionein)	serum albumin $\rightleftharpoons$	lmm Cu^{2+} complexes	$\rightleftharpoons [Cu(H_2O)_6]^{2+}$
Zinc α_2 macroglobulin (Metallo-thionein)	serum albumin $\rightleftharpoons$	lmm Zn^{2+} complexes	$\rightleftharpoons [Zn(H_2O)_6]^{2+}$

(d) exceedingly low concentrations of the aquated metal ion. These concentrations, other than for calcium, are far too low for nature to have found an active role for them.

Examples of these four broad categories are listed in *Table 3.5*.

It is the last three areas – labile protein, low molecular mass complexes, and aquated metal ions – which are modelled by using computer simulation programs such as ECCLES or HALTAFALL to solve the algorithms relating the mass balance characteristics of the system as expressed in terms of equilibrium constants such as partial pressure constants, activity constants, solubility products [24–26].

The databases of physical chemical constants are, as expected, stored in computers and the Speciation Research Group in Cardiff has the largest database of such constants in the U.K., most of which have been verified by compatibility exercises or assessed by peer review [24].

The scale of the modelling is noteworthy. For example, in blood, in which more than a dozen metal ions interact with 20 or more amino acids as well as other inorganic and organic ligands, the models run to many thousands of complexes. Our widely used model of blood plasma contains some

Table 3.6. THE PERCENTAGE DISTRIBUTION OF THE METAL IONS Ca^{2+}, Cu^{2+}, Fe^{3+}, Mg^{2+}, Mn^{2+} AND Zn^{2+} AMONGST LOW-MOLECULAR-MASS (lmm) LIGANDS IN HUMAN BLOOD PLASMA AS FOUND BY COMPUTER SIMULATION

Only the most prevalent few lmm complexes are listed [25].

Complex	*Percentage of the total metal in the lmm fraction*	*Complex*	*Percentage of the total metal in the lmm fraction*
Ca carbonate · H^+	7.8	Mg carbonate · H^+	5.1
Ca citrate · OH^{2-}	3.2	Mg citrate$^-$	3.9
Ca phosphate$^-$	2.8	Mg lactate$^+$	3.4
Cu histidinate · glutamate	19.7	Mn carbonate · H^+	23.1
Cu (histidinate)$_2$	13.1	Mn citrate$^-$	8.4
Cu histidinate · serinate	12.1	Mn carbonate	2.3
Cu histidinate · threoninate	11.4	Zn (cysteinate)$_2^{2-}$	35.9
Cu histidinate · valinate	5.8	Zn cysteinate · histidinate$^-$	16.5
Cu histidinate · glycinate	5.5	Zn citrate · OH^{2-}	7.2
Fe citrate$(OH)_2^{2-}$	99.6	Zn histidinate$^+$	3.7

8,000–10,000 complexes. *Table 3.6* lists the main low-molecular-mass species for a few of the essential trace metals.

The non-invasive nature of the technique and the ability to produce results rapidly permits rationalization of clinical researches and planning of side-effect experiments using other models in a more effective manner. Thus, the decision-making processes are aided.

BIOLOGICAL FLUIDS PREVIOUSLY SPECIATED

The first fluid to be studied two decades ago was that of blood plasma [25–29]. This was investigated, not only because it has such a central role to the whole of life's biochemistry in man, but also it is fortunate that it is such an invariant fluid, being relatively constant from patient to patient. The pH of blood plasma is tightly buffered at a value of 7.4 and the concentrations of most of the essential low-molecular-mass species present in plasma are buffered. The metal ions are actually buffered by the labile proteins acting as a circulating 'reservoir' for such cations and, in addition, cells act as useful buffers for many concentrations of biochemicals such as amino acids. A typical list of the major trace metal complexes present in blood plasma are shown in *Table 3.6*. There are several

hundred less prevalent low-molecular-mass metal complexes present that are included in a full computer simulation printout of plasma, but for simplicity these are omitted from *Table 3.6*.

Later work compared milk from human and bovine sources and discussed the nutritional overtones for the feeding of infants [30,31]. The presence of aluminium as an impurity in human and bovine milk has been modelled to reveal the different bioavailabilities [32].

Saliva (which interacts with drugs, food, dentifrices and all orally administered products), intestinal fluids and perspiration have also been investigated [10]. Through careful chemical manipulation of speciation, complexes can be designed with antiplaque activity or to provide calcium fluoride species for remineralisation of carious lesions [5].

The presence of metals or ligand agents (for example, pharmaceuticals) as impurities disturbs the existing steady-state equilibrium. The mobilizing ability inherent with each pharmaceutical can now be expressed quantitatively in terms of a Plasma Mobilising Index as defined by May and Williams [28]. Thus, the likely trace metal side-effects, and even the *modus operandi*, of pharmaceuticals can be modelled using computer simulation.

Some metal ions have the ability to undergo redox reactions between different oxidation states. This is particularly important for the ferrous and ferric states of iron in the body and polluting metal ions such as plutonium [11,34]. In addition, some pharmaceutical agents hydrolyze or metabolize to form related products [33]. A whole series of papers has been published concerning these different aspects ([8,35] and references therein).

The desirable objectives include the ability to correlate biological response with that of the chemical speciation prevailing [36,37]. This enables optimization of the desirable biochemical reactions and minimization of likely side-effects. Furthermore, species that may be subject to excretion can be identified and this information may provide evidence for the need for trace element supplementation therapy.

The field of computer simulation of speciation is expanding rapidly. Over and above the biofluids already mentioned, gastric, pancreatic and bile juices [38], river waters, groundwaters, sea waters, food, drink, and health-care agents are all being investigated. New journals covering speciation are being published [39] and Governments are moving towards the situation where legislation will no longer involve just the total amounts which are permissible but rather some definition of the speciation present will be mandatory.

The following sections indicate how such speciation analyses may well be applied to TPN fluids and how administration of such fluids may interact with the normal speciation chemistry of blood plasma.

MINERALS AND NITROGEN INTERACTIONS IN TPN FLUIDS

Metal ions in TPN fluids can interact with phosphate groups and other anions to form precipitates which either block in-line filters or can lead to thrombosis within the circulation. The same metal ions are able to form metal-ligand complexes with the anions of amino acids administered by the TPN route. Berthon *et al.* [40], using computer simulation methods, have considered how best to match TPN fluid speciation with that of blood plasma. The approach is one of matching up the respective free ion concentrations between blood plasma and TPN fluid so that the minimum disturbance occurs upon mixing. Some difficulties may be expected in assessing the total trace element status of the patient, as speciation models of patient metal-ligand complexing can be significantly different from those for a normal human. Ideally, blood plasma from each patient ought to be analyzed using column liquid chromatography coupled to an amino acid analyzer.

Alternatively, when such data are not available, the computer can scan a wide range of concentrations looking for the worst possible case of speciation disturbance or of precipitation. Finally, in order to encourage healing, it is sometimes necessary to have metal concentrations in plasma in positive balance. Conversely, the range of amino acids administered ought not to upset the metal ion speciation in the blood plasma. With metal-depleted plasma of the seriously ill patient, it may be necessary to iteratively raise amino acid concentrations. It is noteworthy that such simulation approaches, particularly studying zinc excretion through the urine, have been validated by animal experiments [41]. Certainly, TPN administration without having metal ions present causes copious excretion of important metals such as zinc. The implementation of metal administration through TPN is a means of ensuring that (a) excretion of essential metals from plasma is not encouraged and (b) the overall level of trace metals in plasma can be gently raised to promote healing – a positive nutritional status resulting in more rapid healing of damaged tissues.

The administration of relatively large amounts of a reducing amino acid such as cysteine can cause copper ions to be reduced to the cuprous state and to form precipitates. It has been suggested that replacing much of the cysteine with another amino acid such as methionine can circumvent this situation. Clearly, a more quantitative assessment of redoxing is highly desirable.

Critics may claim that such models are an oversimplification, but a good model merely attempts to mimic the essential features of the system, setting aside the mass of lesser detail. This permits clinicians to adjudicate more objectively on the most important aspects.

METAL ION AND DRUG INTERACTIONS

Most pharmaceuticals have electron-donor groups which are responsible for their pharmacological activity. This is matched by the electron-acceptor properties of metal ions in solution and so it is not surprising that the two tend to come together whenever possible to form complex species. Trissel's review lists several hundred solutions containing drugs which may be injected intravenously [20]. Clearly, many of these drugs have the potential to interact with metal ions present in the TPN fluid or in blood plasma.

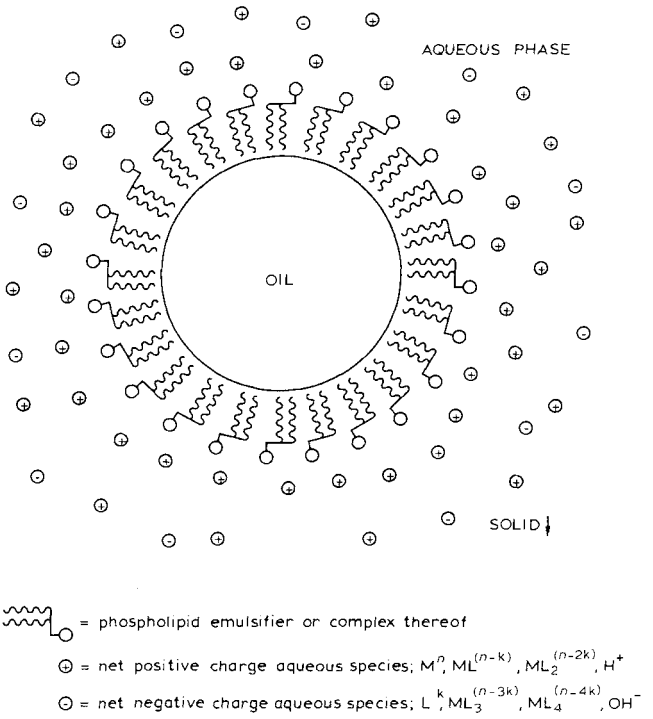
Furthermore, pharmaceutical chelating agents have been added to TPN stock solutions in order to suppress an undesired side-reaction. For example, versenate (EDTA) is added to Aminoven solution in order to discourage metal ions therein from polymerizing and precipitating. TPN speciation modelling has shown that the EDTA complexes a significant percentage of the ferric and manganous ions present.

LIPIDS AND COLLOIDS

In general, intravenous emulsions consist of dispersions of fat droplets in a continuous aqueous phase (*Scheme 3.3*). Blood is also colloidal in nature being a dispersion of corpuscles in serum [42]. It is possible that agents present for the stabilization of one emulsion system may well influence the stability of others; for example, anticoagulants, while preventing the aggregation of corpuscles, adversely affect the stability of the fat emulsion used in TPN infusions.

The patient requires both essential fatty acids (linoleic, linolenic and arachidonic) and energy sources. The use of lipid emulsions of soybean oil, safflower oil, cottonseed oil may achieve these objectives. An appropriate emulsifier is required, but the number of acceptable surfactants for intravenous use is limited by problems of toxicity [43]. Purified egg yolk phospholipids such as phosphotidylcholine are often used to produce stable intravenous emulsions with acceptable particle size distributions in the range 0.1–1.0 μm [44]. After administration, these particles break down and release triacylglycerols, which are then hydrolysed by lipoprotein lipase in a manner similar to the utilization of chylomicra produced following the enteral absorption of fats.

Problems arise with the stability of soya oil lipid emulsions when calcium and heparin are coadministered, heparin being used to suppress blood coagulation. It has been suggested that the negatively charged phosphate groups of the lipid emulsifying agent form crosslinks via the positively charged calcium ions onto



Scheme 3.3. Representation of how chemical speciation in the aqueous phase of an emulsion interacts with the emulsifying agent.

adjacent oil droplets [45]. The phenomenon of creaming associated with such interactions is to be avoided in TPN solutions [46].

PROGRESS WITH TPN CHEMICAL SPECIATION STUDIES

Firstly, we have recently produced computer software for marshalling data concerning compounding materials so that the clinical pharmacist can present the clinician with an audit of amounts administered compared with recommended daily amounts [47]. This is designed to be used with a desktop computer.

Secondly, solution problems arising from calcium precipitation have been examined. Plots of calcium and phosphate concentrations exhibit precipitation boundaries (based upon solubility products) which can be used to quantify the

factor by which a compounding solution risks precipitation [48–50]. An example is shown in *Figure 3.1.* These speciation predictions have been validated with laboratory experiments, which reproduce the computed curves.

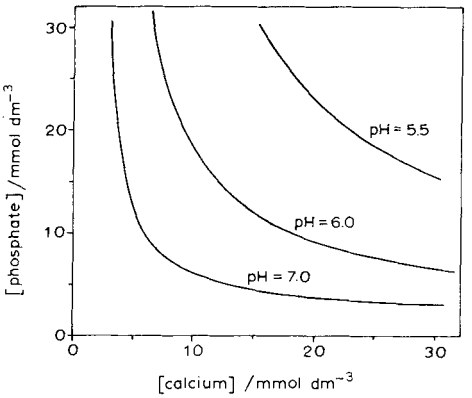


Figure 3.1. Calcium and phosphate solubility limit curves for several pH values at 25 °C and at an ionic srength of 150 mmol dm^{-3}. Precipitation of $CaHPO_4 \cdot 2H_2O$ is predicted for concentrations above and to the right of the curves.

Thirdly, aluminium and silica frequently occur as impurities in TPN solutions. This gives the possibility of precipitation. As aluminium is ubiquitous (the third most abundant element in the Earth's crust), man's biggest exposure is through the oral route. However, highly charged complexes are not absorbed across the GI tract [38,50,51]. In the absence of TPN, plasma levels of aluminium may be approximately 5 μg dm^{-3}. However, typical paediatric TPN regimes can contain 2.8 μmol dm^{-3}, which can raise plasma levels to 37 μg dm^{-3}. Usually, fully functional kidneys can excrete from this circulating pool, but in neonates and patients with compromised renal function, the dangers are best avoided by using ingredients which have a lower aluminium content [50]. Silica tends to co-deposit with aluminium in plaques [52] and we are currently modelling the ability of silica to bind aluminium in TPN and plasma.

Finally, the pH values of TPN solutions is a critical factor in determining stability. Surprisingly, many scientists cannot accurately determine pH and so we have simplified approaches and theory in a recent publication [53].

The influence of aqueous solutions upon the emulsion particles is pivotal to a full consideration of TPN speciation. Theories of emulsion stability were produced before there was a speciation rationalization of aqueous chemistry. Recent research papers from our laboratories have (a) discussed the applica-

bility of the Deryaguin-Landau and Verwey-Overbeek theory as an explanation for the Schulze-Hardy rule for colloid aggregation [54], (b) produced a stochastic computer simulation of emulsion coalescence [55] and (c) attempted to validate new theories by particle distribution tests on TPN solutions [56].

PROBLEMS FOR THE FUTURE

The risks of the following need to be quantified: solution coloration, precipitation, pH changes associated with degradation of glucose solutions, Maillard reactions between glucose and amino acids, and redox problems between copper and sulphur-containing amino acids.

CONCLUDING REMARKS

Unlike many other chapters in this series, this article has looked towards future developments rather than surveying past science. Each of the foregoing aspects that we have described may be assessed, quantified by speciation analysis, and combined into an overview for the clinician and hospital pharmacist in order to target their biological sampling, monitoring and, above all, their decision-making processes. The use of desktop computers is one obvious way of achieving many of these objectives. To date, there have been several reports of such computers being used to accumulate patient data and formulation choices, but only in terms of total amounts of materials. Speciation and bioavailability have yet to be introduced in order for the clinical pharmacist to optimize the formulations presented to the clinician, to quantify the likely side-effects, and to ensure that adequate safety margins are built into these formulations for optimum patient benefit [57–60].

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4 Anti-Ulcer Drugs of Plant Origin

D.A. LEWIS, Ph.D., D.Sc. and P.J. HANSON, D. Phil.

Pharmaceutical Sciences Institute, Aston University, Aston Triangle, Birmingham, B4 7ET, U.K.

INTRODUCTION

Drugs are discovered in a number of ways, sometimes by a chance observation. A well-known example is Fleming's observation in 1929 that *Penicillin notatum* produced a substance that hindered the growth of certain bacteria. This led ultimately to the discovery and development of the penicillins and indirectly to the discovery of other antibacterial agents.

Drugs are found by screening compounds against an animal model of a human disease. The usual starting point is a compound with some pharmacological activity against the model and this is followed by screening analogues of the compound in the hope that their biological properties are improved. This approach has been used extensively in cancer research in an attempt to find new antitumour drugs. Sometimes a drug that fails a screening test for one disease may succeed in another. Such a drug is azidothymidine (Retrovir) which, having failed an anti-cancer screen, is at present an approved drug for treating AIDS. This type of screen is expensive and time-consuming and has a low success rate. Empirical methods such as the general screening of large numbers of compounds for biological activity are slowly being replaced by the more scientific approach of drug design. This approach demands a knowledge

of the aetiology of the disease and our knowledge of the biochemistry of diseases has been so improved by technological advances that a molecular approach can now be made to design drugs for certain diseases. The recent science of computer graphics is making a useful contribution to this approach. Unfortunately, with some diseases, and peptic ulceration is one, our knowledge of the aetiology is still incomplete. Although drug treatment for the patient has improved, peptic ulcers with their complications still carry a significant mortality. Consequently, new drugs are still needed.

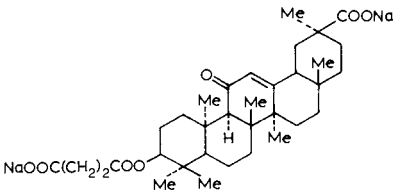
Plants provide an alternative strategy in the search for new drugs. The exploitation of the old herbal books led to the discovery of the first modern anti-ulcer drug, carbenoxolone. Despite this early success, plants are still largely unexploited as sources of new drugs. Medicinal plants offer the chemist and the pharmacologist a rich source of new molecules to explore as future drugs or as biochemical tools to unravel aetiology. This review describes both established and experimental anti-ulcer drugs from plants and attempts to relate structural features in active compounds to the pathology of peptic ulceration. The existence of any relationships are discussed at the end of the review.

Historically, many medicinal plants listed in ancient pharmacopoeias have a reputation for treating gastric disorders. Crude drugs derived from such plants contain only low concentrations of active substances and in consequence are often largely ineffective. In fact, until 1965, when carbenoxolone became generally available, there were no drugs capable of healing ulcers. The standard treatment for peptic ulcer in the U.K. before 1965 was bedrest and advice on diet and on improving one's lifestyle in showing moderation in smoking and alcohol intake. About the only drugs used were simple antacids with a neutralizing effect on stomach acidity and relief from pain. The effect of the simple antacids available at the time rarely lasted for more than 2 h. An interesting natural treatment based on antacids was used by North American indians (Ojibwas) who made a 'tea' of the inflorescences of the hop (*Humulus lupulus*) which supposely acted like sodium bicarbonate in decreasing acidity [1].

A significant advance in the treatment of peptic ulcer was made in the 1960's following a phytochemical investigation of the roots and rhizomes of liquorice. Extracts of liquorice (*Glycyrrhiza glabra*) were widely used in seventeenth century Europe in clearing 'inflamed stomachs'. Other *Glycyrrhiza* species were used in far earlier times in China in the preparation of crude drugs used for treating stomach disorders.

In 1962, Doll and his colleagues published a paper [2] entitled 'Clinical trial of a triterpenoid liquorice compound on anti-ulcer activity'. This established the

efficacy of the triterpenoid saponin glycyrrhizic acid (glycyrrhizen) which is an active anti-ulcer component of liquorice extracts. This substance is also present in the roots and leaves of *Abrus precatorius* which is found throughout the tropics and is used as a substitute for liquorice in the Malay peninsula and Indonesia. Other *Abrus* species such as *A. melanospermus* and *A. pulchellus* have been used in south-east Asia as a liquorice substitute. Glycyrrhizic acid on boiling is hydrolysed to glycyrrhetinic acid. A drug, carbenoxolone sodium (Biogastrone, Bioral, Duogastrone, Neogel, Sanodin (1)), based on glycyrrhetinic acid was marketed; it is the water-soluble sodium salt of the hemisuccinate of glycyrrhetinic acid.



(1)

Before considering the mode of action of carbenoxolone sodium, it is necessary to understand the pathology of peptic ulcer formation. Peptic ulcer is a convenient term which covers both gastric and duodenal ulcers. Ulcers are fairly common and about 10% of the population carry ulcer scars [3]. In the U.K., duodenal ulcers are more common than gastric ulcers, but the distribution of gastric and duodenal ulcers varies in other countries. Gastric ulcers are more common than duodenal ulcers in coastal regions of Norway, Australia and Samoa. Dietary differences may be responsible for this variation. Peptic ulcers are produced by the self-digestion of the stomach or duodenal wall by gastric juice. The integrity of the gut wall depends on a balance between aggressive and defensive mechanisms. The factors which can upset this balance are either environmental or genetic. Certainly, ulcers run in families, where the risk of individuals increases with adverse environmental conditions. These conditions include mental or physical stress, chronic fatigue, smoking, excess alcohol, infections and drugs, especially anti-inflammatory drugs which inhibit prostaglandin (PG) synthesis. It is likely that several factors interact to cause ulceration.

AGGRESSIVE AND DEFENSIVE FACTORS IN ULCER FORMATION

Some of the factors thought to act as aggressors and defenders of the integrity of the gastric epithelium are shown in *Figure 4.1* and brief comments will be made on the roles of acid, pepsin, mucus and bicarbonate.

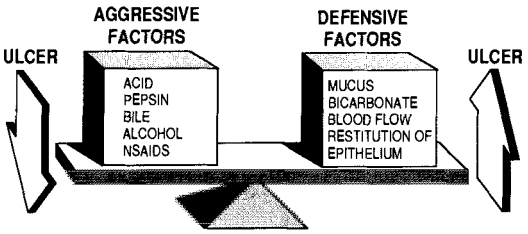


Figure 4.1. The balance of aggressive and defensive factors in peptic ulcer formation.

There is a well-known dictum 'no acid, no ulcer'. Indeed, inhibition of acid secretion by pharmacological blockade of the histamine H_2 receptor using drugs such as cimetidine or ranitidine cures 70–80% of peptic ulcers in 4 weeks [4]. Although removal of acid facilitates the healing of ulcers, it remains unclear to what extent acid is a cause of the ulceration. Another problem is that, although histamine H_2 antagonists cure the ulcer, they do not cure the underlying disease. Consequently recurrence of ulceration after cessation of treatment is very common [4]. Of the plant products discussed in this review only cyanidanol-3, narinogenin, possibly catechin and atropine act by inhibiting acid secretion, although their mode of action is not that of H_2 antagonists.

Pepsin can digest the gastric epithelium and also the surface coating of mucus (see below). Separation of the role played by the various forms of pepsin from that of acid in the genesis of gastric ulcer is complicated because pepsin is only active in the presence of acid. In gastric and duodenal ulcer patients, there is a greater proportion of pepsin 1 than in normal subjects. This result could be significant because pepsin 1 appears to digest mucus more readily than the major form of the enzyme, pepsin 3 [5]. Of the plant products mentioned in this review, carbenoxolone has been reported to inhibit pepsin secretion [6].

Mucus is an insoluble viscoelastic gel which forms a continuous layer over the surface of the stomach. In man the average thickness of this layer is 180 μm [5]. The main constituent of mucus is a high-molecular-weight glycoprotein. The gel-like properties of the mucus depend on the extent of polymerization of

this glycoprotein. Mucus is impermeable to large molecules like pepsin, and therefore prevents this enzyme from attacking the cells lining the stomach. Pepsin can itself attack the surface of the mucus layer. It causes the dissolution of the mucus by breaking up the polymeric structure of the glycoprotein. The mucus layer is permeable to acid. Bicarbonate is secreted from the surface cells into the unstirred layer of mucus where it neutralizes the acid diffusing in the opposite direction from the lumen of the stomach (Figure 4.2) [5]. This process enables the pH of the epithelial cell surface to be maintained near neutrality, provided that the pH in the lumen does not drop below about 1.5. If there were no unstirred layer, the acid in the bulk phase would easily neutralize all the bicarbonate secreted by the surface cells. Mucus does not prevent the access of damaging agents like bile salts, non-steroidal anti-inflammatory drugs (NSAIDs) or alcohol to the epithelium. However,a gelatinous coat made up of mucus and fibrin may act as a protective cap under which repair of the epithelium can occur after damage. A number of the plant drugs to be discussed appear to affect mucus synthesis and/or secretion. These effects are probably mediated via effects on PGs, and it is therefore important to discuss the actions of these agents in some detail.

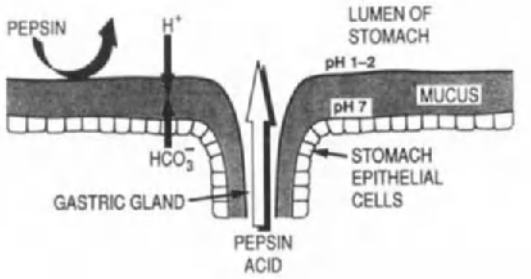


Figure 4.2. The layer of mucus which protects cells lining the surface cells of the stomach wall.

PROSTAGLANDINS AND THE MAINTENANCE OF THE GASTRIC MUCOSA

There is evidence, albeit somewhat controversial, that PGs are involved in maintenance of the integrity of the gastric epithelium. Firstly, the NSAIDs inhibit cyclooxygenase (Figure 4.3) and therefore PG production in the gastric mucosa. This action has been linked with the ulceration induced by these agents [7]. Secondly, neutralization of endogenous PGs by specific PG antibodies produced ulceration of the gastric mucosa in rabbits [8]. Antibodies against

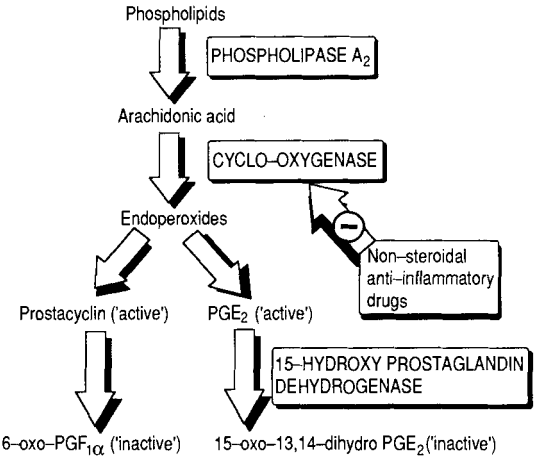


Figure 4.3. The formation and breakdown of the probable major prostaglandins of gastric mucosa.

inactive PG metabolites such as 13,14-dihydro-15-oxo-PGE_2 were ineffective, thus implying that it was not the formation of immune complexes *per se* which was inducing ulceration.

PGs of the E series and prostacyclin (PGI_2) are inhibitors of acid secretion [9]. At lower, non-secretory doses, these compounds can prevent the gross haemorrhagic lesions induced by the intragastric administration of agents like 100% ethanol or aspirin to rats. These findings suggested that PGs might in some way enhance defensive mechanisms in the gastric mucosa. Consequently considerable effort was put into the production of stable orally-active PG analogues for the treatment of peptic ulcer. The results have been disappointing [10]. Generally, the agents were no more effective than histamine H_2 receptor antagonists, caused more side-effects than the histamine antagonists, and indeed only seemed to act at doses at which they inhibited acid secretion. There are two possible explanations of these findings. Firstly, administration of exogenous PGs may not mimic the effects of endogenous PGs. In this context, it may be pertinent that many of the plant products to be discussed may affect endogenous PG production. Alternatively, the acute damage models referred to above and in more detail later may be of little relevance to the pathology of peptic ulcer and its healing [11].

Many suggestions have been made of the mechanisms by which PGs may maintain the integrity of the gastric mucosa under normal circumstances and in the face of acute injury [9]. The following considerations may be of particular importance. PGs stimulate the secretion of mucus and of bicarbonate. They

therefore strengthen the mucus-bicarbonate barrier. As mentioned above, alcohol and aspirin penetrate this barrier, and it is thought that prevention of microvascular stasis and maintenance of mucosal blood flow may at least be part of the mechanism by which PGs reduce the damaging effects of these agents [12].

PLANT DRUGS

An examination of the phytochemical literature for anti-ulcer molecules reveals that anti-ulcer activity is not confined to one class of compound. Such activity is usually discovered by screening various plant molecules against one or more animal models. Biochemical information on the role of plant drugs in preventing ulceration is of increasing importance. For instance, it is valuable to understand the interaction of drugs with PG metabolism. Since our knowledge of the pathology of ulceration is still incomplete, animal models are still of paramount importance in assessing anti-ulcer activity. Both established plant drugs and their derivatives and experimental drugs are discussed below.

TRITERPENOID COMPOUNDS

CARBENOXOLONE SODIUM

The biochemistry and pharmacology of this drug (1) has been extensively investigated for its mode of action. Like many plant drugs, its efficacy is based on its ability to strengthen defensive factors. It is an excellent stimulant of mucus synthesis [13]. This is due at least in part to increased *N*-acetyl-neuraminic synthesis [14]. Carbenoxolone sodium maintains the PG content of gastric mucosa at high levels. This is due to its inhibitory action on the catabolic enzymes 15-hydroxy-PG-dehydrogenase and Δ^{13}-PG-reductase. The drug also raises cyclic AMP levels by inhibiting mucosal phosphodiesterases [15]. It is probable that these high PG levels promote the action of factors which defend the mucosa against ulceration. The mechanisms have been mentioned earlier. Carbenoxolone has also been reported to inhibit pepsin secretion [6]. The pharmacology of carbenoxolone has been reviewed [16].

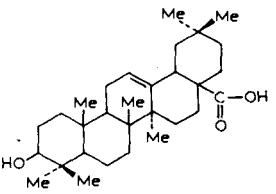
Carbenoxolone sodium is not free from side-effects which mimic the action of excess aldosterone and show as weight gain, oedema, hypertension and hypokalaemia (potassium loss). These side-effects have been found with liquorice extracts and the natural triterpenoids, namely, glycyrrhizic and

glycyrrhetinic acids. The similarity of the triterpenoid structure to that of steroids may be responsible for these steroid-like side-effects.

Other anti-ulcer substances are present in liquorice and a deglycyrrhetted preparation (deglycyrrhizinised liquorice, Caved-S, Rabro) is marketed free from steroid-like side-effects. The residual active anti-ulcer compounds in this preparation are flavonoids or their analogues. These are discussed below. The efficacy of deglycyrrhizenised liquorice is less than that of carbenoxolone.

OTHER TRITERPENOIDS

Oleanolic acid (2) which is present in such species as *Prosopis glanulosa* (flowers), *Calendula* species, (marigolds), *Helianthus* species, and *Solidago* species, has been patented as an anti-ulcer compound after its activity against ulcers induced by aspirin, indomethacin, reserpine and tetragastrin was demonstrated [17]. Since aspirin and indomethacin lower PG levels by inhibiting their synthesis without affecting their breakdown, oleanolic acid may, at least in part, promote its action by restoring PG levels. Lupeol acetate (3), taraxerol (4) and ursolic acid (5) were reported by Gupta *et al.* [18] to inhibit stress-induced ulcers in rats; ursolic acid and lupeol acetate reduced ulceration induced by pyloric ligation. These three triterpenoids are present in *Spilanthes ocymifolia* (lupeol acetate), *Taraxacum officinale* (taraxerol) and the leaves of *Psychotria adenophylla* (ursolic acid). An examination of a crude extract from the medicinal herb *Glechoma hederacea* showed that the active anti-ulcer



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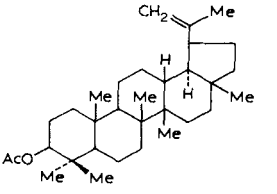

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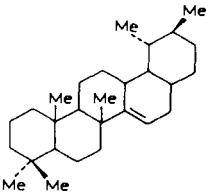

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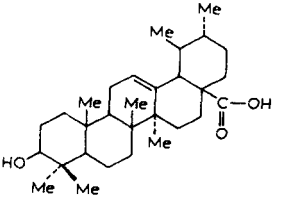
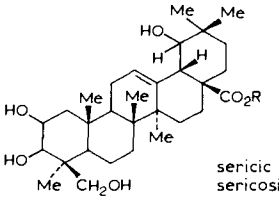


(5)

compound in this plant was 2-α-hydroxyursolic acid, which inhibited stress-induced ulcers in mice [19]. All the above triterpenoids also have anti-inflammatory activity.

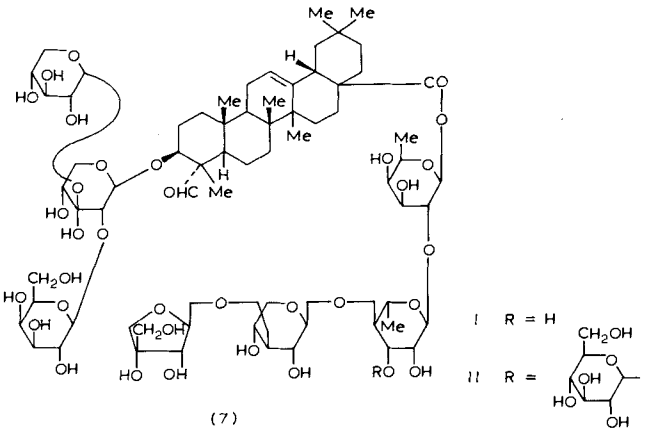
Sericic acid and its glycoside sericoside (6) are anti-inflammatory and anti-ulcer substances extracted from the roots of *Terminalia sericea* [20].



(6)

TRITERPENOID SAPONINS

Anti-ulcer activity has been associated with triterpenoid saponins from various sources. The saponin fraction was isolated as an ethanol extract from the leaves of *Pyrenacantha staudtis*, a Nigerian medicinal plant. The extract gave protection against gastric lesions induced in rodents by serotonin, indomethacin, cold restraint stress and pyloric ligation [21]. Two quillaja saponins [22], I and II (7), and a saponin Gypenoside (8) from *Gynostemma pentaphyllum* [23] have been patented in Japan for anti-ulcer activity. Gypenoside was extracted from the aerial parts of *G. pentaphyllum* by warm water and the solution was concen-



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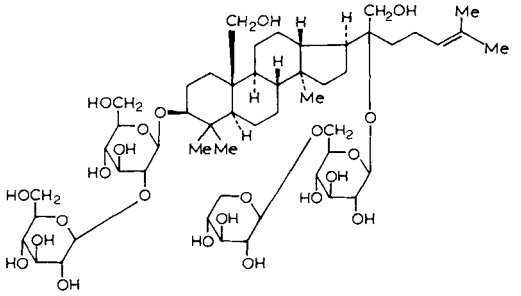


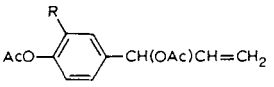
(8)

trated *in vacuo*. The material was obtained in a crude form by spray drying and redissolved in water. The aqueous solution was fractioned by column chromatography using a styrene-polymer packing. The saponin fraction was eluted with methanol and rechromatographed on an alumina column to clean up the saponin fraction. The saponins were finally separated on a silica-gel column.

OTHER TERPENOIDS

ACETATES

Phenols often accompany terpenes in essential oils (for example, clove oil prepared from the fruit of *Eugenia aromatica* contains both eugenol and its acetate). Essential oils are prepared from leaves, flowers and fruit (seeds) and many have carminative properties. It is of interest that two phenolic acetates, 1′-acetoxychavicol acetate and 1′-acetoxyeugenol acetate (9), have been identified as the anti-ulcer compounds in the fruits of *Alpina galanga* and *A. conchigera* [24] which are used in Chinese traditional medicine for the treatment of ulcers. The mode of action of these compounds is unknown.

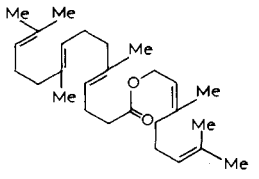


1′- acetoxychavicol acetate : R = H
1′- acetoxyeugenol acetate : R = OMe

(9)

GEFARNATE

This compound (geranyl farnesyl acetate, Gefanil (10)) consists of three isoprene units (farnesol) linked to two isoprene units (geraniol) by an acetate grouping. The drug is a mixture of two stereoisomers of 3,7-dimethyl-2,6-octadienyl 5,9,13-trimethyl-4,8,12-tetradecatrienoate. Gefarnate appears to be a dietary factor in some species. Various foods such as greens, whole grains and certain fats were withheld from the diet of chicks in some experiments carried out in the 1940's. It was found that the chicks developed ulcers and the missing dietary constituent was called an 'anti-gizzard erosion factor' [25]. Later, the factor was described as vitamin U when its presence in raw vegetables such as cabbage inhibited the development of histamine-induced ulcers in guinea-pigs [26]. The factor was finally identified as gefarnate, as a constituent of various fresh vegetables, including white-headed cabbage [27]. The drug was widely used in Italy but was not as active as carbenoxolone sodium. In a trial, 80.6% of patients treated with carbenoxolone showed improvement compared with 51.7% with gefarnate [28]. Gefarnate has low toxicity as shown by an LD_{50} value in mice of 2.8 g/kg [28].

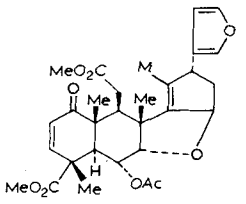


(10)

The mode of action of the drug has not been as extensively investigated as that of carbenoxolone but it promotes the synthesis of mucus and acid mucopolysaccharides in gastric mucosa [29]. The drug, like carbenoxolone, promotes the action of factors which defend the mucosa against ulceration. However, it has regenerative properties in healing ulcers in animals [30].

NIMBIDIN

Nimbidin is a crude product isolated from the oil of seeds of *Azadirachte indica* (Neem tree). It is a mixture of tetranortriterpenoids, some of which have been individually characterized. These are nimbin (11), nimbolide (12), nimbolidin A and B (13) and 17-epiazadiradione (14) (reviewed in [31]). It has anti-inflammatory [32] and anti-ulcer activity at 20–40 mg/kg [33]. Its anti-ulcer effect



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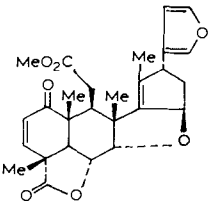
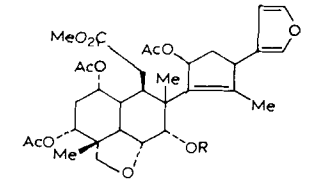
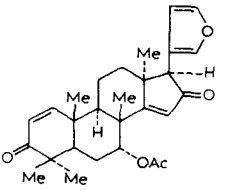


(12)



nimbolidin A: R = PhCO
nimbolidin B: R = MeCH=CMeCO

(13)



(14)

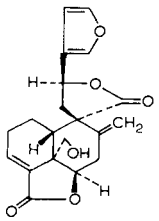
was demonstrated as a protective effect against gastric lesions induced by aspirin, stress and serotonin and cysteamine- and histamine-induced duodenal lesions in rodents. It also had a healing effect on acetic-acid-induced ulcers in rats and dogs [33].

PLAUNOL B

Two diterpene lactones plaunol A and B were isolated from *Croton sublyratus* (see plaunotol). Plaunol B (15) but not plaunol A was found to inhibit ulcer formation in the Shay rat model by 85% at 10 mg/kg [34].

PLAUNOTOL

Plaunotol ((2*E*,6*Z*,10*E*)-7-hydroxymethyl-3,11,15-trimethyl-2,6,10,14-hexadecatetraen-1-ol) (16) is an acyclic diterpene alcohol present in the Thai medicinal plant 'Plan-noi' (*Croton sublyratus*). The plant is the source of extracts used for the treatment of gastric disorders, and plaunotol is the active anti-ulcer compound present. This compound has not yet been fully evaluated, but is of interest since it, like gefarnate, contains isoprene units, and its activity may be related to increased PG levels. The compound raises PGE_2 and PGI_2 levels in rat stomach mucosa, but it does not affect cyclo-oxygenase activity [35]. It activates cellular phospholipases [35] which would raise the arachidonic acid



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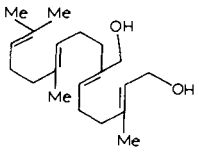


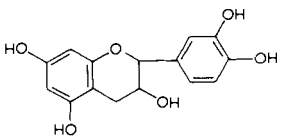
(16)

pool (*Figure 4.3*). Metabolites of plaunotol inhibit gastric mucosa 15-hydroxy-PG-dehydrogenase *in vitro*, suggesting another mechanism by which PGE_2 and PGI_2 levels might be sustained [36].

FLAVONOIDS

CATECHINS, METHYL CATECHIN AND CONDENSED TANNINS

Catechin (17) which is a commercially available flavonoid naturally present in trees such as beech and oak in the U.K. was found to be the active ulcer principle in the leaves of *Artocarpus integra*, which are the basis of a herbal drug in Indonesia. The flavonoid inhibits stress-induced ulcers in water-immersed mice [37]. In a separate study, condensed tannins which are polymers of catechin were examined for anti-ulcer activity [38]. Both (+)-catechin and 3-*O*-methylcatechin were found to protect against ulcers in Shay pyloric-ligated rats, restrained rats, and drug-induced ulcers using aspirin, phenylbutazone and reserpine. The mechanism suggested was histidine decarboxylase inhibition based on the close structural resemblance of the catechins to cyanidanol-3 [39]. The source of the condensed tannins was *Lindera umbellata* stems, which have a medicinal reputation. Trimer A (18) and its isomer Trimer B were found to suppress peptic activity *in vitro* probably by binding to the protein. Antipeptic activity was also found *in vivo* in pyloric-ligated mice and the two trimers and proanthocyanidin B-2 protected mice against stress-induced gastric lesions [38].



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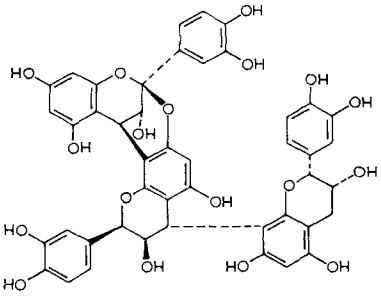


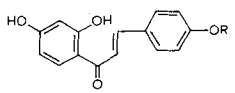
(18)

CYANIDANOL-3

(+)-Cyanidanol-3 (2*S*,3*R*) is a stereoisomer of (+)-catechin (2*R*,3*S*) and its anti-ulcer activity is due to its inhibiting action on histidine decarboxylase [40]. This inhibits the formation of histamine which stimulates the production of gastric hydrochloric acid. This mechanism is the closest found in nature to that of cimetidine and ranitidine, which are synthetic blocking histamine (H_2) receptors.

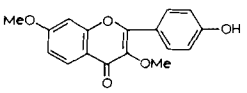
DEGLYCYRRHIZINIZED LIQUORICE

Removal of glycyrrhizen from liquorice decreases but does not abolish anti-ulcer activity. Consequently, a deglycyrrhizinized liquorice (Caved-S, Rabro) was marketed in an attempt to avoid the steroid-like side-effects of carbenoxolone. The active compounds in the deglycyrrhetted preparation have been identified and are mainly flavonoids or flavonoid analogues. Flavonoids include isoliquiritigenin (2′,4′,4′-trihydroxychalcone) and its glycoside isoliquiritin (chalcones which are flavonoid intermediates) (19), kumatakenin (also found in *Alpina kumatake*) (20), licoflavonol (21), the isoflavone licoricone (22), licuzid (chalcone) (23) and the coumestan glycyrol (24) [41–43].



isoliquiritigenin: R = H
isoliquiritin : R = $C_6H_{11}O$

(19)



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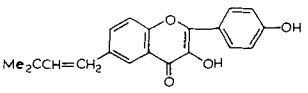

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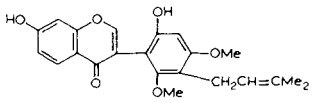

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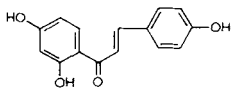

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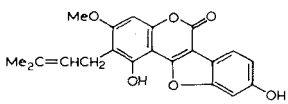

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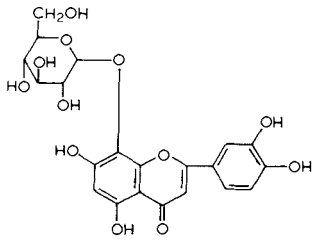
HYPOLAETIN-8-GLUCOSIDE

This flavonoid (25) was prepared from the aerial parts of *Sideritis mugronensis*, a medicinal herb found in Spain and other parts of southern Europe. The herb is reputed to have an anti-inflammatory and anti-ulcer actions and hypolaetin-8-glucoside was found to possess anti-ulcer properties [44] against cold-restraint induced ulcers in the rat. ED_{50} values obtained were cimetidine, 23.9 ± 11.8 mg/kg; hypolaetin-8-glucoside, 57.3 ± 11.7 mg/kg. Therefore, it has about half the activity of cimetidine but is metabolized completely to phenolic acids and conjugates which are readily excreted. It is therefore probably non-toxic. When tested on rats at 600 mg/kg, it produced no toxic effects. Insufficient of the drug was available for a toxic ceiling to be determined. Extracts of *S. mugronensis* have been shown to have low toxicity [45].

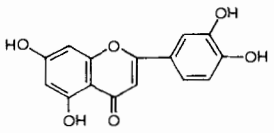


(25)

The flavonoid raises PG levels and therefore enhances defensive factors in the stomach. It stimulates PG synthesis but is inactive against 15-hydroxy-PG-dehydrogenase. Many flavonoids and particularly glycosides stimulate PG synthesis and the structural features of this activity have been reviewed [46].

LUTEOLIN AND APIGENIN

Acacetin, apigenin and luteolin (26) were examined for anti-ulcer activity against histamine-induced ulcers in the guinea-pig and after pyloric ligation in the rat. Luteolin was active in inhibiting gastric erosions but apigenin was less effective. Acacetin was not active in the two models examined. The doses used

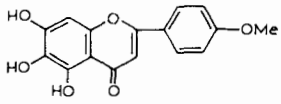


(26)

were 10 mg/kg day for 5 days in the histamine model and the same dose for 12 days prior to pyloric ligation in the rat. The presence of a free hydroxy group in the uncondensed benzene ring was regarded as necessary for anti-ulcer activity [47].

4'-*O*-METHYLSCUTELLAREIN

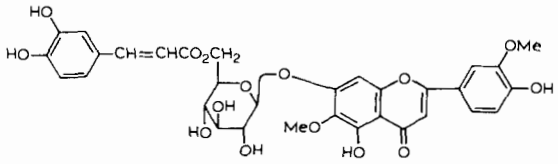
This flavonoid (27) present in *Scutellaria* species and *Clerdendron inerma* inhibits reserpine-induced gastric lesions in mice [48].



(27)

MYRICETIN, GNAPHALOSIDE, RUTIN, HYPEROSIDE AND QUERCETIN

In an experimental screen of thirteen naturally occurring flavonoids, myricetin (28) and gnaphaloside (29) were found to have significant anti-ulcer activity against cold-stress-induced ulcers in mice predosed with reserpine and against



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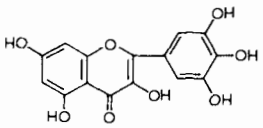

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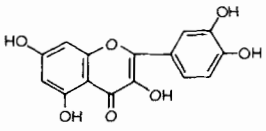
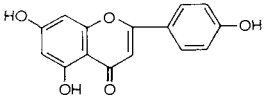


(30)

stress-induced ulcers in immobilized mice. It was suggested that an association of these anti-ulcer flavonoids with NSAIDs might decrease the risk of gastrointestinal damage [49]. A structural requirement for anti-ulcer activity in flavonoids was reported to be the presence of two *ortho*-hydroxy groups as found in myricetin and quercetin. Quercetin (30) and its glycoside hyperoside (quercetin-β-D-galactoside) have been reported to possess anti-ulcer activity [50].

NARINGENIN AND GOSSYPIN

Naringenin (31) and gossypin (32) both exhibit anti-inflammatory actions but also inhibit ulcer formation in animal models [51]. The mode of action of naringenin is by inhibiting histidine decarboxylase [52] (cf. cyanidanol-3).



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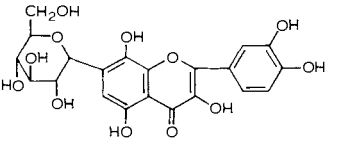
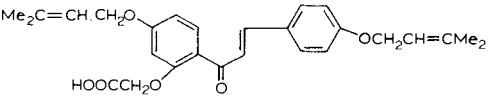


(32)

SOLON

Solon (sofalcone, SU-88,2′-carboxymethoxy-4,4′-bis(3-methyl-2-butenyloxy)chalcone, (33) was one of a number of isoprenyl and isoprenoxyl derivatives synthesized as analogues of sophoradin (34), a isoprenyl substituted chalcone present in the medicinal plant *Sophora subprostrata* [53]. The plant is the source of the Chinese drug 'Guang-duo-gen' which is used for the treatment of digestive



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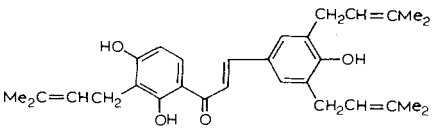


(34)

disorders. Sophoradin was found to be an active anti-ulcer compound present in the drug when tested against pyloric-ligated rats and water-immersed stressed rats. Solon is active against gastric ulcers produced by aspirin, water immersion, restraint stress or ethanol [54]. A description of these animal models is given later.

Solon has been used in the clinic [55,56], where it was found that the drug accelerated the healing of ulcers, particularly when a deficiency of PG in the gastric mucosa was noted. It also has a stimulating action on gastric mucin acylation [57]. In rats, it was found that when solon was administered before ulcer-producing agents, such as ethanol or aspirin, it promoted an increase in gastric mucosa PG content which was related to a decrease in damage caused by the ulcerogens [54]. The increased PG levels were related to increased mucus and alkaline secretion in mucosa and also to an elevated blood flow in the tissue [54]. It seems likely that the increased PG levels associated with solon are due to its inhibiting effect on the PG-metabolizing enzyme, 15-hydroxy-PG-dehydrogenase [54].

ALKALOIDS

Although many alkaloids are poisons, used responsibly they are extremely useful drugs. *Corydalia* tubers are used as sources of medicines in China and Korea and dehydrocorydaline, (+)-corydaline and di(tetrahydropalmatine), which have been identified as present in the tubers, have been found to have good pain-killing and anti-ulcer activity [58]. Alkaloids such as atropine, *Belladona* alkaloids and hyoscine are anti-cholinergic drugs which inhibit parasympathetic innervation consequently reducing secretion and motility of the stomach and duodenum. They are sometimes used as a supplement to antacid therapy in ulcer treatment. Although there is little evidence to suggest they heal ulcers, their use is justified in that, by delaying gastric emptying, they may prolong the action of antacids in the stomach. At high doses, anti-cholinergic drugs inhibit basal and meal-stimulated gastric acid production. The use of the drugs is limited by the high incidence of side-effects associated with therapeutic doses.

ATROPINE

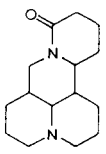
This alkaloid from deadly nightshade (*Atropia belladona*) has an anti-ulcer action in animals. In vagally-innervated dogs, it acts by inhibiting gastric secretion after gastric stimulation by the well-known stimulants 2-deoxy-D-glucose, gastrin and histamine [59].

BERBERINE ALKALOIDS

These alkaloids are found in *Berberis* species and their pharmacological properties including anti-ulcer inhibition have been reviewed [60].

MATRINE AND OXYMATRINE

The roots of *Sophora subprostrata* are used in the treatment of fever, inflammation and peptic ulcer in Chinese traditional medicine. Active anti-inflammatory and anti-ulcer compounds were found to be matrine (35) and oxymatrine (36). These alkaloids inhibited gastric secretion in rats in a dose–response manner at 10–50 mg/kg and inhibited ulcer formation at 250 mg/kg [61]. This plant also produces the anti-ulcer chalcone sophoradin, whose mode of action involves enhancing PG levels, as mentioned above.



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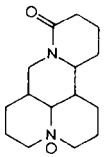

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STEROID-TYPE ALKALOIDS

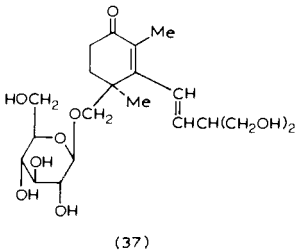
A pregnane-type steroid alkaloid fraction isolated from the medicinal plant *Pachysandra terminalis* was found to have a preventative effect on restraint and water immersion stress-induced ulcers in mice. Compounds identified were pachystermine A, pachysamine A, epipachysamine A, pachysandrine A and spiropachsine [62].

MISCELLANEOUS COMPOUNDS

GLYCOSIDES

Recently, three glycosides with potent anti-ulcer activity against rat models [63] have been isolated from the stem bark of *Cinnamomium cassia* and their structures have been determined. The stem bark is used in a prescription 'Goraison' in Chinese medicine to treat ulcers. The glycosides are

cassioside (4*S*)-2,4-dimethyl-3-(4′-hydroxymethyl-1′-butenyl)-4-(β-D-glucopyranosyl)methyl-2-cyclohexen-1-one (37); cinnamoside, (3*H*)-4-[(2′*R*,4′*S*)-2′-hydroxy-4-1′-(β-D-apiofuranosyl-(1 → 6)-β-D-glucopyranosyl)-2′,6′,8′-trimethylcyclohexylidene]-3-buten-2-one (38) and 3,4,5-trimethoxyphenol-β-D-apiofuranosyl (1 → 6)-β-D-glucopyranoside.



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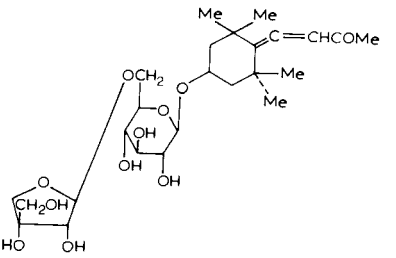
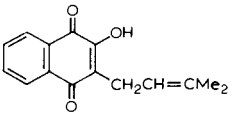


(38)

The mode of action of these compounds has yet to be investigated but cassioside has a dihydroxylated isoprenyl group which is closely related to the isoprenyl group present in many anti-ulcer compounds and will be discussed together with other common structural features later.

LAPACHOL

Lapachol (2-hydroxy-3-[3-methyl-2-butenyl]-1,4-naphthoquinone) (39) which has anti-ulcer properties [64] was isolated from the powdered roots of *Tectona grandis* (common teak). The tree is common in India, Burma, Java and the Malay peninsula. Extracts of the tree are used in various formulations in India and south-east Asia to treat stomach disorders. The mode of action of lapachol is unknown.



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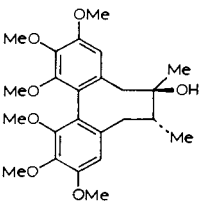


(40)

LIGNAN

The fruits of *Schizandra chinensis* are used in the Far East and particularly China in traditional medicine for the treatment of stomach disorders. A dibenzocyclooctadiene lignan, isoschizandrin (40) was found to be an active anti-ulcer constituent which inhibited stress-induced ulcers in rats [65]. The mode of action has yet to be investigated.

LINOLEIC ACID

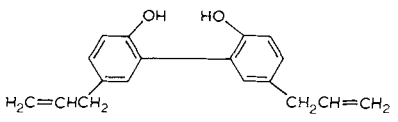
This acid (41) was administered at 40 mg/kg per day for 10 days i.m. and 280–440 mg/kg orally to rats in a series of anti-ulcer experiments. The models used were hydrocortisone-induced gastric ulcers and cold-restraint-induced ulcers. The acid had a anti-ulcer effect in both models [66]. The mode of action suggested was that linoleic acid increased PG levels in gastric mucosa. The acid, which is present in fish oils, is also a plant product since it occurs in high concentrations in seeds. Some seed extracts such as 'evening primrose oil' are a rich source of linoleic acid [67].

$$Me(CH_2)_4CH{=}CHCH_2CH{=}CH(CH_2)_7COOH$$

(41)

MAGNOLOL

Magnolia bark is used in several oriental crude drugs associated with the treatment of peptic ulcer. The active substance is magnolol (42) which has been shown to inhibit ulcer formation in water-immersed stressed rats [68].



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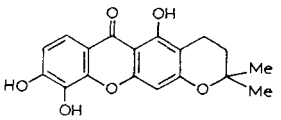

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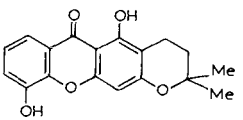


(44)

XANTHONES

Two xanthones, jacoreubin (43) and 6-desoxyjereubin (44), extracted from *Calophyllum inophyllum* and *Mesua ferrea* were found to have anti-ulcer activity against restraint-induced ulcers in rats [69]. Both substances were active at 50 mg/kg i.p. compared with gum acacia as control.

MEDICINAL PLANTS USED TO TREAT STOMACH AILMENTS

The number of medicinal plants subjected to a full phytochemical investigation is but a small fraction of such plants which have been reported in the literature. Even smaller is the number of phytochemicals that have been subjected to a full or even partial pharmacological screen. Many plants have a long history of use in traditional medicine. The early settlers in North America learnt many herbal remedies from the indiginous Indians [1]. They found that the red alder (*Alnus rubra*) was used in western North America to treat indigestion and stomach problems, whilst the hazel alder (*A. rugosa*) was used for the same purpose in eastern North America where this species was more common. The Indians of western Washington used a 'tea' (a hot water infusion) of dewberry leaves (*Rubus macropetalus*) to treat stomach ailments. Other Indian remedies recorded include the use of the roots of the black haw (*Viburum prunifolium*) and the southern black haw (*V. rufidulum*) to prepare a tea for treating stomach problems. The Catawbas Indians located in eastern North America used the green or dried root of shrub yellowwood to treat ulcerated stomachs and stomach pains. They also used the roots of *Gentiana catesbaei* and other Gentian species to treat stomach pains. The early settlers adopted many of these remedies, including golden seal (*Hydrastis canadensis*) whose roots were used for stomach ailments. In the south-west United States, greasewood (creosote bush) branches were boiled by the Indians to extract a gum which was drunk as a hot decoction for stomach troubles. Other parts of the world have a rich history of medicinal plants. This particularly applies to tropical countries with a rich flora. The rich traditional medicine of South and Southeast Asia has been reviewed [70]. Infusions of the rhizome of *Acorus calamus* have been used as an anti-dyspeptic agent and for the treatment of general stomach discomfort in Indonesia and parts of the Malay peninsula. The activity has been located in a sesquiterpene fraction. Common teak (*Tectona grandis*) was used in India, Burma, Java and the Malay peninsula in traditional medicine in the treatment of stomach-ache long before its active anti-ulcer substance lapachol was identified. Also in parts of India and West Africa the

pulp of the plantain banana (*Musa* species) is used to treat stomach ulcers as part of the traditional medicine of the regions. Extracts of the dried banana pulp were found to both inhibit and cure aspirin-induced ulcers. The extract contained substances (soluble in aqueous alcohol) which promoted mucus formation and induced mucosal cell healing [71]. Dried banana pulp powder has recently been marketed in India ('Musa-Pep') and, on a smaller scale, as a 'health product' in the U.K.. Recently, Goel and colleagues [72] have shown that ethanol extracts of the pulp of *Musa sapientum* var. *paradisiaca* gave a concentration-dependent increase in PG accumulation in human gastric and colonic mucosa *in vitro*. Another crude natural product available is an enriched anthocyanoside extract from bilberry (*Vaccinium myrtillus*). This preparation is claimed to have several medicinal uses including an anti-ulcer action. This claim is based on the ability of the anthocyanosides to inhibit lesions induced in various rat models (stress-induced, pyloric ligation, reserpine, phenylbutazone, acetic acid) [73]. A histological examination of the stomachs of rats on anthocyanoside therapy showed an increase in the amount of gastric mucus present.

Traditional Chinese medicine which is still currently practised has many crude drugs derived from indigenous plants as described above. The anti-ulcer plants of Brazil have recently been reviewed [74].

ANIMAL MODELS FOR EVALUATING ANTI-ULCER DRUGS

Although still widely used, animal models provide little information on the mode of action of anti-ulcer drugs and also suffer from the disadvantage that animal models are often not representative of the pathology of ulcers in humans. Modern evaluations of anti-ulcer drugs usually include biochemical data such as the effect of the drug on PG levels. Restraint-type models are based on stress-induced ulceration, whereas ulcers induced by aspirin and other NSAIDs drugs (for example, indomethacin, phenylbutazone) are based on cyclo-oxygenase inhibition (lowered PG levels). Salicylate toxicity may also contribute to aspirin-induced lesions [75]. Some animal models which have been previously mentioned as screens for anti-ulcer phytochemicals are described below. Experimental methods of evaluating anti-ulcer drugs, including animal models, have been reviewed [76].

COLD-RESTRAINT INDUCED ULCERS IN THE RAT

In this model, rats are deprived of food but not water for 24 h prior to the administration of test compounds. After dosing, the animals are restrained

individually in small cylindrical cages 4–5 cm in diameter for 4 h at 4°C. The animals are then killed and the stomachs removed. Each stomach is opened by cutting around the lesser curvature. After washing with saline, erosions and ulcers are examined under a 3× magnifier and scored. A suitable scoring scheme is described below.

SHAY PYLORIC-LIGATED RATS

Each rat is laparotomied under ether anaesthesia and the pylorus is ligated. The drugs under test are administered individually to each rat immediately after ligation. The animals are killed 18 h later by an overdose of ether and the stomachs are removed. Each stomach is opened by cutting round the greater curvature. Lesions in the forestomach are examined and scored as a sum of the area of each lesion.

WATER-IMMERSED AND RESTRAINT-STRESS RATS

The drug is administered to the rat and the animal is placed in a small (stress) cage and immersed in water at 23°C to the xiploid level. After 7 h, the rat is killed and its stomach removed and inflated with 1% (v/v) formalin solution and the whole stomach placed in formalin solution for 5 min. The stomach is then cut open along the greater curvature and examined for erosions and ulcers in the glandular region. The ulcer index is calculated as the sum of the lengths of the lesions in the stomach.

ASPIRIN-INDUCED EROSIONS

This model is used in our laboratory and our model will be described [71].

Aspirin can be used either as a prophylactic method or a curative method for evaluating drugs. In the prophylactic method each rat is housed in a separate cage and the drug is supplied mixed with a paste of 14 g of normal rat food. Preliminary experiments with rats of 200 g body weight established that over a period of 24 h the rat will eat the entire supply of food plus drug. The animal is then fasted for 48 h before being dosed by gavage with 150 mg of aspirin suspended in 1 ml of water. 5 h later, the rat is killed and the stomach is removed and opened by dissection along the greater curvature. After washing with cold saline, the ulcers and erosions are scored by a system based on that of Robert and Nezamis [77,78]. In the curative method, the rat is fasted for 48 h after normal food and then dosed by gavage with 150 mg/kg of aspirin suspended in 1 ml of water. 5 h later the rat is allowed access to 14 g of ground

food mixed with the drug under test. After 48 h, the animal is killed and the stomach is removed and examined for ulcers and erosions as described for the prophylactic method. Other ulcer-producing drugs, for example, NSAIDs or ulcerogens such as acetic acid or 100% (v/v) ethanol, with minor modifications to the above procedure, can be substituted for aspirin.

SCORING SYSTEM

Many scoring systems have been used. A modification of the Robert and Nezamis scoring procedure used in our laboratories is described [71].
Deep linear ulcers more than 10 mm in length, 4 points each (maximum 4)
Medium linear ulcers less than 10 mm in length, 2 points each (maximum 14)
Circular ulcers 1–2 mm diameter, 1 point each
Circular ulcers less than 1 mm diameter, 0.5 points each
Microscopically visible erosions as fraction of 24 cm^{-2}, 2 points (maximum 2)
Fraction of stomach showing haemorrhage, 2 points (maximum 2)
Fraction of stomach showing transparency to back lighting, 2 points (maximum 2)

In practice, in using the system to score the aspirin model, we found the maximum score did not exceed a value of 20 [71].

STRUCTURAL FEATURES RELEVANT TO ANTI-ULCER ACTIVITY

A cursory glance at the structures of drugs with anti-ulcer activity reveals only that activity is not confined to any one class of chemical compound. However, within individual classes of compounds there are some structural features that have been related to anti-ulcer activity. Several flavonoids have anti-ulcer activity which is present in flavonoids with *ortho*-dihydroxy-substitution [52] or the presence of a free hydroxy group in the uncondensed benzene ring [47]. The anti-ulcer activity of the flavonoids is not confined to a single target. Of the compounds mentioned above, cyanidanol-3, (+)-catechin and naringenin inhibit histidine decarboxylase which, by blocking histamine synthesis, would reduce gastric acid production. Sophoradin and its chalcone analogues, including the clinically used drug solon, inhibit 15-hydroxy-PG-dehydrogenase, which would maintain beneficially high PG levels. Hypolaetin-8-glucoside also maintains high PG levels but this flavonoid stimulates cyclo-oxygenase activity and therefore raises PG levels. The structural requirements for cyclo-oxygenase stimulation or inhibition and arachidonic acid metabolism by flavonoids have

been reviewed [46,79]. Flavonoid glycosides stimulate cyclo-oxygenase even when the parent aglycone inhibits the enzyme. Most flavonoids are present as glycosides in plants and this may explain why some plant extracts exhibit anti-ulcer activity. Although some flavonoid aglycones inhibit cyclo-oxygenase *in vitro*, there is no evidence that flavonoids are ulcerogenic *in vivo*. This is in contrast to NSAIDs, which are powerful cyclo-oxygenase inhibitors and powerful ulcerogens. It is possible that flavonoids may be multivalent in their anti-ulcer action. Quercetin, the aglycone of rutin, inhibits cyclo-oxygenase activity but has some anti-ulcer activity [52,80]. However, quercetin, like many other flavonoids, is a phosphodiesterase inhibitor [81]. The effect of phosphodiesterase inhibition *in vivo* would be to raise cyclic AMP levels. Cyclic AMP stimulates mucus secretion from mucosa epithelial cells. Catechin tannins inhibit pepsin action by their tanning action on proteins which would inactivate pepsin. The isoprenyl group has been associated with anti-ulcer activity [28]. This group is present in other anti-ulcer compounds and in particular, within the flavonoids, certain liquorice root flavonoids and chalcones related to sophoridin, including sophoridin itself. The group is discussed below. Other possibilities relating to the anti-ulcer activity of flavonoids remain to be fully explored. Undoubtedly ulcer formation is inflammatory in nature, although the detailed pathology is unclear. A large number of flavonoids have anti-inflammatory activity [82] and although this property has been related to PG inhibition it has also been related to the strong inhibition of lipoxygenase, a characteristic property of many flavonoids [46,82]. Lipoxygenase activity is responsible for the formation of the powerful inflammogens, the leukotrienes. Flavonoids are also anti-oxidants and therefore may also act as scavengers of superoxide and other active oxygen species which have been shown to be destructive agents in inflammatory processes. Clearly, a considerable amount of work remains to be carried out on the anti-ulcer activity of the flavonoids.

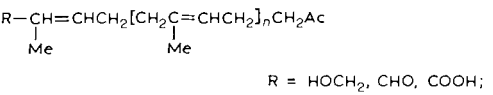
Of the triterpenoids, carbenoxolone has been the most intensively researched. Several mechanisms have been proposed to explain its anti-ulcer action. These have been previously mentioned but the inhibition of the PG-metabolizing enzymes 15-hydroxy-PG-dehydrogenase and Δ^{13}-PG reductase is of interest, since carbenoxolone shares this property of inhibiting PG metabolism with Solon and other related isoprenyl chalcones.

The alkaloids are probably prohibited by their toxicity as potential anti-ulcer drugs and their activity appears to be related to an anti-cholinergic effect as illustrated by atropine.

Of the other compounds mentioned in this review, little is known of their mode of action. However, a point of interest is the appearance of unsaturated alkyl chains in many anti-ulcer compounds. One such group is the isoprenyl

group present in gefarnate, plaunol, some liquorice flavonoids, sophoridin and derivatives (as isoprenyloxy in Solon), lapachol, cassioside, gypenoside saponin and 1′-acetoxyeugenol acetate. Although the group is chemically stable, it could possibly be metabolized in mammals, either by oxidation at a terminal methyl group or by epoxidation at the double bond.

Although not strictly relevant to an article on plant drugs, it is of interest to note that a European patent was taken out on a series of anti-ulcer polyprenyl ketones (45) [83] produced from a broth of *Nocardia* species (filamentous bacteria). These compounds contain unsaturated structural features including a substituted isoprenyl group common to many anti-ulcer compounds. The isoprenyl group, which is quite common in plants, seems worthy of further investigation in mammalian metabolism.



(45)

There is a rich abundance of plants reputed in traditional medicine to possess anti-ulcer properties, of which only a few have been considered. Most plants have yet to be evaluated or at best have only been partially evaluated. It seems likely that plants will continue to be a valuable source of new molecules which may, after possible chemical manipulation, provide new and improved anti-ulcer drugs.

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5 Heterosteroids and Drug Research

HARKISHAN SINGH, M. Pharm., Ph.D., DHARAM PAUL JINDAL, M.Pharm., Ph.D., MANGE RAM YADAV, M.Pharm., Ph.D. and MANOJ KUMAR, M.Pharm., Ph.D.

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

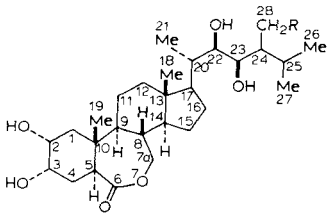
INTRODUCTION

Heterosteroids may have heteroatom(s) as part of the steroid nucleus, or the heteroatom(s) may be extranuclear, forming part of a fused or spiro ring system, an attached group, or a side-chain. The hydroxy- or oxosteroids are not considered in this review.

A comprehensive review on heterosteroids and drug research was published in 1979 [1]. During the last decade there has been sufficient progress in the field to justify updating the review. In this contribution, the arrangement and style remain broadly as before, so that it may be convenient to read this chapter as a complement to the earlier presentation.

Biologically active synthetic and natural products will be referred to later in this review. However, some interesting work on selected natural heterosteroids may be mentioned here. In 1979, plant growth promoter brassinolide (1) was isolated from bee-collected *Brassica napus* Linn. (rape) pollen [2]. This has been a discovery of major significance. The B-homo-7-oxa-5α-6-oxo lactone system, 2α,3α,22,23-tetrahydroxy functions and the 22*R*,23*R*,24*S*-configurations are important structural features for its activity as plant growth

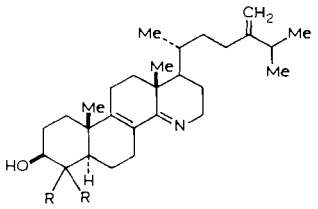
promoter. Brassinolide and related compounds, termed as brassinosteroids, are ubiquitous in the plant kingdom. The literature on brassinosteroids was surveyed in two reviews which appeared simultaneously [3,4]. Two independent syntheses of brassinolide were reported in 1980 [5–7], and later there have been other reports on its synthesis [8–12]. Several synthetic analogues of brassinolide have been prepared. The synthetic brassinosteroid 28-homobrassinolide (2) [13–16] is as highly active as brassinolide in the rice-lamina inclination test [17]. The 7-aza analogue of homobrassinolide has been synthesized [18]. Various aspects of brassinosteroids continue to be examined [19–25].



(1) R = H
(2) R = Me

In the previous review, a mention was made of A25822B (3), a representative of the antifungal antibiotic azasteroids [1]; related to it is A25822 factor A (4). A synthesis of the latter starting with ergosterol has been reported [26]. 15-Aza-D-homo-8,14,22-ergostatrien-3β-ol, a relative of (3) has been prepared [27]. Another kind of antifungal heterosteroid is represented by viridin, demethoxyviridin (5) and wortmannin, the biosynthetic aspects of which have been examined [28–30].

A sustained research of 15 years on marine worm *Cephalodiscus gilchristi* has resulted in the isolation and structure elucidation of a powerful cell growth



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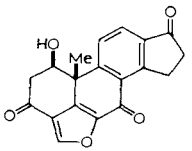

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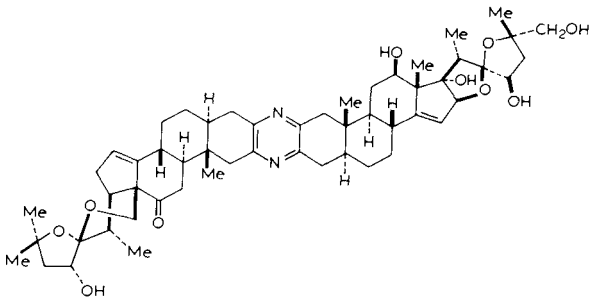


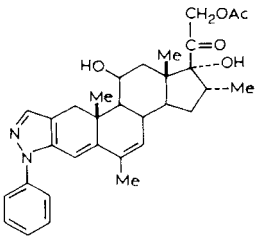
(6)

inhibitory substance designated as cephalostatin 1 (6) [31]. The structure is rather unusual.

ANTI-INFLAMMATORY HETEROSTEROIDS

Trends in the design of anti-inflammatory steroids have been recently highlighted [32]. Certain of the leads have heterosteroidal characteristics. Cortivazol (7) and nivazol (8) have the [3,2-*c*]pyrazole structural component. The 3-keto group is absent from both of them. Cortivazol is a potent non-halogenated corticosteroid. Steroidal pyrazoles such as (7) had been described as extremely potent anti-inflammatory steroids [33,34]. Later, (7) was shown to possess high affinity for hepatoma tissue culture cell receptors [35]. A recent study shows that cortivazol binds to the glucocorticoid receptor in the cytosol from CEM C7 cells (a human acute lymphoblastic leukaemia line) in a fashion consistent with interaction with at least two sites [36].

Nivazol (8) lacks not only the 3-keto group, but is also devoid of 11β-hydroxy group, and 20,21-ketol system. Still nivazol has been shown to produce glucocorticoid effects in the rat [37]. It was observed that in rhesus monkey it exerted



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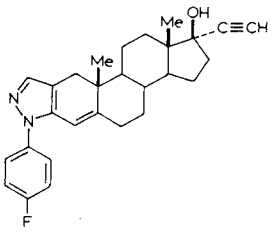
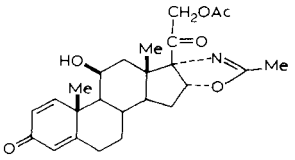


(8)

central glucocorticoid activity (inhibition of the hypothalamic-pituitary-adrenal axis) but showed no peripheral glucocorticoid action. Suppression of ACTH is the predominant action of nivazol in the primate. A similar activity profile in humans is suggested. It has been observed that nivazol and its 11β-hydroxy derivative WIN 44577 (an agent with about 10-times the glucocorticoid activity of nivazol in the rat) bind competitively to the glucocorticoid receptor from the liver and anterior pituitary from both rat and monkey; however, no marked interspecies differences in receptor binding affinities have been found [38]. In another study, cortivazol and nivazol were as active as hydrocortisone in raising blood pressure in sheep [39].

Work continues on steroidal pyrazoles as potential anti-inflammatory agents. 11β,21-Dihydroxy-20-oxo-2′-phenyl-2′*H*-2,4-pregnadieno[3,2-*c*]pyrazol-17α-yl propionate, when tested for vasoconstrictive activity in male volunteers, exhibited activity equal to betamethasone 17-valerate [40].

Deflazacort (9) has a 2′-methyloxazoline ring fused at the 16α,17α-positions of the D-ring [41,42]. It is also a non-halogenated bone-sparing glucocorticoid. It inhibited synthesis of collagen in different rat-bone cell populations and rabbit articular chondrocytes [43]. *In vitro* studies on the compound show it to be as potent an immunosuppressive agent as prednisolone [44]. Steroidal [16α-17α-*d*]isoxazolidines have also been examined [45,46]. The analogue (10) showed higher topical anti-inflammatory activity in mice than did beta-methasone 17-valerate [45].



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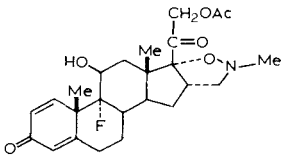
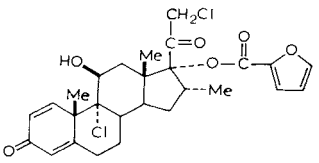


(10)

Certain of the 17-heterocyclic aromatic esters of corticosteroids have shown high topical anti-inflammatory potencies on testing in mice by a modification of the Tonelli croton oil-ear assay [47,48]. For example, 17α-hydroxy-16α-methyl-9α,11β,21-trichloro-1,4-pregnadiene-3,20-dione 17-(2′-furoate) [47] and 9α,21-dichloro-11β,17α-dihydroxy-16α-methyl-1,4-pregnadiene-3,20-dione 17-(2′-furoate) (mometasone fuorate, Elocon) (11) [48] were 8- and 6-times as potent as betamethasone 17-valerate, respectively. Mometasone furoate as 0.1% ointment and cream has been clinically evaluated and found to be a very efficacious, long-acting anti-inflammatory agent. The 17-(methylthio)acetate of 6α-methylprednisolone had increased activity [49].



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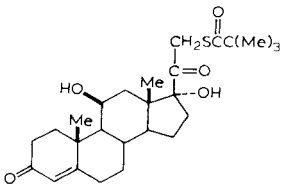
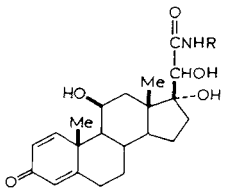


(12)

Tixocortol pivalate (12) is a pivaloyl thiol ester derivative of hydrocortisone [50]. It possesses local anti-inflammatory activity. In humans, it has not shown systemic glucocorticoid effects when used in very high doses [51], nor does it exhibit immunosuppressive effects on lymphocytes [52]. A comparison of its pharmacokinetics with hydrocortisone in the rat shows that tixocortol pivalate has a faster rate of metabolism combined with a larger volume of distribution and a low oral bioavailability [53].

Other 21-substituted corticosteroids with sulphur-containing moieties have been studied. In rats 21-carboxyethylthio-17α-hydroxy-4-pregnene-3,20-dione was found to be as active as prednisolone [54]. 21-Ethyl or methylthio-6α,9α-fluoro-11β-hydroxy-16α,17α-isopropylidenedioxy-1,4-pregnadiene-3,20-diones were found to be more potent than betamethasone 17-valerate as topical anti-inflammatory agents on testing for vasoconstrictive activity in humans [55].

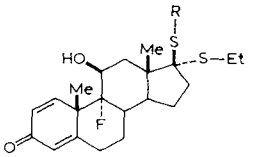
The 'antedrug' concept of Lee and Soliman [56], that is, that a compound may be locally active but hydrolysed to an inactive compound upon entry into systemic circulation, has been followed in the design and study of 11β,17,20-trihydroxy-3,21-dioxo-1,4-pregnadiene-21-*N*-substituted carboxamides (13) [57–59]. On evaluation with the croton oil-induced ear oedema bioassay in the rat, for each pair of the *N*-substituted carboxamides, the *R* isomer was more active than the corresponding *S* isomer [58]. The amides with high local anti-inflammatory activity exhibited systemic activities, unlike the corresponding esters of steroidal 21-oic acids, which are devoid of systemic activities.



(13)

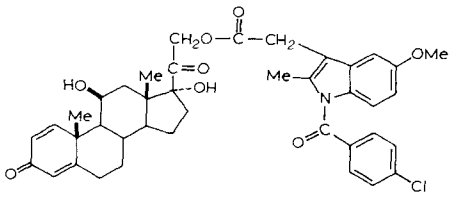
Another type of modification of 20,21-ketol system is exemplified by 11β,17α-dihydroxy-21-morpholino-1,4-pregnadiene-3,20-dione-21-thione, which showed potent activity in the carrageenin oedema test and in the granuloma pouch test in rats [60].

There are certain other heterosteroids known which do not have the ketol function but have anti-inflammatory activity. Among these are some androstene-17-thioketals [61,62]. Tipredane (SQ-27,239) (14) and related SQ-28,300 (15) show moderate activity in topical anti-inflammatory assays using rats. They exhibit favourable separation of local activity from systemic effects on the thymus and hypothalamic-pituitary-adrenal axis. A mention may also be made of anti-inflammatory activity observed in preliminary tests on 17β-amino-3β-methoxy-5-androstene hydrochloride and related derivatives [63] and 17β-(2-aminooxazol-4-yl)steroids and their 2′-*N*-acetyl derivatives [64].



(14) R = Me
(15) R = CH_2CH_2F

Indopred (16) is a conjugate of prednisolone with non-steroidal anti-inflammatory drug, indomethacin. It shows greater local and topical anti-inflammatory effects than prednisolone in rats, with reduced levels of systemic side-effects [32,65].

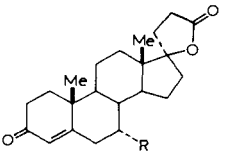


(16)

There is a mention of anti-inflammatory activity of some 8-aza-16-oxagonane-12,17-dione derivatives [66,67].

HETEROSTEROIDAL ANTI-MINERALOCORTICOIDS

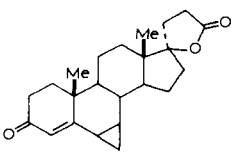
New spironolactone (17)-related aldosterone antagonists have been prepared. The clinical usefulness of spironolactone is limited because it also has anti-androgenic action and that contributes to its side-effects. SC-25152 (18) has considerably reduced affinity for the androgen receptor of human and rat prostate but exhibits similar affinity for the mineralocorticoid receptors of human and rat kidney [68]. Bioassays in the rat [69] showed that SC-25152 has reduction of anti-androgenic activity to one-tenth of spironolactone at doses giving equal antimineralocorticoid activity. An efficient stereoselective methodology leading to a formal total synthesis of 19-norspironolactone has been worked out [70].



(17) R = $-SC(=O)Me$

(18) R = $-C(=O)-OMe$

A note may be made of the work on aldosterone antagonists carried out at Schering, Berlin. A stereo-selective synthesis of aldosterone antagonist prorenone (19) has been described [71]. Spirorenone (20) has 6β,7β;15β,16β-dimethylene groups [72]. It was found to be more than 5-times as potent as spironolactone in enhancing sodium excretion in the rat [73]; the activity has been confirmed in man [74]. Pharmacokinetic studies have been carried out in man [75,76]. The pharmacokinetic profile resembles spironolactone in many respects [76]. A metabolite of spirorenone has been identified as its 1,2-dihydro derivative, which is active. A study in rats showed that spirorenone was 8.6-times as potent as spironolactone, but it possessed *in vitro* a lower affinity



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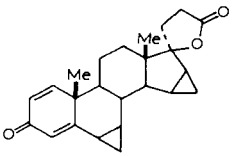
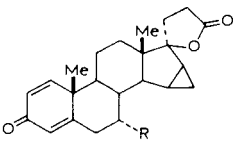


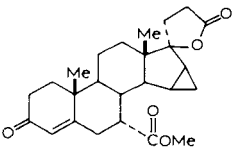
(20)

for the mineralocorticoid receptors [77]. There are associated progestational side-effects with spirorenone; it has greater activity than spironolactone in the Clauberg test [78], and the 1,2-dihydro derivative, to which it is converted in man [76] as well as in monkey [79], has more progestational activity [80]. The 1α,2α-methylenederivative 3-oxo-1α,2α: 6β,7β: 15β,16β-trimethylene-17α-pregn-4-ene-21,17-carbolactone has a similar aldosterone antagonistic activity compared with spirorenone but does not show decreased endocrinological side-effects [81].

Following the above leads, other 15,16-methylene-17-spirolactones [82,83] have been studied. Mespirenone (21) in animal experiments has shown higher antialdosterone activity and a lower antiandrogenic activity. Increase in antimineralocorticoid and absence of progestational activity at doses antagonizing the renal actions of aldosterone have also been demonstrated in human volunteers. ZK-97,894 (22) is a biologically active metabolite of mespirenone. The other derivative of interest is ZK-91,587 (23) [82]. The latter binds almost exclusively to the Type I receptor in the rat hippocampus [84].



(21) R = —SCMe
(22) R = —SMe

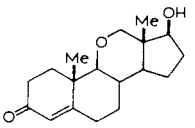


(23)

ANABOLIC HETERO DERIVATIVES

Some nucleo-heterosteroids, androstanes with fused heterocycles and some other modifications have been studied.

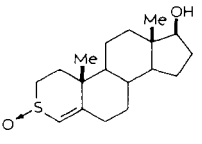
Continuing the work on design of steroidal hormones having the 11-carbon atom replaced by an oxygen atom, Engel and associates [85] have synthesized 11-oxatestosterone (24). The modification leads to diminished androgenic and



(24)

anabolic activities. 11-Aza-19-nortestosterone lacked anabolic or androgenic activity [86]. 2-Formyl-17α-methyl-3-oxa-1,4-androstadiene-11α,17β-diol was reported to have good anabolic activity [87].

The 3-sulphinyltestosterone analogue (25) of testosterone has been prepared [88]. The product obtained was a 2 : 1 mixture of the sulphoxides. No biological data are given for the products. 2-Thia-A-nor-5α-androstan-17β-yl acetate (26) has been known to have potency of the order of testosterone; however, introduction of 7β-methyl group abolishes the activity [89]. *N*-Cyano-2-aza-A-nor-5α-androstan-17β-yl acetate (27) retains androgenic-myotrophic activity [90]. On *in vitro* and *in vivo* study in the rat prostate gland, 2-selena-A-nor-5α-androstan-17β-ol (28), which has significant androgenic activity, was found to selectively complex with the specific receptors of 5α-dihydrotestosterone in the cytosol and was retained in the nuclei in an unaltered form [91].



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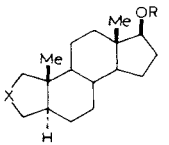


(26) X = S, R = Ac
(27) X = NCN, R = Ac
(28) X = Se, R = H

Ring A-fused heterocycle stanozolol, 17α-methyl-2′*H*-5α-androst-2-eno-[3,2-*c*]pyrazol-17β-ol is a well known anabolic agent. Anabolic steroids exert major changes in plasma lipoproteins. Stanozolol decreases high density lipoproteins and apolipoproteins A-I and A-II, and increases low-density lipoproteins [92,93]. With use of the drug there has been observed reduction of lecithin-cholesterol acyltransferase, apolipoprotein D and the Lp(a) lipoprotein [94]. A study has shown that stanozolol elevates δ-aminolaevulinic acid synthase activity and thereby porphyrin production without affecting hepatic monooxygenase activity [95].

Stanozolol improved hand blood-flow in patients with idiopathic Raynaud's syndrome or scleroderma which did not always correlate with fibrinogen levels or fibrinolytic activity [96]. Increased fibrinolytic activity has been found in rats [97] and humans [98]. The main action of stanozolol on fibrinolytic mechanism is through its effects on extrinsic plasminogen activator activity and the plasminogen HRG system [99].

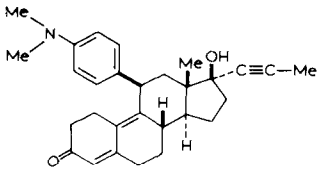
Chinese workers have indicated that 17β-hydroxy-7α,17α-dimethyl-4-androsteno[2,3-*c*]-1′,2′,5′-oxadiazole, chemically related to furazabol, has greater anabolic and androgenic activities than stanozolol [100]. Certain

estreno [2,3-*d*]dihydroisoxazolediols are claimed to have pituitary gonadotropin inhibitory and anabolic activities [101].

A study of androgenic and myotrophic activities in castrated male rats has shown that the silyl ethers, dimethylditestosteroxysilane and diphenylditestosteroxysilane, act as latentiated derivatives of testosterone [102]. 3-Oxo-4-androsten-17β-yl azidocarboxylate was claimed to have favourable anabolic-to-androgenic ratios [103].

MIFEPRISTONE

Mifepristone (RU-38,486; RU-486; Mifegyne) (29), a product of Roussel Uclaf, is currently a subject of major studies. It has been described as another milestone in the history of steroids. It was first synthesized in 1980 [104,105]; there are later reports on its synthesis [106,107]. It is a 19-nor steroid substituted in 11β-position, and possesses antiprogestin [108–110] and antiglucocorticoid activities [111,112].



(29)

ANTIPROGESTATIONAL ACTIVITY AND FERTILITY CONTROL IN WOMEN

Mifepristone showed strong binding activity on rabbit uterine progestin receptors and rat thymus glucocorticoid receptors, weak affinity for the rat prostate androgen receptors, and negligible affinity for the mouse uterine oestrogen and rat kidney mineralocorticoid receptors [113]. Several receptor binding studies have been carried out on the compound. A mention may be made of some recent work on binding to the rat ovary progesterone and glucocorticoid receptors [114], calf uterus progesterone receptors [115,116], and rabbit uterus cytosol progesterone receptors [117]. Mifepristone may have direct effect on human ovarian steroidogenesis. It has been shown *in vitro* that the compound decreases human granulosa cell progesterone production and inhibits human ovarian 3β-hydroxysteroid dehydrogenase-isomerase [118], and directly inhibits human ovarian 17-hydroxylase activity but does not affect aromatase

activity or oestradiol production [119]. *In vitro* inhibition of pituitary gonadotropin has been demonstrated [120]. There are on-going studies in animals [110] and a selective reference may be made to study of its effect on ovarian activity in rat [121], luteolysis of the *corpora lutea* of rat pregnancy [122], inhibition of egg development and implantation in rats [123], inhibition of production of progesterone-induced PGE_2 binding sites in the endometrium of ovariectomized rats [124], inhibition of cell proliferation in the mouse uterus [125], mouse preimplantation development [126], interference with embryonic transport and development in the genital tract in mice [127], inhibition of nidation in guinea-pigs [128], relatively more of antiprogestin than anti-glucocorticoid activity in immature female dog [129], induction of menstruation in primates by a local action on endometrium [130], blockade of the midcycle gonadotropin surge and ovulation in monkeys [131,132], blockade of ovulation and non-competitive anti-oestrogenic activity at the endometrial level in monkeys [133], cervical dilation, labour induction, and early delivery in near-term monkeys in combination with oxytocin [134] and termination of early pregnancy in long-tailed macaque [135].

A preliminary clinical study showed that mifepristone, given in early pregnancy, resulted in abortion in 9 of 11 women [136]. It also induced menses at the midsecretory phase of the cycle. Mifepristone is orally active and is now considered to be a drug of interest for fertility regulation in women [110,137,138]. The anti-glucocorticoid activity of the drug has no clinical relevance at the doses used for fertility control purposes. Its action is particularly significant in the endometrium, where it prevents the initiation and progression of pregnancy in the first weeks (contragestive effect). It can be used for voluntary interruption of pregnancy between 6 and 10 weeks, induction of menstruation during fifth week of amenorrhoea, and post-coital contraception. It is being studied for once-a-month menses inducer. The drug can be used for therapeutic interruption at a late stage of pregnancy. The clinical evaluation of the drug continues [139–151]. The decrease in oestradiol level in normal women during treatment with antiprogesterone mifepristone indicates the possibility of its direct action on the ovary [140]. The drug has no major effect on menstrual cycle events when given at the time of natural progesterone withdrawal which occurs before menses in nonpregnant women [144]. A comparative study of mifepristone and epostane (see later), a progesterone synthesis inhibitor, confirms that both compounds are potent abortifacients in early human pregnancy [152]. Studies have been made for use of mifepristone in combination with prostaglandin derivatives. Single oral dose of mifepristone in combination with a small dose of gemeprost (a PGE_1 analogue) through vaginal pessary effectively induces early abortion [153–156]. In a trial carried out in

China, a combination with (±)-15-methylprostaglandin-$F_{2\alpha}$-methyl ester has been used [157].

ANTIGLUCOCORTICOID ACTIVITY

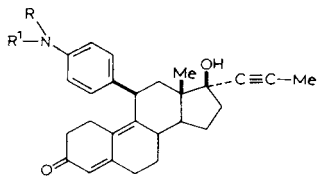
The other feature of mifepristone is its antiglucocorticoid activity. The antagonistic action against glucocorticoids has been demonstrated *in vitro* using different test systems, for example, mouse fibroblasts [158], rat kidney fibroblasts and Burkitt lymphoma cells [159], human skin fibroblasts [160], human leukaemic cell lines [161], rat hepatoma cell lines [162–164], mouse mammary epithelial cells [165], human pituirary tumour cells and normal rat pituitary cells [166], and murine *in vitro* antibody response and human autologous mixed lymphocyte reaction [167]. It is suggested that *in vivo* the antagonistic activity of RU-486 stands at the level of receptor transformation [168]. The drug inhibited the induction of α-glycosidases by hydrocortisone in suckling rats [169], stimulated the oxygen consumption and brown adipose activity in the rat [170], and exhibited anabolic-anti-catabolic activity in rats [171]. The latter activity is therapeutically relevant in diseases characterized by muscle atrophy. RU-486 has been evaluated successfully in the treatment of Cushing's syndrome [172]. The peripheral effect of the drug has also been of interest. It antagonized the suppressive effects of dexamethasone on the inflammatory response in rats [173], lowered intraocular pressure in rabbits on local application [174], and inhibited the steroid-induced cutaneous vasoconstriction in humans [175]. It inhibited the dexamethasone-induced leucocyte changes in man [176].

ANTICANCER POTENTIAL

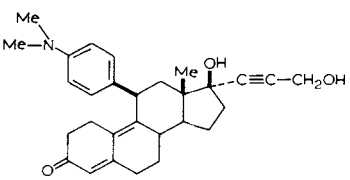
Mifepristone showed growth-inhibitory effects on progesterone receptor-positive human breast cancer cell lines [177–179]. Preliminary trial in the treatment of breast cancer showed growth-inhibitory effects in some patients [180,181]. The antiglucocorticoid properties appear to be responsible for some observed side-effects [181]. Antiprogestins constitute a new form of endocrine therapy for human breast cancer. Some recent *in vitro* studies are of interest. Unexpectedly, it has been observed that RU-486 stimulates growth of T47D human breast cancer cells [182]. RU-486 binds with high affinity in a human endometrial cancer cell line and shows a dual effect acting both as a progestin antagonist and weak agonist on the cells [183].

PHARMACOKINETICS

An early report on the pharmacokinetics of mifepristone in humans and animals, using a radioreceptor assay, showed that after an oral dose the peak concentration of the drug was reached in 2 to 3 h and the plasma elimination half-life of the drug was 10 to 20 h [184]. The gastrointestinal absorption of the drug was low (less than 25%) and the therapeutic results on subcutaneous and intramuscular administration were no better than oral administration. There have subsequently been several pharmacokinetic studies on the drug [185–192]. A study implying quantitation in human plasma by high-performance liquid chromatography and radioimmunoassay after column chromatography showed that circulating plasma levels of RU-486 were not significantly affected by increasing the oral dose [185]. Maybe there are other mechanisms partly involved in the antisteroidal action of the drug. The metabolites can be active in humans and show different affinities to progestin and glucocorticoid receptors [184]. In rats, eight metabolites have been found in the bile, and four of these have been identified in humans. The routes of metabolism, either alone or combined, are through mono- and di-*N*-demethylation followed by *N*-acetylation, and oxidation of the methyl of the 17α-propynyl to an alcoholic function. An investigation based on measurement of the plasma concentrations of RU-486, and its monodemethylated (RU-42633) (30), didemethylated (RU-42848) (31) and alcoholic nondemethylated (RU-42698) (32) metabolites up to 72 h following oral ingestion of 100 mg of RU-486 in five female volunteers has been described [187]. The peak plasma level of the drug occurred within 1 h after ingestion of the drug and at this point significant levels of the metabolites were also present in the plasma. After the initial redistribution within 6 h, the plasma concentrations of RU-486 and three of its metabolites measured remained stable for 24 h. The binding affinities of the metabolites to human progesterone and glucocorticoid receptors suggested an important role of the metabolites, along with the parent compound, as regards



(30) R = H, R¹ = Me
(31) R = R¹ = H

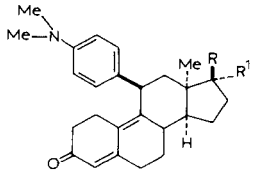


(32)

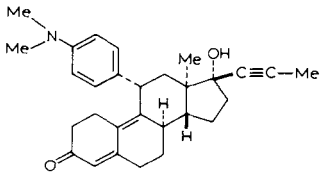
the antisteroidal action of mifepristone. Another study suggests that the RU-486 receptor reactivity in man is due primarily to the drug and secondarily to the metabolites (30) or (31) [188]. It is stated that an administration during the morning hours does not appear to disrupt the circadian rhythmicity of ACTH or hydrocortisone; a prolonged pharmacologic action of RU-486 can be achieved by a single-dose administration [190].

STRUCTURAL MODIFICATIONS

The success with mifepristone (29) has prompted the use of the molecule as a model in designing different related analogues. Attempts have been made to dissociate the antiprogestational and antiglucocorticoid activities observed in the prototype. The Schering Company, Berlin, has actively pursued the work [193–199]. Interestingly, the change of C/D-*trans* fusion in RU-486 to a *cis* junction as in (33) and (34) left the biological activity essentially unaltered [193,194]; the C-13 epimers generally exhibited lower antiglucocorticoid activity as compared with the corresponding compounds in the natural series. The enantiomer (35) of RU-486 (29) showed neither antiprogesterone nor antiglucocorticoid properties [195]. On continued research, two important compounds which have emerged from the Schering group are lilopristone (ZK-98,734) (36) [196] and onapristone (ZK-98,299) (37) [197,198]. Lilopristone has a minor variation of the lead compound, RU-486 (29), having a Z-configurated 17α-(3-hydroxy-1-propenyl) group; in onapristone, there is



(33) R = OH, R^1 = —C≡C—Me
(34) R = —C≡C—Me, R^1 = OH



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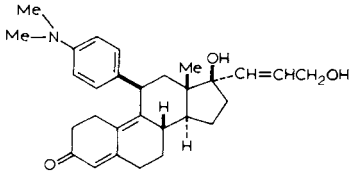

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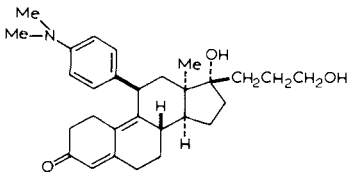
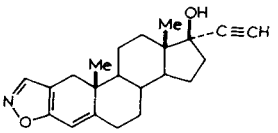


(37)

configurational inversion at C-13 and C-17 and it has a 17β-(3-hydroxypropyl) group. Studies have been made on binding of lilopristone to human uterine progesterone receptors [199], luteolytic action in the rat [200], and abortifacient action in the common marmoset [201]. The antiprogestational activity of lilopristone and onapristone has been studied at different stages of pregnancy in the guinea-pig [202]. Onapristone has high affinity for progesterone receptors in the endometrium [203], and induces menstruation [204] in cycling bonnet monkey. Clinical trials for fertility regulation are recommended for lilopristone [201] and onapristone [204]. The tumour-inhibitory activity of onapristone in mammary tumour models has been shown [205,206].

DANAZOL

Danazol (38), an analogue of ethisterone, was first synthesized in 1963 [207]. It has a complex pharmacology [208–210]. Danazol prevents the midcycle surge of luteinising hormone (LH) and follicle-stimulating hormone (FSH), does not significantly suppress basal LH or FSH in gonadally intact human beings, and in castrated animals it can prevent the compensatory increase in LH and FSH. It binds to androgen, progesterone, and glucocorticoid receptors, but does not bind to oestrogen receptors. It binds to sex-hormone-binding globulin and corticosteroid-binding globulin. Danazol inhibits multiple enzymes of steroidogenesis. It inhibits cholesterol cleavage enzyme, 3β-hydroxysteroid dehydrogenase, 17β-hydroxysteroid dehydrogenase, 17,20-lyase, 17α-hydroxylase, 11β-hydroxylase and 21-hydroxylase. It does not inhibit aromatase. It increases the metabolic clearance rate of progesterone. To say that danazol is a 'selective antigonadotropin' does not seem to be appropriate. The known metabolites of danazol have the isoxazole ring cleaved off and are more closely related to ethisterone.



(38)

The pharmacology outlined above may explain the use of danazol in a wide variety of disorders. It has found its widest application in the treatment of endometriosis [209–212]. The treatment produced significant improvement in the symptoms, signs and laproscopic findings of endometriosis. Danazol con-

tinues to be examined extensively in its role in the treatment of endometriosis [213–223].

Danazol has also been found to be effective in the treatment of benign breast disease [211,224], precocious puberty [211,225], hereditary angioneurotic oedema [211,226,227], menorrhagia [228], certain kinds of infertility in women as in endometriosis [229], and gynaecomastia [230]. It has been studied as a male and female contraceptive agent [211].

Danazol has profound effects on lipid metabolism. A study in women showed that during danazol treatment the mean levels of total cholesterol and triacylglycerol, decreased slightly, while no significant changes in low-density proteins were seen [215]. However, a marked reduction in mean levels of high-density lipoprotein cholesterol and triacylglycerol occurred. Patients with pretreatment hyperlipoproteinaemia experienced a substantial fall in plasma lipids and lipoprotein and had normal lipoprotein profiles during treatment.

OTHER HORMONAL, ANTIHORMONAL AND/OR ANTIFERTILITY HETEROSTEROIDS

There are several other hormone-related heterosteroidal studies. Certain of the aspects are covered here. The inhibitors of biosynthesis of steroids are dealt with in the next section.

ANDROGEN ANTAGONISTS

Cyproterone acetate (6-chloro-1α,2α-methylene-3,20-dioxo-4,6-pregnadien-17α-yl acetate) is a well-known antiandrogen. The pharmacological basis for clinical use of antiandrogens has been documented [231]. Cyproterone acetate acts in all target organs for androgens and affects all functions which are governed or influenced by androgens under physiological or pathophysiological conditions. The accessory gland function and spermatogenesis are inhibited. There is loss of libido in most species. The onset of puberty and bone maturation is delayed. Sebacious gland function and lipogenesis are inhibited. Cyproterone acetate is a highly effective progestogen and it inhibits gonadotrophin secretion. The proven indications for cyproterone are prostatic carcinoma, precocious puberty, hypersexuality and sexual deviations, and androgenization in women (hirsutism, acne, seborrhoea oleosa, alopecia androgentica).

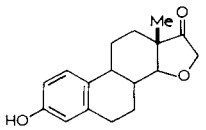
Spironolactone (17) has antiandrogenic side-effects on long-term or high-dose therapy. Several spiro-oxazolidinones were synthesized for aldosterone-blocking activity [232]. None of the compounds possessed any significant

endocrine activities, excepting the compound 3-oxo-13β-ethyl-4,9(10),11-gonatriene-17S-spiro-5′-(2′-oxo-3′-methyl)oxazolidine, which on subcutaneous administration showed antiandrogen activity on CFY male rats and Swiss mice, only somewhat weaker than cyproterone acetate. However, no activity was observed on oral administration.

Interestingly, 17α-chloro-17β-(benzylsulphinyl)-1,4-androstadiene-3,11-dione (R configuration at sulphur) showed no antiandrogenic effects in animals, but inhibited sebaceous gland activity in hamsters [233].

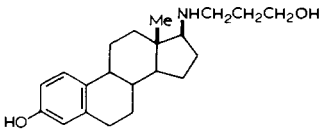
OESTROGENIC ACTIVITY

Certain studies carried out on hetero analogues of oestrogens may be mentioned. Synthesis of (±)-2-azaoestradiol 3-methyl ether and its 11β-methyl homologue [234] and 13-aza-equilenin analogues [235] have been reported. It has been observed that oestrogenic activity of orally administered 15-oxa-oestrone (39) is 12-times greater than that of oestrone by the uterotropic assay in rats [236]. The steroidal coumarins 3-hydroxy-6-oxa-1,3,5(10),8(9)-estratetraene-7,17-dione and 3,17β-dihydroxy-6-oxa-1,3,5(10),8(9)-estratetraen-7-one showed low oestrogenicity on subcutaneous administration in mice [237].



(39)

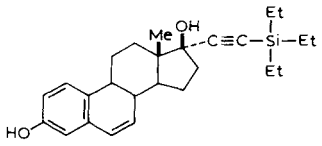
17α-(3-Amino-1-propyn-1-yl)-1,3,5(10)-estratriene is a weak oestrogen [238]. N-(3-Hydroxy-1,3,5(10)-estratrien-17β-yl)-3-hydroxypropylamine (prolame) (40) is a derivative which has prolonged anticoagulant and brief oestrogenic effects in animals [239,240]. 3-Hydroxy-17β-[(1-methyl-1,4-dihydropyridin-3-yl)carbonyloxy]-1,3,5(10)-estratriene and 3-hydroxy-17-[(1-methyl-1,4-dihydropyridin-3-yl) carbonyloxy-19-nor-17α-pregna-1,3,5(10)-trien-20-yne are examples of delivery systems exerting central oestrogenic activity as measured by serum LH suppression in rats [241].



(40)

17β-Amino-1,3,5(10)-estratrien-3-ol and 3-hydroxy-1,3,5(10)-estratrien-17-one 17-hydrazone showed low binding to the cytosolic receptor of immature rat uterus, possibly because of nearly complete protonation of the nitrogen at pH 7.5 [242]. 3-Hydroxy-17β-(*p*-nitrophenyldithio)-1,3,5(10)-estratriene, an affinity labelling agent, has been used to show that one of the thiol groups essential for the binding with oestradiol resides at the oestrogen binding site on the receptor of rabbit uterine cytosol [243].

There is a separation of oestrogenic from antifertility activities by introduction of trialkylsilyl groups to the ethynyl side-chain of ethynyloestradiol and analogues [244,245]. On oral administration in rats, compound (41) has twice the potency of ethynyloestradiol as an antifertility agent but has only 2% of oestrogenic activity of ethynyloestradiol [245].



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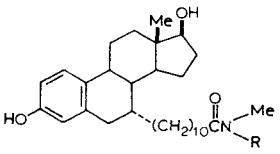
ANTIOESTROGENS

Design of an antioestrogen which may be devoid of oestrogenic activity is an attractive goal of research. There is an interesting discovery from ICI Pharmaceuticals (UK) in this context. 7α-Alkylamide analogues of oestradiol have been shown to be oestrogen antagonists with no intrinsic oestrogenicity in rat uterotrophic-antiuterotrophic tests [246–249]. The most potent compounds are (42) and (43) with oral ED_{50} levels of 6 mg/kg and 2 mg/kg, respectively [249]. The butylamide (42) is a potent inhibitor of the growth of breast cancer MCF-7 cells [247,248] and ZR-75 cells [246,247] *in vitro* and also inhibits the growth of DMBA-induced mammary tumours in rats [250].

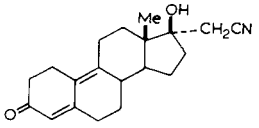


(42) R = —Bu
(43) R = $-CH_2CF_2CF_2CF_3$

As the dialkylaminoethoxy side-chain is required for antioestrogenic activity of triphenylethylene derivatives, oestradiol analogues substituted at 17β-position with dialkylaminoethoxy, dialkylaminoethylamino or dialkylaminoethylthio groups were synthesized, but the compounds obtained had decreased binding to oestradiol receptors, decreased uterotrophic activity, and no antiuterotrophic activity in mouse and rat [251].

STS-557

At the Central Institute of Microbiology and Experimental Therapy, Jena (G.D.R.), 17α-CH_2X-substituted 4,9(10)-gonadienes with progestational activity were synthesized. [252,253]. Of the compounds prepared, STS-557 (44) was the most potent. In McPhail assay using immature rabbits, it showed progestagenic activity about 10-times higher than levonorgestrel. More recently, syntheses of dienogestril (44) [254] and 7α-methyldienogestril [255] have been described. STS-557 also possesses antiprogestational and contragestational properties in rabbits [256]. It is an effective interceptive in the mouse, rat and baboon but not in the guinea-pig [257]. In another report on evaluation of STS-557 as a postcoital contraceptive in baboon, it was pointed out that is was not sufficiently effective if the breeding period was longer than 6 h [258]. It inhibited the fertility of male rabbits without impairing their sexual behaviour [259,260]. In male rats, while at high doses it is antispermatogenic on account of its progestational-antigonadotrophic property, at low dosage its weak antiandrogenic nature fails to interfere with epididymal function [261]. STS-557 has shown a complex metabolic pattern; in the dog and rat the two metabolites identified are 17α-cyanomethyl-11β,17-dihydroxy-4,9-estradien-3-one and 17α-cyanomethyl-1,3,5(10),9(11)-estratetraene-3,17-diol [262].



(44)

OXIMINOSTEROIDS

There has been interest in antifertility activity of certain oximinosteroids. Norgestimate (ORF-10131; (+)-13-ethyl-3-oximino-18,19-dinor-17α-pregn-4-en-20-yn-17-yl acetate) is known to have progestational and antifertility

activity. A biotransformation study in women has shown that the metabolism of norgestimate following oral administration includes rapid hydrolysis of the oxime and acetate moieties, 4,5-double bond reduction, polyhydroxylation and conjugation [263].

ORF-9326 (2α-chloro-3-*p*-nitrophenoxyimino-5α-androstan-17β-yl acetate) possesses antifertility activity in laboratory animals. The major route of excretion for the compound and/or its metabolites in dog and rat is biliary, whereas in monkey and rabbit it appears to be renal [264].

It is suggested that the anti-implantation action of norethisterone 3-oxime is exhibited by its effect on the oviduct in rabbits [265].

MISCELLANEOUS STUDIES

A mention may be made of the reported affinity of 7α-butylthio-3,20-dioxo-4-pregnen-17α-yl acetate against tritiated progesterone and receptor from rabbit uterus cytosol [266], inactivity of 18-cyanoprogesterone and related compounds in Clauberg and anti-Clauberg tests in rabbits [267], and post-coital activity of certain 17β-*N*-substituted carbamoyloxy-1,3,5(10)-estratrienes in baboons [268,269].

Some heterosteroids have been tested as male antifertility agents. 17β-Hydroxy-17α-methyl-5α-androstano[3,2-*c*]pyrazole 17-methyl ether is claimed to have contraceptive activity in the male [270]. 22,25-Diazacholesterol dihydrochloride (SC-12937) inhibits spermatogenesis in bandicoot rat [271]. Solasodine, a steroidal alkaloid, impaired spermatogenesis in dog [272]. 1-Methyl-A-nor-2,3-diaza-1,5(10)-estradien-17-one and 1-methyl-17α-ethynyl-A-nor-2,3-oxaza-1(10),3(5)-estradien-17β-ol have shown a high affinity for androgen binding protein [273].

HETEROSTEROIDAL INHIBITORS OF STEROID HORMONE BIOSYNTHESIS

A relatively recent approach to new drug development is the design of inhibitors of steroid hormone biosynthesis [274]. Such agents may be more selective in their actions. The suicide substrates and the pseudo-irreversible or very tight binding inhibitors may be more promising in achieving selectivity. Suicide substrates are inocuous until transformed by their target enzyme into highly reactive alkylating groups.

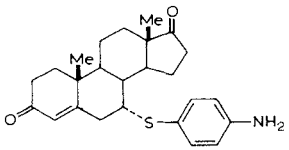
Certain heterosteroids have proved to be active in inhibiting some steroid biosynthetic enzymes. An account of such heterosteroids is given under the

headings: aromatase inhibitors; 5α-reductase inhibitors; and 3β-hydroxysteroid dehydrogenase inhibitors. Earlier, under mifepristone and danazol, mention was made of inhibitory activity of the drugs on some enzymes.

AROMATASE INHIBITORS

Aromatase (oestrogen synthetase) inhibitors, by suppressing the oestrogen biosynthesis, can have potential use in the treatment of oestrogen-dependent mammary carcinoma and even benign prostatic hyperplasia.

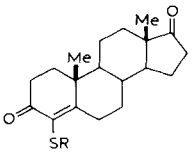
4-Androstene-3,17-dione is the precursor of the biosynthesis of oestrone, and as such analogues corresponding to it have been studied as aromatase inhibitors (review: [274a]). Several 7α-substituted 4-androstene-3,17-diones were found to be effective competitive inhibitors [275]. The most active compound in the series is 7α-(4-aminophenylthio)-4-androstene-3,17-dione (UM-583; 7α-APTA, (45)). Its *in vitro* activity as an aromatase inhibitor has been ascertained employing microsomal fraction of human placental tissue [275], human mammary tumour homogenates [276], MCF-7 human mammary cell line [277], and *in vivo* it has been found to be effective in the DMBA-induced rat mammary carcinoma [277,278]. 7α-(4-Bromoacetamidophenylthio)-4-androstene-3,17-dione competitively inhibits microsomal aromatase, covalently binds to the enzyme complex, and produces an irreversible inactivation of enzyme activity [279].



(45)

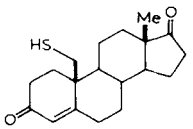
4-Hydroxy-4-androstene-3,17-dione is an aromatase inhibitor and appears to have clinical significance in the treatment of breast cancer [280–282]. As such, it was expected that there should be interest in the isostere (46) which has been claimed to be an aromatase inhibitor [283]. 4-(Substituted-thio)-4-androstene-3,17-dione derivatives possess considerable *in vitro* aromatase inhibitory activity; the most potent compound being 4-phenylthio derivative (47) [284].

There is a loss of the C_{19}-methyl group during aromatization of ring A, and so the hetero changes in the methyl group have been the other course followed in the design of aromatase inhibitors. Several such derivatives obtained



(46) R = H
(47) R = Ph

showing human placental aromatase competitive inhibition include 19-methylthio-4-androstene-3,17-dione and 19-azido-4-androstene-3,17-dione, competitive inhibitors [285]; 10β-amino-4-estrene-3,17-dione and 19-amino-4-androstene-3,17-dione derivatives, showing poor activity [286]; (19*R*)- and (19*S*)-isomers of 10β-oxiranyl-4-estrene-3,17-dione, powerful competitive inhibitors [287,288]; and (19*R*)- and (19*S*)-isomers of 10β-thiiranyl-4-estrene-3,17-diones, showing even greater stereoselectivity in binding than the oxiranes [288,289]; the 10-oxiranyl and 10-thiiranyl heteroatoms appear to coordinate with the haem iron of the aromatase system [288]. 17β-Hydroxy-10-methylthio-1,4-estradien-3-one [290], 17β-hydroxy-10β-mercepto-4-estren-3-one and 19-mercapto-4-androstene-3,17-dione (48) [291], 10β-mercapto-4-estrene-3,17-dione and 2α-mercapto-4-androstene-3,17-dione [292] are suicide substrates of human placental aromatase. A bidentate mechanism through which (48) inactivates both the ferric and ferrous forms of aromatase has been suggested [292].



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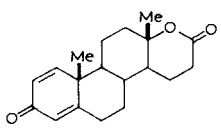


(49)

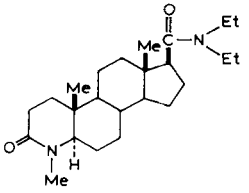
Testolactone (49) is a suicide substrate of aromatase [293]. It produces pronounced decrease of serum oestrone levels in postmenopausal women with metastatic breast cancer [294]. A study in male rats shows that the majority of oestradiol is not testicular in origin but is derived from the adrenal gland [295].

As oestrogens may play a role in the pathogenesis of benign prostatic hyperplasia in man, aromatase inhibitors may be of interest in non-surgical treatment of the disease [296,297]. A clinical study with the aromatase inhibitor testolactone has given encouraging results [298].

5α-REDUCTASE INHIBITORS

5α-Reductase, present in many androgen-sensitive tumours such as the prostate, the seminal vesicle, the epididymis and skin, catalyses conversion of the major circulating androgen testosterone in adult males to more active metabolite 5α-dihydrotestosterone. The latter is implicated in the pathogenesis of certain androgen-dependent conditions such as benign prostatic hyperplasia, acne, male pattern baldness and female hirsutism. Thus, 5α-reductase inhibitors may be of interest for minimizing certain androgen-responsive conditions.

Merck Sharp & Dohme Research Laboratories (U.S.A.) discovered 17β-*N*,*N*-diethylcarbamoyl-4-methyl-4-aza-5α-androstan-3-one (4-MA) (50), a potent reversible 5α-reductase inhibiting heterosteroid [299,300]. It strongly inhibits the 5α-reductase-mediated conversion of testosterone to 5α-dihydrotestosterone both *in vitro* and *in vivo*. It has high affinity for 5α-reductase and moderate affinity for the androgen receptor [301]. In porcine granulosa cells, it inhibits 3β-hydroxysteroid dehydrogenase [302]. In laboratory animals, it is relatively nontoxic, antagonizes certain androgen dependent actions but is without significant oestrogenic, progestational, gonadotropin inhibiting, androgenic or antifertility activities [303]. It is effective in reducing the size of the sex accessory organs in rats and dogs [304]. It has a marked effect on the behaviour of intact male hamsters and on castrated males maintained on testosterone [305]. It has been found to be effective in reducing the size of the enlarged dog prostate [306,307].



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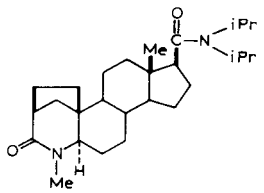
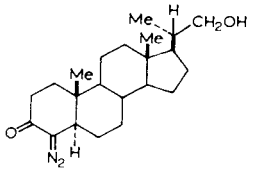


(51)

Several analogues of the 5α-reductase inhibitor (50) been studied [308–313]. A series of A-ring heterocyclic steroids have been examined [308]; the 5α-reductase inhibitory activity resides uniquely with 3-oxo-4-aza-5α-steroids optimally substituted with methyl group at the 4-position. The variation of 17β-functional groups of the inhibitors has shown species differences. Those 5α-reductase inhibitors with 17β-diethylcarbamoyl, 17β-di-isopropylcarbamoyl, 17β-*t*-butylcarbamoyl, 17β-*s*-butylcarbonyl functional groups, are approxi-

mately equipotent as inhibitors of the rat and human enzymes, whereas they are only 0.1–15% as potent as inhibitors of the dog enzyme [310]. The bridged analogue (51) has been prepared as a potential 5α-reductase inhibitor [314].

(20*S*)-4-Diazo-21-hydroxy-20-methyl-5α-pregnan-3-one (MDL-18,341) (52) has been designed as an irreversible inhibitor of 5α-reductase, at the Merrell Dow Research Institute (U.S.A.) [315–317]. The diazoketone produces both competitive inhibition and time-dependent inactivation of the 5α-reductase from rat prostate *in vitro* [316]. It has been shown that MDL-18,341 produced a pure 5α-reductase deficiency, in which testosterone-mediated androgenic effect (growth of the levator ani muscle) was not affected, while 5α-dihydrotestosterone-mediated effects (growth of the ventral prostate, seminal vesicles and preputial glands) were reduced when testosterone was the androgen administered to castrate rats [317].



(52)

The structural parts of both (50) and MDL-18,341 (52) were combined in 20-(hydroxymethyl)-4-methyl-4-aza-5α-pregnan-3-one-related compounds but they produced only weak inhibition of 5α-reductase from rat prostate [311].

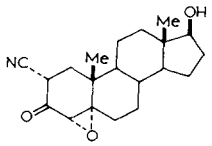
The 5α-reductase inhibitory activity of 17β-carboxy-4-androsten-3-one benzylanilide and some non-nitrogenous steroids has been examined within MCF-7 human breast cancer cells [318].

3β-HYDROXYSTEROID DEHYDROGENASE INHIBITORS

The utility of such agents may be evident from the following accounts of the inhibitors trilostane, epostane and azastene.

Trilostane

Trilostane (WIN-24540) (53) is an orally active inhibitor of steroid biosynthesis [319]. It is an inhibitor of adrenal 3β-hydroxysteroid dehydrogenase activity. It inhibits adrenal, ovarian and placental steroidogenesis when administered orally to rhesus monkey [320]. By inhibiting 3β-hydroxysteroid dehydrogenase



(53)

activity, it causes an increase in circulating levels of pregnenolone. It is an interceptive agent in the rat and monkey [319,320], interrupting pregnancy after implantation. However, it does not appear to be a useful interceptive, as higher doses are needed to terminate pregnancy in the rhesus monkey than required for reducing adrenal steroidogenesis [320]. Trilostane is effective in inhibiting aldosterone synthesis and raising potassium levels in diuretic-treated animals [321,322]. Clinically, trilostane is effective in the treatment of primary aldosteronism [323,324]. It can correct diuretic-induced hypokalaemia by lowering aldosterone secretion [325]. The reduction in aldosterone may enhance the antihypertensive effects of diuretic therapy. It has been considered useful in the treatment of Cushing's syndrome [326], but there is a report to the contrary [327]. It has been investigated in the treatment of low-renin essential hypertension [328].

Studies in rats [329] and man [329,330] have shown that trilostane treatment may decrease testosterone synthesis [329]; the human testicular 3β-hydroxysteroid dehydrogenase is inhibited. This action may be kept in view during clinical use of trilostane. In rats, high doses of trilostane induce compensatory adrenal hypertrophy [319,331]. A study in healthy volunteers shows that low-dose trilostane treatment induces latent adrenal insufficiency [332].

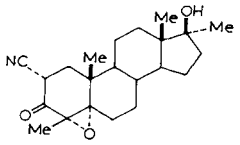
The main metabolic products of trilostane in rats have been identified as 4α,5-oxido-3,17-dioxo-5α-androstane-2α-carbonitrile (M-1), 3α,16α-dihydroxy-4α,5-oxido-17-oxo-5α-androstane-2α-carbonitrile (M-2), and 3α,16α,17β-trihydroxy-4α,5-oxido-5α-androstane-2α-carbonitrile (M-3) [333,334]. The structures of the two other metabolites, M-4 and M-5, have not been conclusively assigned [333]. The unconjugated metabolites are predominantly excreted in the urine, whereas conjugated ones are excreted in the bile. Trilostane and its metabolite (M-1) have been estimated in human blood plasma by HPLC [335,336]. A comparative study using HPLC and quantitative cytochemical assays has been made on the bioavailability and metabolism of trilostane in normal subjects [337].

Trilostane-related compounds have been studied. The methylation of C-4 or C-4 and C-17 changes this relatively selective adrenal inhibitor to compounds having increased ovarian-placental inhibitory activity with decreased adrenal

inhibitory activity [338]. Mineralocorticoid inhibitory activity has been claimed for 4α,5-oxido-3,20-dioxo-5α-pregnane-2α,16α-dicarbonitrile [339].

Epostane

Epostane (WIN-32729) (54) like trilostane (53) is also a 3β-hydroxysteroid dehydrogenase inhibitor. It is claimed to exert preferential effect on the gonadal 3β-HSD enzyme rather than on the enzyme in the adrenal cortex [340]. It is a potent interceptive agent in rats and rhesus monkeys. It inhibited ovulation and plasma progesterone levels in rats [341]. Local inhibition of the conversion of pregnenolone to progesterone by human placenta and chorio-decidua has been observed [342]. In sheep there was lowering of progesterone concentrations and there appeared to be no effect on peripheral oestradiol concentrations [343]. In goats in late pregnancy, a direct inhibition by epostane of luteal function was found [344]; there was reduction of circulating concentrations of progesterone by about 80% within 6 h of treatment, and these low levels were maintained for at least 50 h and were associated with premature expulsion of the foetal kids. Epostane is an inhibitor of ovarian and placental steroidogenesis in early human pregnancy, measured as effect on circulating progesterone, and can induce vaginal bleeding and abortion [345,346]. It has been shown to be more selective than trilostane [347].

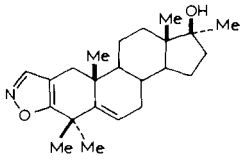


(54)

The 7α-methyl derivative of epostane is reported to have interceptive activity [348]. 3-Acetoxy-17β-hydroxy-4,4,17-trimethyl-2,5-androstadiene-2-carbonitrile has been claimed to terminate pregnancy in rat [349].

Azastene

Azastene (55), from the Sterling-Winthrop Research Institute (U.S.A.), is an orally active interceptive agent in rats [350] and monkey [351,352]. It is a competitive inhibitor of 3β-hydroxysteroid dehydrogenase, reducing production of progesterone and glucocorticoids without hormonal effects. Very early in pregnancy it acts as a luteolytic agent in that it inhibits progesterone produc-



(55)

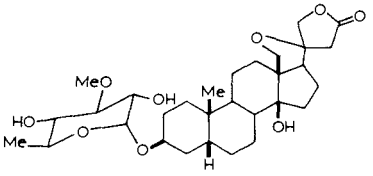
tion stimulated by chorionic gonadotropin and later in pregnancy it acts by inhibiting placental progesterone production. Involvement of prostaglandins is also suggested on the basis of experiments in rats and rabbits [343,354].

HETEROSTEROIDS ACTING ON THE CARDIOVASCULAR SYSTEM

CARDIAC GLYCOSIDES AND RELATED ASPECTS

Digitalis glycosides continue to be of interest as cardiotonic agents. Attention has been drawn to gitaloxin (16-formylgitoxin), a cardiac glycoside occurring naturally in *Digitalis purpurea* [355]. It is exceedingly potent and inhibits Na^+/K^+-transporting ATPase. It appears that the therapeutic activity of *D. purpurea* depends substantially on the content of gitaloxin. A detailed investigation of the latter is suggested. A study of a series of gitoxigenin C-16 acetates, formates, methoxycarbonates and 16-ones has shown that 16β-formate group increases the biological activity 30 times (inhibition of Na^+/K^+-ATPase), and a 16-one decreases the activity [356].

An unusual oxygenated cardenolide, 18,20-oxido-20,22-dihydroneriifolin (56), has been isolated from *Thevetia thevetoides* Schum [357]. The glycoside has oxygenated C-18. The complete stereochemistry of *Asclepiadaceae* glycosidogomphoside (57) has been determined [358,359] and its metabolism has been studied [360]. A convenient synthesis of bufadienolide bufalin (58) ad α'-isobufalin (60) has been worked out [361]. A new synthesis of azabufalin



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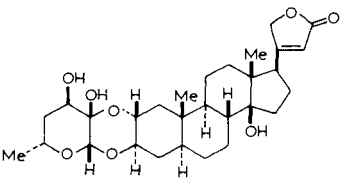
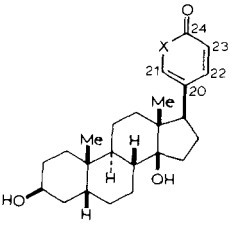
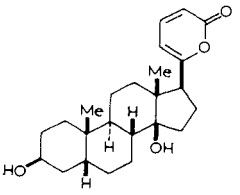


(57)

(59) has been reported [362]. From the derivatives of hellebrigenin prepared at Degussa (Frankfurt, F.R.G.) [363], hellebrigenin 3β-dimethylacrylate (D12316; acrihellin) (61) has a favourable pharmacological profile and has been chosen for clinical evaluation [364–366].



(58) X = O
(59) X = NH



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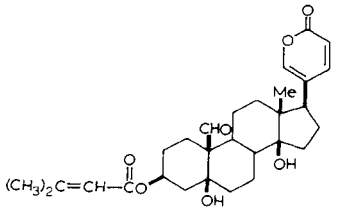
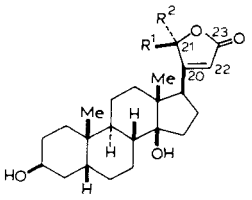


(61)

Through recognition of Na^+-K^+-transporting ATPase as the cardiac glycoside receptor, it has become possible to draw a relationship between the chemical structure and biological activity of cardiac glycosides at the molecular level. The major conclusions which are evident from a variety of approaches have been summed up [367]: the lactone side-chain in cardiac glycosides is not a pharmacophoric substructure as had been long believed; the steroid nucleus (5β,14β-androstane-3β,14-diol) in cardiac glycosides is the lead structure serving as the structural determinant for specificity of action; the lactone and sugar side-chains, although dispensable substructures, contribute much to thermodynamic selectivity; and the electrostatic fields of effector and receptor dominate the long-to-medium-range interaction. Use is made of computer graphics to study cardiac glycoside–receptor interactions [368].

21-Methyldigitoxigenin, having an additional chiral centre in the lactone ring, has been synthesized; both (21*R*)- (62) and (21*S*)- (63) epimers have been obtained [369]. The glucoside of (21*R*)-methyldigitoxigenin (62) is only very slightly less potent than digitoxin, but it has a reduced toxicity. The glucoside of the epimer (63) is less active. The possibility of there being two separate

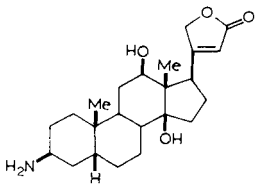


(62) R^1 = Me, R^2 = H
(63) R^1 = H, R^2 = Me

receptors for the inotropic effect and toxicity of digitalis glycosides has been commented upon.

Digitoxigenin 3-aminoacetate possessed inotropic activity with low *in vivo* potency and short duration of action [370].

In isolated guinea-pig atrial preparation, the 3β-amino isostere (64) of digoxigenin was as active as its 3-hydroxy counterpart (digoxigenin), while the 3α-isomer was inactive [371]. The related compounds with changes in the butenolide ring were also prepared. 3β-Amino-17β-(3-furyl)-5β,14β-androstane-12β,14-diol and 17β-(3-furyl)-5β,14β-androstane-3β,12β,14-triol possessed appreciable activity. 3β-Amino-17β-(4-pyridazinyl)-5β,14β-androstane-12β,14-diol 12-monoacetate exhibited a weak activity. The corresponding studies have been carried out in relation to the aglycone digitoxigenin [372].



(64)

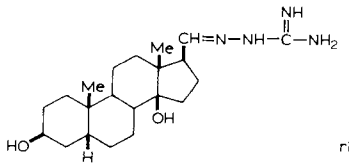
The butenolide moiety of digitoxigenin was replaced by other 5- or 6-membered cyclic Michael acceptor systems [373]. The analogues possessing 17β-(2-oxo-3-pyrrolin-4-yl)-, 17β-(21-methyl-2-oxo-3-pyrrolin-4-yl)- or 17β-(2,5-dihydro-2-oxothiophen-4-yl)- groups showed weak to moderate inotropic activity in atria of guinea-pigs and inhibition of bovine kidney Na^+/K^+-ATPase.

SC-4453 is a digoxin analogue having 17β-(pyridazin-4-yl)-instead of the lactone ring of cardenolides. It inhibits Na^+/K^+-ATPase within the same range of concentrations as digoxin [374]. SC-4453, like digoxin, stimulated the sodium pump in guinea-pig isolated left atria at low concentrations and inhibited at high concentrations [375]. A pharmacokinetic study in guinea-pig

showed that following intravenous bolus doses, plasma concentrations diminished biexponentially and followed a two-compartment open model [376].

17β-(Pyrid-3-yl)-14β-androst-4-ene-3β,14-diol inhibited Na^+/K^+-ATPase-induced activity in guinea-pig cardiac muscles [377]. Calculations have been made on molecular structure and electrostatic potentials of cardiotonic steroids, including pyridazinyl and pyridyl derivatives [378].

One of the compounds having no heterocycle attached to position 17 and possessing a positive inotropic effect is prednisolone 3,20-bisguanylhydrazone [379,380]. It inhibits Na^+/K^+-ATPase *in vitro*; its enzyme-inhibiting action develops rapidly and the inhibition observed is reversible. It binds to a phosphorylated form of Na^+/K^+-ATPase. Certain steroidal 17β-formyl guanylhydrazones have been studied [381]. All the guanylhydrazones, regardless of their steroid system, inhibited Na^+/K^+-ATPase to about the same extent, and only (65), which has the same steroid system as digitoxigenin, gave rise to a positive inotropic effect on the isolated guinea-pig atrium.



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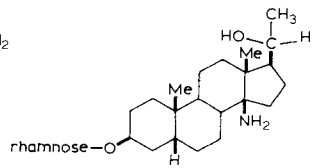


(66)

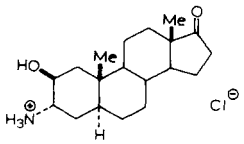
Replacement of the lactone ring of digitoxigenin with open-chain bioisosteres, such as 17β-carbomethoxyethylene and 17β-cyanoethylene, gives compounds with potent digitalis-like activity; however, an AB-*cis*, BC-*trans*, CD-*cis* steroid system is necessary to elicit the activity [382].

14β-Aminosteroid derivatives were claimed to have cardiotonic activity [383]. There has been interest in LND-623 (3β-rhamnosyloxy-14-amino-5β,14β-pregnan-20β-ol) (66) and its close derivative LND-796 [384]. They exert strong positive inotropic effects [385–388] and inhibit Na^+/K^+-ATPase activity [389]. LND-623 has been found to have greater potency and lower toxicity than digitalis [384,390]. The electrophysiological, toxic and inotropic effects have been studied in both normal and partially K^+-depolarized guinea-pig isolated ventricular muscle [391]. The mechanisms involved in the inotropic effects of these aminosteroids are discussed.

There is a mention of cardiotonic and short-duration hypotensive activities of certain 8,16-diazasteroids [392].

ANTIARRHYTHMIC AGENTS

The antiarrhythmic activity of the Organon compound amafolone (Org-6001) (67) has been studied in different test systems. It has antiarrhythmic properties in the ischaemic pig heart which compare favourably with those of lignocaine [393]. It is rapidly absorbed after oral administration and is long-acting. It did not reduce very early ischaemic damage in the isolated rat heart as detected by lactate dehydrogenase release, whereas it antagonized dysrhythmias evoked by reperfusion [394]. It produces the membrane stabilization (class I); in anaesthetized rats, it increased the ventricular fibrillation threshold (VFT) of normal myocardium and in lower doses reduced the postligation fall in VFT [395]. It had a short-lasting effect against aconitine-induced arrhythmias in rats or strophanthin-induced arrhythmia in guinea-pigs but it did increase survival of rats with $CaCl_2$-induced arrhythmias [396]. *ent*-16α-Methylamino-3-methoxy-1,3,5(10)-estratrien-17β-ol and its antipode with natural steroid configuration showed equal potency as antiarrhythmic agents [397].



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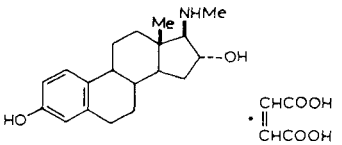

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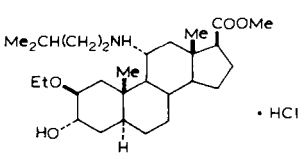
Org-7797 (68) is a new antiarrhythmic agent [398]. It is currently undergoing clinical evaluation. It is suggested that the drug owes its antiarrhythmic effects to inhibition of the fast inward sodium current in cardiac cells (class I action). It has been classified as a Ic antiarrhythmic agent, as in the porcine cardiac tissue, its electrophysiologic profile is very similar to that of propafenone [398]. Substitution of the 3-position in 17β-amino- or 17β-amino-16α-hydroxyestratrienes with 2-hydroxy-3-(isopropylamino)propyl or 2-hydroxy-3-(t-butylamino)propyl ether groups (moieties corresponding to class II antiarrhythmics) markedly reduced the class I activity while conferring noncompetitive β-adrenoceptor blocking activity [399]. However, such substitution did not offer any advantage over the parent compounds.

CCI-22277 (69) is from a series of 11α-alkylamino-steroids examined at the Glaxo Group Research (U.K.) [400]. It is more active than lignocaine, disopyramide and amafolone (67) in the rat aconitine test. It has been characterized as primarily a class I antidysrhythmic drug at low concentrations, with additional class IV action at much higher concentrations [401].



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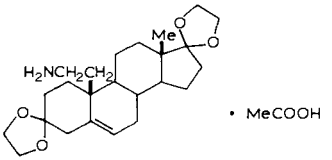

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The research group of G.D. Searle & Co. (U.S.A.) has examined the cellular electrophysiological properties of the aminosteroid SC-35135 (70) [402]. Its properties are similar to those of amafolone and CCI-22277, and resemble class Ic antiarrhythmics. The arrhythmogenic activity of (70) has prevented further development of this compound.

Cardenolides and bufadienolides having ajmaline moiety linked through 3β-OH have been reported to relieve cardiac arrhythmia [403]. Strophanthidin-3β-*O*-acetyl-2′-*N*(b)-ajmaline chloride increased the survival rate in rats with $CaCl_2$-induced arrhythmias [404].

ANTILIPEMIC ACTIVITY

During the last decade there have appeared only a few reports on antilipemic activity of heterosteroids. 2-Methyl-2,3-diaza-A-nor-19-nor-17α-pregn-1-(10),3(5),9(11)-trien-20-yn-17β-ol is claimed to lower serum cholesterol level in rats [405]. Another estrane derivative is 1,3,5(10)-estratrieno[17,16-*c*]pyrazol-3-ol, which lowers cholesterol level in rats with alimentary hyperlipemia [406] and has a weak oestrogenic activity [406,407]. 16α-Methylamino-3-methoxy-1,3,5(10)-estratrien-17β-ol hydrochloride lowered plasma cholesterol levels in rats, but its enantiomer had no such effect [397].

Antilipemic activity of anabolic heterosteroid oxandrolone (17β-hydroxy-17α-methyl-2-oxa-5α-androstan-3-one) has been confirmed in the retired breeder rat, an animal known to undergo premature aging and to develop hypercholesterolaemia [408]. Its effect on total serum cholesterol is limited to the low and high density lipoproteins. Furazabol, chemically 17β-hydroxy-17α-methyl-5α-androstano[2,3-*c*]-1′,2′,5′-oxadiazole, another anabolic agent, decreased the serum level of cholesterol in normal rats without significantly affecting the triacylglycerols [409]. A mention has been made under danazol about its profound effect on lipid metabolism.

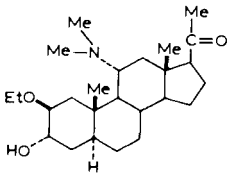
20,25-Diazacholesterol, a desmosterol reductase inhibitor, caused an accumulation of desmosterol in the central nervous system and of 7-dehydrodesmosterol and desmosterol in peripheral nervous system in rats [410]. 25-Hydroxy-23-thiacholesterol is claimed to inhibit conversion of hydroxymethyl-

glutaryl CoA into mevalonate by aortic smooth muscle cells [411]. There is a mention of anticholesterolemic activity of 3-aza-A-nor-3(5)-cholestene and 3-(2-hydroxyethyl)-3-aza-A-nor-5β-cholestane [412].

A passing reference may be made to U-18666A (3β-(2-diethylaminoethoxy)-5-androsten-17-one hydrochloride), which inhibits the enzymatic reduction of desmosterol to cholesterol and is cataractogenic [413]. It is suggested on the basis of study in rats that the development and/or progression of the cataract induced by the compound may be related to a selective block in formation of γ-crystallin secondary to alteration in the levels of Na^+ and K^+ ions in the lens.

CENTRAL NERVOUS SYSTEM ACTIVITY OF SOME HETEROSTEROIDS

From studies carried out on 11-dialkylamino steroids at Glaxo Research Laboratories (U.K.) there resulted the general anaesthetic, minaxolone (71) [414]. In part it is structurally related to alphaxalone (3α-hydroxy-5α-pregnane-11,20-dione). Minaxolone is basic and the citrate is readily soluble in water. The results in animals [415] and early clinical studies [416–418] were encouraging for use of the drug as an intravenous anaesthetic. It showed high total body clearance in sheep [419] and man [420]. However, long-term animal toxicity and certain adverse clinical effects [421] led to withdrawal of the drug from clinical studies.



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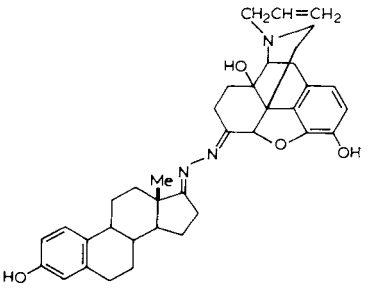

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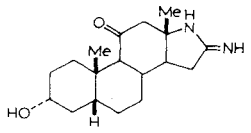
Certain of the analogues corresponding to 11,13,15-triaza-[422], 11,13,15-triaza-B-homo [423], 11,13,15-triaza-D-homo- [424], and 11,13-diaza-15-thia-D-dihomo- [425] steroidal systems inhibited reserpine-induced hypothermia in mice. Analgesic activity has been observed in some 8,13-diaza-oestrone analogues [426] and related systems [427].

There has been interest in γ-selective opiate antagonists of a non-peptide nature; a mixed azine between oestrone and naloxone is the lead compound (72) [428,429]. Syntheses and stereochemical determinations of several antagonist and agonist analogues of (72), mixed azines between steroids and opiates, have been described [428].



(73)

RU-5135 (3α-hydroxy-16-imino-17-aza-5β-androstan-11-one) (73) has a high affinity for γ-aminobutyric acid (GABA) and glycine receptors *in vitro* [430]. It appears to be a GABA receptor antagonist. It causes convulsions but is devoid of any hormonal activity in rodents. It has been shown to induce epileptiform activity [431]. In a study on isolated preparations, it has been found that on rat cuneate nucleus, it antagonizes muscimol and shares a common site of action with bicuculline, and on the rat optic nerve, it antagonizes glycine and shares a common site of action with bicuculline, and on the rat optic nerve, it antagonizes glucine and shares a common site of action with strychnine [432].

Epipachysamine-A, a steroid alkaloid, prevented 2-deoxy-D-glucose- or thyrotrophin-releasing hormone-stimulated gastric acid secretion in anaesthetized rats; the effect apparently is due to the influence on the central nervous regulatory mechanism in the gastric acid secretion [453].

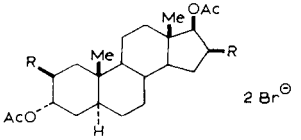
NEUROMUSCULAR BLOCKING AZASTEROIDS

The work pertaining to pancuronium (74) [434] and chandonium (75) [434–436] has been reviewed. Interest continues in these and related azasteroidal quaternary ammonium compounds.

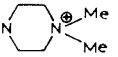
PANCURONIUM ANALOGUES

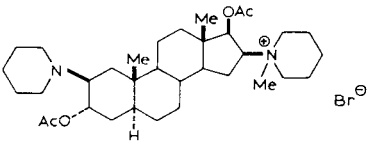
A notable discovery after pancuronium from the Organon Laboratories is of the potent nondepolarizing neuromuscular blocking agent vecuronium bromide (Org NC 45; Norcuran) (76) [437–445] which has short duration and rapid

onset of action and little cumulative effect. It is suggested that quaternary ring D acetylcholine fragment is intrinsically suited to skeletal muscle nicotinic receptors and is relatively unsuited to cardiac muscarinic receptors. It is highly selective and possesses a wide margin between neuromuscular and vagal blocking doses. It has low propensity to release histamine and possesses negligible ganglion-blocking activity.



(74) R = 

(75) R = 



(76)

The deacetoxy analogues of pancuronium and vecuronium have been examined for their activity in anaesthetized cat [446]. The results showed that the greater neuromuscular blocking potency of pancuronium and vecuronium is lost after removal of one or both of the acetylcholine moieties. The block was faster in onset and shorter in duration.

The clinical pharmacology of vecuronium has been reviewed [447]. The potency of vecuronium appears to be slightly greater than that of pancuronium. It has a more rapid onset and considerably shorter duration of action and faster recovery rate. The usual clinical dose varies from 80 to 100 $\mu g\ kg^{-1}$. Several clinical studies have been carried out [448–451]. Vecuronium is considered to have little or no cumulative effect after repeated administration [452,453]. No adverse cardiovascular side-effects are reported [454]. In a recent study it was shown that vecuronium did not influence heart rate, or systolic or diastolic arterial pressure [455]. It has been noted that vecuronium may increase the risk of bradyarrhythmias [456–458]. This may be explained by the probable absence of vagolytic activity of this agent. Vecuronium seems to be free of histaminergic properties [459–461].

A pharmacokinetic study [462] in man showed elimination half-life for vecuronium as 31 min, which may explain shorter duration of action, and the more rapid rate of recovery as compared with pancuronium. The elimination half-life for pancuronium has been found to be 110 min [463] in man.

The short duration of neuromuscular blockade induced by vecuronium is

explained by the more rapid clearance and shorter elimination half-life as compared with pancuronium [464]. The lack of a significant alteration in vecuronium pharmacokinetics in the absence of renal function in animals [465] and humans [466] suggests that non-renal clearance perhaps via hepatic mechanisms may play an important role in the elimination of vecuronium. In accordance with this concept, neuromuscular blockade induced by vecuronium has been found to be prolonged by liver exclusion in cats [467,468]. Further, in rats more than 40% of the dose of vecuronium was recovered in the bile as compared with 8% in the urine [469]. Thus, in humans vecuronium may be eliminated primarily by the liver, and its effect may be prolonged in patients with liver disease. Vecuronium exerts a prolonged neuromuscular blockade in patients with cirrhosis [470,471]. It has prolonged effect in patients with cholestasis which is caused by a delay in its elimination [472]. It has been demonstrated, however, that alcoholic liver disease does not alter the pharmacokinetics or the duration of action of vecuronium after a dose of 0.1 mg kg^{-1} is administered [473].

The disposition and effect of vecuronium are only marginally disturbed by renal failure. The pharmacokinetic parameters of patients with or without renal failure were found to be similar [474]. No metabolites of vecuronium were detected in the plasma. 20% of vecuronium was excreted unchanged in the urine and 5% as the 3-hydroxy derivative. In another study, vecuronium has been reported to produce a longer neuromuscular blockade in patients with renal failure during an isoflurane anaesthesia [475].

A study of the distribution and kinetics in rats and mice has shown that [^{14}C]vecuronium accumulates rapidly in the liver [476]. Both unchanged and metabolized vecuronium was excreted with bile into the intestine and stomach. Blood-brain barrier and placenta were permeable only to a small degree.

Pipecuronium bromide (RGH-1106, Arduan) (75) [477], an analogue of pancuronium (74), was discovered at the laboratories of Gedeon Richter Ltd. (Budapest, Hungary). Pipecuronium is a nondepolarizing blocker and in animal experiments [478,479] it has shown activity 2–4-times that of pancuronium and duration of action is twice as long as that of pancuronium bromide in equiactive doses. It produces no histamine release and is stated to cause no influence on the cardiovascular system. Studies in rats using labelled substances showed that it is rapidly taken up by the liver and to a lesser extent by the kidneys [480]. It has been demonstrated in pigs that the liver plays a major role in the clearance of pipecuronium [481], so in patients with severe hepatic or multiorgan failure the duration of neuromuscular blockade by the drug may be significantly increased. A pharmacokinetic study [482] indicated it to have a biological average half-life of 41 min. It showed a poor binding to plasma

proteins. The rate of hepatic elimination of the compound and/or its metabolites reached 6% after 4 h. The dihydroxy metabolite and possibly a monoacetate are present in plasma and bile. Pipecuronium showed mixed type of inhibitory effect both on acetylcholinesterase and serum cholinesterase [483].

Safety tests [484] and clinical studies [485–487] showed pipecuronium to be a potent muscle relaxant with medium duration of action. It is claimed [488] that the drug does not cause circulatory side-effects, even in patients with severe heart diseases. In a clinicopharmacological comparison, pipecuronium was found to be 20% more potent than pancuronium [489]. It caused a mild bradycardia, in contrast to the heart rate increasing effect of pancuronium. It has been concluded that administration of pipecuronium is accompanied by a haemodynamic stability [490]. It may be a clinically useful alternative to pancuronium for those in whom tachycardia is best avoided. Pipecuronium and vecuronium, when combined with sufentanyl-O_2 anaesthesia, provide similar and minimal changes in haemodynamics of patients scheduled for coronary surgery [491].

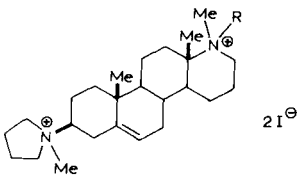
In humans, the plasma clearance of pipecuronium was found to be higher than that of pancuronium [492]. A colorimetric method of assay was used, and the plasma concentrations were measured for 60 min after injection of pipecuronium. In a recent study, comparison of pharmacokinetics and duration of action of pipecuronium and pancuronium has been made, using capillary gas chromatographic assay, the plasma concentrations being measured for 6 h following administration [493]. The durations of action of pipecuronium and of pancuronium were similar, despite pipecuronium having larger steady-state volume of distribution and greater plasma clearance than pancuronium.

CHANDONIUM AND ANALOGUES

Additional data have been collected on chandonium (77), discovered at the Panjab University (Chandigarh, India), and related agents. Saturation of the 5,6-double bond in chandonium and increase in the onium bulk diminishes the potency [494]. The analogues HS-627 (78) and HS-626 (79) possessing acetylcholine-like and choline-like moieties are equipotent with chandonium iodide as neuromuscular blocking agents in anaesthetized cat [495,496]. The X-ray diffraction studies showed the N^+-N^+ distance in chandonium (77) to be 1.029 nm [497] and in HS-626 (79) 1.033 nm [498].

Several other chandonium-related compounds have been synthesized [499–502], but none is better than chandonium.

The toxicity studies have been carried out at the Central Drug Research Institute (CDRI), Lucknow (India). The CDRI is engaged in clinical and



(77) R = Me
(78) R = CH_2CH_2OAc
(79) R = CH_2CH_2OH

developmental studies with the drug. The drug is at phase III of clinical testing. The results are encouraging. It is a well-tolerated, safe and effective neuromuscular blocking agent, having rapid onset and short duration of action in patients.

Spectrofluorimetric [503] and spectrophotometric [504] methods of analysing chandonium iodide have been developed. The injection of chandonium in normal saline withstands autoclaving [504]. There is no change in potency on the usual and accelerated shelf storage. Even exposure to light has no deterimental effect. *In vitro* protein binding studies using labelled chandonium has shown some binding of the drug to plasma proteins and red blood cells [505]. The studies carried out in rat [506] and monkey [507] have shown a rapid disappearance of the drug from the blood, low protein binding, and a limited distribution of the drug to selective tissues. The drug is mainly excreted unchanged in urine.

Some interesting inferences are evident from the molecular orbital calculations which have been carried out on structural models corresponding to chandonium iodide (77) and certain other azasteroidal neuromuscular blockers [508]. The conformations apparent from the calculations are similar to those evident on crystallographic analysis of azasteroids. Further, the calculations show a high degree of delocalization of positive charge.

ANTINEOPLASTIC HETEROSTEROIDS

The antineoplastic potential of mifepristone, danazol and antioestrogens, and the design of aromatase inhibitors as anticancer agents have been described earlier. A mention has been made in the introduction about cephalostatin 1 (6), a natural product, which is being evaluated against neoplastic and other biological systems [31].

Certain nucleoheterosteroids have been examined for their antineoplastic

activity. Some steroid lactams were found to be active [509–511]. 3β-Hydroxy-17a-aza-D-homo-5α-androstan-17-one, 17β-acetamido-3-aza-A-homo-4a-androstene-4-one, 3,17a-diaza-A,D-bishomo-4a-androstene-4,17-dione were active in Ehrlich ascites tumour and L1210 leukaemias. The analogue 3,11-diaza-1,3,5,7,9,13-gonahexaen-12-one showed slight activity against P-388 leukemia in mice [512] and 2,6-dithia[3,4]benzo-D-homo-3,5(10),8,14-estratetraen-17-one was active against fibrosarcoma in rats [513]. 7β-Methyl-2-thia-A-nor-5α-androstan-17β-yl acetate reduced the final breast tumour size in animals [89].

Later references are made to some nucleo-azasteroidal nitrogen mustards and nitrosoureas and nucleo-oxasteroidal lactones.

STEROIDAL NITROGEN MUSTARDS AND NITROSOUREAS

Estramustine phosphate sodium (Estracyst) (80) [514] is of oral use in the palliative treatment of patients with metastatic and/or progressive carcinoma of the prostate. The pharmacological, pharmacokinetic, clinical and other features of the drug have been reviewed [514]. It is a conjugate of oestradiol and the carbamate of nitrogen mustard. The mechanism of action of the drug is not fully understood. In humans, the drug is rapidly dephosphorylated, releasing estramustine, which is readily oxidized to estromustine (17-keto congener of estramustine) [515]. Thus, on hydrolysis both oestradiol and oestrone are released to exert their hormonal effect on the prostatic tumour [516]. However, estramustine itself does not compete with oestradiol for the oestrogen receptor site [517], indicating a lack of classical hormone effect. Estramustine does not induce DNA strand breaks either, indicating a lack of alkylating activity, in spite of the non-nitrogen mustard moiety of estramustine [518]. However, the observation that estramustine inhibits mitosis in prostate tumour cells in culture [519] implicates an involvement of microtubules in its mode of action. Estramustine inhibits microtubule-dependent pigment granule movement in squirrel fish erythrophores [520], causes a rapid disruption of cytoskeletal organization in the fish epithelial and human prostate tumour cells [521], and has inhibitory effect on the assembly of isolated bovine brain microtubules and on fast axonal transport in frog sciatic nerve [522]. It is suggested that estramustine phosphate may exert its cytotoxin effect by interaction with microtubules in the intact cell also.

A mention may be made of some other carcinostatic nitrogen mustard carbamates: 17-oxo-17a-oxa-D-homo-5α-androstan-3β-yl *N,N*-bis(2-chloroethyl)carbamate [523] and 3α-bis(2-chloroethyl)carbamoyloxy-3α-12α-dihydroxy-5β-cholan-24-oic acid [524]. 3β-Oleoyloxy-5-cholen-24-yl *N,N*-bis(2-chloroethyl)carbamate has been developed as a cytotoxic compound, capable

of reconstitution with low-density lipoprotein for delivery to the cancer cells [525].

Prednimustine (chlorambucil 21-ester of prednisolone) [1] is a representative of steroid esters prepared using the carboxylic acid, chlorambucil. In laboratory animals there are formed chlorambucil, dechlorinated chlorambucil and prednisolone on administration of prednimustine [526,527]. Formation of the chlorambucil ester of cholesterol has also been observed [526]; the occurrence of transesterification is rather interesting.

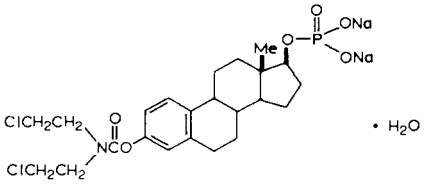
The other cytotoxic esters prepared with chlorambucil include KM-2210 (17β-ester of oestradiol benzoate) [528,529], the 17β-ester of 17β-hydroxy-3-aza-A-homo-4a-androsten-4-one [510,530,531] and the 17β-ester of 17β-hydroxy-4-aza-5-androsten-3-one [532].

Examples of studies on certain other esters carrying nitrogen mustard moiety in the carboxylic acid component are: 17a-aza-D-homo-17-oxo-5α-androstan-3β-yl *p*-bis(2-chloroethyl)aminophenoxyacetate [533,534] and its 17a-methyl analogue [535], 17a-aza-D-homo-17-oxo-5α-androstan-3-yl *p*-bis(2-chloroethyl)amino phenylacetate [536–538], 17a-aza-D-homo-17-oxo-5-androsten-3β-yl *p*-bis(2-chloroethyl)aminophenylacetate [539], 3-aza-A-homo-4-oxo-4a-androsten-17β-yl *p*-bis(2-chloroethyl)aminophenylacetate [540], 4-aza-3-oxo-5-androsten-17β-yl *p*-bis(2-chloroethyl)aminophenoxyacetate [541].

The nitrogen mustard moiety is directly linked to the steroid nucleus in 2/4-bis(2-chloroethyl)amino-3-hydroxy-1,3,5(10)-estratrien-17-ones [542] and they get irreversibly bound to the cytoplasmic oestrogen receptor of the rat uterus [542]. 6α-Bis(2-chloroethyl)amino-1,3,5(10)-estratriene-3,17β-diol has been prepared [543].

The design of *N*-(2-chloroethyl)nucleoazasteroids was an approach to have nitrogen mustard type derivatives, but out of the compounds prepared only 4-(2-chloroethyl)-4-aza-5α-androstan-17β-ol showed some activity against P388 leukaemia [544].

Preparation of steroidal nitrosoureas is another course which has been followed to generate potential anticancer agents. Two such analogues in the estrane series are the compounds (81) and (82) [545]. Both have free 3-OH. They inhibited the growth of the DMBA-induced transplantable rat mammary tumour. They were shown to compete with oestradiol for binding to cytosolic oestrogen receptor in rat uterus [546]. The binding possibly is irreversible. The compound (81) had higher RBA. An earlier study showed that (82) and (83) possessed a relatively high affinity for calf and lamb uterine oestradiol receptors [547]. The nitrosoureas in androstane and pregnane series displayed a very low relative affinity for progesterone receptors. 17β-(*N*-2-Chloroethyl-*N*-nitrosoureyl)-5-androsten-3β-ol is claimed to inhibit L-1210 leukaemia [548].



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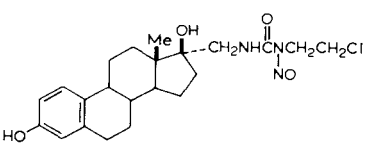

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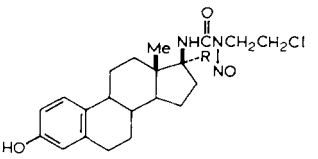


(82) R = H
(83) R = CN

N-Methyl-(2-chloroethyl)-*N*-nitrosocarbamoyl-3β-amino-5α-cholestanes [549] and 3-(*N*-2-chloroethylcarbamoyl)-3-aza-A-homo-5α-cholestane were cytotoxic to L-1210 [550].

3-Oxo-4-estren-17β-yl *N*-2-chloro-*N*-nitrosocarbamate (LS-1727), a nitrosocarbamate of 19-nortestosterone, did not affect the growth of the androgen-dependent R-3327 rat prostate adenocarcinoma [551].

OTHER HETEROSTEROIDAL SYSTEMS

Epitiostanol (2α,3α-epithio-5α-androstan-17β-ol) and its relation mepitiostane (2α,3α-epithio-5α-androstan-17β-yl 1-methoxycyclopentyl ether) have been of interest in the treatment of breast cancer because of their anti-oestrogenic activity [1]. The status of the drugs was reviewed [552]. A clinical study showed that as compared with fluoxymesterone mepitiostane seems to have less hepatotoxicity [553].

Several studies have centred around modifications in oestradiol and analogues. Some examples are mentioned under aromatase inhibitors and steroidal nitrogen mustards and nitrosoureas.

The conjugate 3-propionyloxy-1,3,5(10)-estratrien-17β-yl α-(5-fluorouracil-1-yl)acetate was claimed to have antitumour activity [554]. Oestradiol 3-carboranylmethyl ether as such has uterotrophic activity in rats and such carborane compounds may be expected to concentrate boron in the cell nuclei

of oestrogen-sensitive cancer cells and thus be useful for thermal neutron capture therapy of cancerous tumours [555]. 2-[2-(3,17β-Dihydroxy-1,3,5(10)-estratrien-17α-ylmethylcarbonylamino)ethyl]ellipticinium bromide, has a DNA intercalating property and low affinity for oestrogen receptor [556]. It inhibits L1210 cell growth; a transport by the oestrogen receptor system is not involved in the antitumour activity. Estyramine [17α-(3-amino-1-propyn-1-yl)oestradiol] and related compounds, prepared for antitumour testing, are weak oestrogens [237]. 3,17β-Dihydroxy-20,21-epoxy-19-nor-17α-pregna-1,3,5(10)-trienes were cytotoxic to mammalian cells in culture [557].

17β-Amino-1,3,5(10)-estratrien-3-ol and related derivatives have low relative binding affinity to oestrogen receptor in cytosol preparation from human breast tumour [558]. 4-Nitroestrone 3-O-methyl ether, a specific inhibitor of oestrogen sulphotransferase, is an active inhibitor of certain DMBA-induced rat mammary tumours [559]. Adenine and adenosine methylene-bridged 4-nitro-oestrones were more active than 4-nitrooestrone 3-O-methyl ether in inhibiting human breast cancer cell line [560]. 4-Nitro-1,3,5(10)-estratrien-17β-ol (3-deoxy compound), an effective inhibitor of porcine endometrial oestrogen sulphotransferase, has been claimed to inhibit the growth of murine mammary tumours [561–563]. Some aminooestradiol-platinum(II) complexes [564] and *N'*-(3-hydroxy-17-oxo-1,3,5(10)-estratrien-2- and 4-yl)thiourea derivatives have shown activity (weak) against MCF-7 breast cancer cell lines [565].

Steroids have been coupled to pyrimidine bases and some nucleosides to give potentially target-specific antitumour agents. X-ray diffraction studies have been carried on 17α-hydroxy-21-(thymin-1-yl)-4-pregnene-3,20-dione and 21-(uracil-1-yl)-4-pregnene-3,20-dione and molecular conformations correlated with differences in receptor affinity [566]. 3α-(Uracil-1-yl)-5-pregnen-20-one [567], some androstane derivatives of 5-fluorouracil [568], and 5'-(prednisolone or prednisone-21-phosphoryl)-1-(β-D-arabinofuranosyl)cytosines [569] have been reported to be active against L-1210 lymphoid leukaemia in mice. Certain 9-(β-D-arabinosyl)adenine conjugates of corticosteroids are potent in inhibiting *in vitro* growth of L-1210 lymphoid leukaemia cells [570].

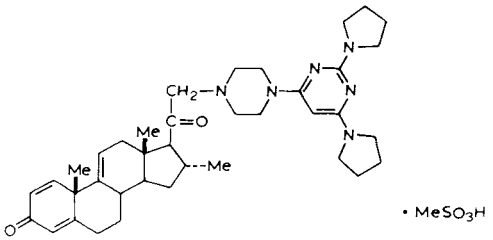
Different α-methylenelactones prepared and tested *in vitro* for cytotoxicity include androstane-3/17-spiro-α-methylene-γ-lactones (active against mouse Ehrlich ascites tumour cells) [571,572], 3β-hydroxy-4'-methylene-16α,17α-androst-5-eno[17,16-*b*]tetrahydrofuran-5'-one (active against HeLa cells) [573], 17β-hydroxy-2-methylene-4-oxa-5α-androstan-3-one and 3β-hydroxy-16-methylene-17a-oxa-D-homo-5α-androstan-17-one (active against human nasopharyngeal carcinoma cells) [574], and 3-hydroxy-16-methylene-17a-oxa-D-homo-1,3,5-estratrien-17-one and derivatives (highly toxic towards HeLa S_3 cells) [575].

SOME MISCELLANEOUS ASPECTS

Certain assorted studies carried out using heterosteroidal systems are covered here, starting with U-74006F, which is currently under development for the treatment of human central nervous system trauma and ischaemia.

U-74006F

At the Upjohn Company (U.S.A.) there were prepared a series of 21-amino steroids [576] which contained many potent inhibitors of iron-dependent lipid peroxidation, out of which U-74006F (84) and U-74500A (85) are the prominent representatives [577]. They inhibited lipid peroxidation in brain homogenates and purified brain synaptosomes under a variety of conditions involving iron. Their response to increasing concentration of Fe^{2+} in lipid peroxidation assays differed qualitatively. U-74006F is being developed for clinical trials in the treatment of head and spinal cord injuries and stroke. This antioxidant is devoid of both glucocorticoid and mineralocorticoid activity in mice or rats and yet blocks the release of arachidonic acid from mouse pituitary tumour cells [578]. It is an effective inhibitor of arachidonic acid-induced vasogenic brain oedema in rats [579]. In cats it attenuated post-haemorrhagic cardiovascular collapse [580], prevented development of post-traumatic spinal white matter ischaemia [581], antagonized post-ischaemic cerebral hypoperfusion [582], and showed a remarkable capacity to promote functional recovery in spinal cord injured cats [583]. It was more potent than methylprednisolone sodium succinate and appeared to be without any side-effects. It has also proved to be potent and effective in promoting early neurological recovery of mice after either moderate or severe concussive head injury [584]. A pharmacokinetic and excretion study using labelled U-74006F shows that it is efficiently cleared by rat liver, metabolised and excreted in bile [585].



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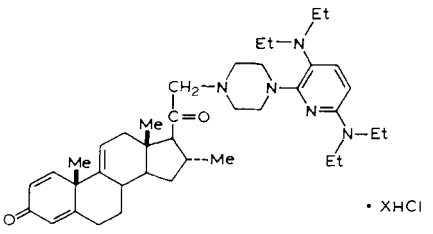

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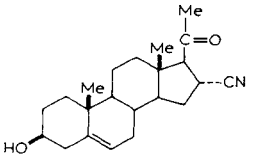
PREGNENOLONE-16α-CARBONITRILE

The catatoxic activity of pregnenolone-16α-carbonitrile (PCN, 86), spironolactone (17), and oxandrolone (17β-hydroxy-17α-methyl-2-oxa-5α-androstan-3-one) was described earlier [1]. Interest in PCN continues. PCN has enzyme-inducing activity. A study in rats showed that PCN induces a form of UDP-glucuronosyl transferase that preferentially conjugates bilirubin and digitoxigenin monodigitoxoside [586,587]. There may be species differences in effects. Unlike the effect in the rat, PCN failed to increase clearance of bilirubin in man [588]. PCN augmented urinary excretion of imipramine- and desipramine-glucuronides in rats but the biliary excretion was not significantly altered [589]. Pretreatment of rats with PCN potentiated the paracetamol-induced depletion of hepatic glutathione [590]. The inhibitory action of ethionine and β-diethylaminoethyldiphenylpropyl acetate on RNA and protein synthesis in rat liver has been shown to be reversed by PCN [591]. PCN acts on the DNA liver cells either directly or via cyclic AMP.



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ASSAY MATERIALS AS RADIOLIGANDS

16α-[^{125}I]Iodooestradiol has been synthesized and shown to have substantial uterotrophic activity and affinity for oestradiol receptors of the rat [592] and bovine [593] uterus. In the human breast cancer cells in culture, it translocates receptor sites and induces a full range of oestrogenic effects [594]. Oestradiol receptors can be measured in human mammary carcinomas using 16α-[^{125}I]iodooestradiol [595]. X-ray study of 16α-iodooestradiol showed that the bond distances and angles of the iodinated oestrogens are similar to those of oestradiol and oestriol [596].

17α-[1(*E*)-Iodovinyl] analogues of oestradiol have also been prepared and they, too, bind to the oestrogen receptor and concentrate in oestrogen target tissues [597,598]. Work on potential radioligands for progesterone receptor has identified 17β-hydroxy-16α-iodo-4-estren-3-one, 17β-hydroxy-17α-[1(*E*)-iodovinyl]-4-estren-3-one and 17β-hydroxy-17α-[1-(*Z*)-iodovinyl]-4-estren-3-

one as excellent competitors, each having a K_i less than or equal to that of progesterone [599]. Synthesis of 17β-hydroxy-17α-[2(*E*)-[^{125}I]iodovinyl]-4-estren-3-one has been carried out; the compound is stable and binds with high affinity to the progesterone receptor [600].

Selenium labelling has also been considered. Out of the D-ring substituted oestrogens prepared, 17α-(methylseleno)ethynyl-17β-oestradiol appeared promising [601]. It possesses 19% of the binding affinity of natural 17β-oestradiol. 21-Phenylselenoprogesterone competed effectively for binding to the progestin receptor [602].

One of the prominent techniques practised today for the determination of biologically active molecules and their metabolites in physiological fluids is radioimmunoassay, which makes use of radioactive isotopes in combination with immunochemical methods. This assay technique is extensively employed in the steroid field. Some heterosteroids are of use as haptens which when covalently bonded to a macromolecule carrier act as antigens. A few examples of haptens may be cited at random: 4-(2-carboxyethylthio)-11-deoxy-corticosterone 21-sulphate [603]; 4-(2-carboxyethylthio)-11α-hydroxy-4-pregnene-3,20-dione, 6β-(2-carboxyethylthio)-11α-hydroxy-4-pregnene-3,20)-dione [604]; 6-(*O*-carboxymethyloximino)-1,3,5(10)-estratriene-2,3,16α-17β-tetraol [605]; 3-hydroxy-1,3,5(10),7-estratetraen-17-one 3-*O*-carboxymethyl ether [606]; and *N*-(2-aminoethyl)-3α-7α-,12α-trihydroxy-5β-cholan-24-amide[607]. amide [607].

Metalloimmunoassay is a non-isotopic approach with potential applications in immunochemical studies. Ferrocenyl-containing derivatives of oestrone, oestradiol and oestriol were prepared and used for the development of procedure for metalloimmunoassay [608]. For example, 17β-hydroxy-1,3,5(10)-estratrien-3-yl ferrocenylmethylcarbamoylmethyl ether was one of the metallo-haptens synthesized.

Photoaffinity labelling is the other recourse to identify biological receptor sites; 3,4-Dimethoxy-1-nitro-1,3,5(10)-estratrien-17-one and 4-bromo-3-methoxy-2-nitro-1,3,5(10)-estratrien-17-one may be useful as possible photoaffinity labels of zero length [609]. 11α-Diazoacetoxyprogesterone, a photoaffinity analogue of progesterone, has been prepared [610].

EFFECTS ON IMMUNE RESPONSE

Deflazacort (9) and 21-deacetyldeflazacort are immunosuppressive agents *in vitro* and, on a molar basis, equally as potent as prednisolone [44]. Earlier effectiveness of deflazacort as an immunosuppressant was seen in normal volunteers [42].

Different 3-aminopregnane derivatives have been claimed to possess immunostimulant properties [611–615]. (20*S*)-20-Hydroxy-5α-pregnan-3α-ylalanine at 0.5 mg/day increased survival time by 50% in mice sensitized with bovine serum albumin [611].

ANTIMICROBIAL ACTIVITY

In the introduction to this chapter a mention was made of antifungal antibiotics A25822B (3), A25822 factor A (4), viridin, demethoxyviridin (5) and wortmannin. The toxicity of A25822B (3) to *Ustilago maydis* sporidia is due to inhibition of ergosterol biosynthesis [616]. Solacongestidine and some other solanum alkaloids have shown *in vitro* antifungal activity [617]. Solacongestidine also prolonged the survival time of mice infected with *Candida albicans*. Some steroidal saponins from *Allium ampeloprasum* L. have shown weak antifungal properties [618].

24-Amino-5β-cholane-3α,7α-diol and related amines have shown antibacterial activity against Gram-positive bacilli [619]. 2-(3α,6α-Dihydroxy-24-nor-5β-cholan-23-yl)-4,4-dimethyl-2-oxazoline is a potent inhibitor of deoxycholic acid 7-dehydroxylase in bacterial cultures [620]. 2′-Amino-5-androsteno-[17,16-*d*]thiazol-3β-yl acetate showed a moderate antifungal and antibacterial activity [621].

Out of the several bufadienolides and some cardenolides tested, scillarenin and 3-*O*-[*N*-t-butoxycarbonyl)hydrazido]succinylbufalin were found to show therapeutic indices of 32 and 16, respectively, against rhinoviruses *in vitro* [622].

Chonemorphine has been identified as the antiamoebic and antitrichomonad principle of *Chonemorpha fragrans* [623]. The *in vitro* and *in vivo* properties of chonemorphine against *Entamoeba histolytica* [624,625] and *Trichomonas vaginalis* [624] have been examined.

DRUG-DELIVERY APPROACHES

Hetero entities have been introduced to create prodrug characteristics in certain steroids. 3-Spirothiazolidines of hydrocortisone and its derivatives [626] and thiazolidine derivatives of progesterone and testosterone [627] were prepared. The thiazolidines readily reverted to their parent steroidal ketones, thus meeting the requirements for a prodrug. The other prodrug approach is represented by the preparation of water-soluble progesterone 3-hydrazones using betaine hydrazide chloride and pyridinium acetohydrazide chloride [628]. Involving 17β-

OH of oestradiol and ethynyloestradiol, 1-methyl-1,4-dihydronicotinoyl esters have been prepared as brain-targeted agents [241].

5-Cholesten-3β-yl 5-carboxypentyl ether succinimido ester and 5-cholesten-3β-yl 6-carboxyhexyl ether succinimido ester were promising among the functionalized cholesteryl derivatives prepared for evaluation of antibody binding to modified phospholipid vesicles [629].

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6 Development of Receptor-Specific Opioid Peptide Analogues

PETER W. SCHILLER, Ph.D.

Laboratory of Chemical Biology and Peptide Research, Clinical Research Institute of Montreal, 110 Pine Avenue West, Montreal, Que., Canada, H2W 1R7

INTRODUCTION

Since the discovery of the enkephalins by Kosterlitz and his collaborators [1], more than twenty endogenous opioid peptides have been detected in mammals. All these opioid peptides are derived from one or the other of three distinct precursor molecules: pro-opiomelanocortin (POMC), the common precursor for β-endorphin, β-endorphin fragments, ACTH and other α-MSH-containing peptides; proenkephalin A, the precursor for met- and leu-enkephalin and several larger peptides, all containing the met-enkephalin sequence at their N-terminus (such as peptide E, peptide F, metorphamide); and proenkephalin B, processing of which generates again leu-enkephalin and several larger opioid

peptides, including the dynorphins, neo-endorphins and leu-enkephalin (for a review, see [2]). Aside from these opioid peptides from mammalian sources, peptides with opioid activity have also been isolated from frog skin (dermorphins [3] and deltorphins [4]) and from tryptic digests of casein (β-casomorphins, morphiceptin [5,6]).

Another major development in the opioid field during the past 15 years was the demonstration of opioid receptor heterogeneity [7,8]. The fact that at least three different classes of opioid receptors (μ, δ, κ) exist is now generally accepted. There is increasing evidence for the existence of receptor subtypes in the case of the μ receptor (μ_1, μ_2) [9], δ receptor [10] and κ receptor (κ_{1a}, κ_{1b}, κ_2, κ_3) [11,12]; however, the concept of opioid receptor subtypes has not yet found general acceptance. Opiates and opioid peptides produce a large spectrum of central and peripheral effects which, aside from spinal and supraspinal analgesia, include tolerance and physical dependence, respiratory depression, euphoria, dysphoria and hallucinations, sedation, appetite suppression and other behavioural effects, effects on gastrointestinal motility, control of release of several peptide hormones and neurotransmitters, hyperthermia and/or hypothermia, cardiovascular effects, effects on tumour growth, effects on the immune response, etc. (for reviews, see [13,14]). It has not yet been possible to establish clear-cut relationships linking specific opioid receptor types to distinct opioid effects. To obtain such correlations, further efforts will have to be made to develop opioid agonists and antagonists with high specificity for the various receptor types and subtypes. The lack of receptor specificity of such classical opiates as morphine is well documented and, unfortunately, none of the opioid peptides generated through processing of the three mammalian precursors shows high receptor selectivity, either (see [2]). For example, the enkephalins do bind preferentially to δ receptors, but also have some affinity for μ receptors and metorphamide displays only moderate preference for μ receptors over δ receptors. The dynorphins show some selectivity for κ receptors, but also interact with μ and δ receptors, albeit with lower affinity. β-Endorphin has high affinity for both μ and δ receptors and may also interact with a specific receptor of its own, the ε receptor [15]. The dermorphins display marked but not yet optimal μ-selectivity, and the various peptides of the β-casomorphin family are either only moderately μ-selective and/or have relatively low affinity (for example, morphiceptin). The only naturally occurring opioid peptides with high receptor specificity are the δ-selective deltorphins [4].

The development of receptor-specific opioids is of great importance, not only for the elucidation of the physiological rôle(s) of the various opioid receptor types but also for the development of potential drugs with minimal side-effects. Considerable progress has been made in recent years in the development of

receptor-selective non-peptide opioid agonists and antagonists and some of the most selective ones will be mentioned here briefly. The fentanyl derivatives sufentanil [16] and ohmefentanyl [17] are highly μ-selective agonists. Among the non-peptide κ agonists, ethylketazocine, bremazocine and tifluoadom are only moderately selective, whereas more recently developed compounds like U50,488 [18], U69,593 [19], ICI 197067 [20], PD 117302 [21] and (−)-(5β, 7β, 8α)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]benzo[*b*]furan-4-acetamide [22] show much improved κ selectivity. Naltrindole [23] and binaltorphimine [24] are antagonists with excellent selectivity for the δ and the κ receptor, respectively.

In the opioid peptide field most of the analogues prepared to date are structurally derived from the enkephalins; however, interesting analogues of the β-casomorphins, dermorphins, dynorphins and β-endorphin have also been developed. Since the natural enkephalins (H-Tyr-Gly-Gly-Phe-Met(or Leu)-OH) are rapidly degraded by various peptidases, initial efforts were aimed at making the molecule more enzyme-resistant and this goal was quickly achieved through substitution of D-alanine in the 2-position of the peptide sequence and through amidation of the C-terminal carboxyl group [25]. Another important development in the late seventies was the demonstration that appropriate structural modifications of the enkephalin molecule can lead to analogues, such as the Sandoz compound FK 33–824 (H-Tyr-D-Ala-Gly-MePhe-Met(O)-ol) (see p. 331) [26], that are able to produce a long-lasting analgesic effect after systemic administration. During the past decade, most synthetic efforts have been aimed at developing receptor-specific opioid peptide analogues and progress made in this area will be the major topic of this review. The focus will be on the most selective and the most frequently used opioid peptide analogues and no attempt will be made to provide a comprehensive review of the more than 1000 analogues of the enkephalins, β-casomorphins, dermorphins, deltorphins, dynorphins and β-endorphin that have been synthesized to date. For more comprehensive information on structure–activity relationships in the opioid peptide field, the reader is referred to several published review articles which contain extensive lists of analogues [27–31].

Various approaches have been taken to develop receptor-selective opioid peptide analogues and the following design principles will be discussed in some detail in this chapter:

(a) Substitution, deletion or addition of natural or artificial (non-proteinogenic) amino acids
(b) Design of bivalent ligands containing two opioid peptide moieties separated by a spacer of appropriate length and interacting simultaneously with two receptor-binding sites

(c) The concept of conformational restriction as achieved primarily through appropriate peptide cyclizations
(d) Peptide bond replacements.

Approaches b, c and d represent relatively new concepts in peptide analogue design that have been used most extensively in the opioid peptide field, but in recent years have emerged as generally applicable design strategies for the development of peptide analogues. Furthermore, a number of irreversible opioid receptor ligands have been prepared through incorporation of chemical (for example, electrophilic) affinity labels or photoaffinity labels into opioid peptides and the receptor selectivities of these derivatives will be discussed here as well. Finally, the receptor selectivity profiles of several opioid peptide analogues that have undergone clinical trials or are being considered as potential future drugs are discussed.

DEVELOPMENT OF PEPTIDE ANALOGUES WITH SELECTIVITY FOR THE THREE MAJOR OPIOID RECEPTOR CLASSES

DETERMINATION OF RECEPTOR SELECTIVITY

In principle, opioid receptor ligands can be characterized either in terms of receptor-binding selectivity or in terms of pharmacological selectivity [32]. The binding selectivity depends only on the relative affinities for the different receptor types, whereas the pharmacological selectivity is determined by three parameters – receptor-binding affinity, intrinsic activity (efficacy) and receptor reserve – which all may vary among different receptor types. Pharmacological selectivities of opioids can be determined with bioassays based on inhibition of electrically evoked contractions of the guinea-pig ileum (GPI), mouse vas deferens (MVD), hamster vas deferens (HVD), rabbit vas deferens (LVD) and rat vas deferens (RVD). Whereas the HVD and LVD contain homogeneous populations of δ and κ receptors, respectively, the opioid receptor populations in the other three tissues are heterogeneous. In the GPI, μ and κ receptors exist and the MVD contains both μ and κ receptors in addition to the predominant δ receptors. In the RVD, μ receptors and, possibly, ε receptors mediate opioid effects. Because of the existing receptor heterogeneity, the ratios of the IC_{50} values determined in the most frequently used GPI and MVD assays are not precise indicators of μ versus δ selectivity. In particular, highly μ-selective ligands will show relatively low $IC_{50}(MVD)/IC_{50}(GPI)$ ratios because they interact not only with the μ receptors present in the ileum, but also with the μ receptors present in the vas. This is, for example, the case with morphiceptin,

which shows an IC_{50}(MVD)/IC_{50}(GPI) ratio of only about 3, in contrast to the much higher δ/μ selectivity ratio ($\sim$ 500) obtained in the receptor-binding assays.

Receptor binding selectivities have been determined by displacement of relatively selective radioligands from receptor sites in membrane suspensions prepared mostly from either rat or guinea-pig brain. These brain tissues also contain heterogeneous populations of opioid receptors. In rat brain, 46% of the opioid receptors are of the μ type, 42% of the δ type and 12% are κ, whereas in guinea-pig brain the proportions are somewhat different: 24% μ 32% δ and 44% κ [33]. It is of great importance to use the most selective radioligands available to obtain the most accurate binding selectivity ratios possible. Whereas early studies had to rely on radioligands with quite limited selectivity, much more selective radiolabels have become available in recent years. Perhaps the most precise selectivity ratios can be obtained by using brain membranes which are enriched in one receptor type and which can be prepared by using a procedure based on receptor alkylation with β chlornaltrexamine in the presence of an appropriate protecting ligand [34]. This method has been used to determine the binding selectivity profiles of about 40 opioid ligands [32,35]. In binding competition experiments, the IC_{50} for displacing the radioligand is often taken as affinity measure for the ligand being tested; however, more correctly, the apparent dissociation constant (K_i) should be calculated from the determined IC_{50} value and from the dissociation constant and concentration of the radioligand, according to the equation of Cheng and Prusoff [36]. Accordingly, selectivity ratios are expressed either as IC_{50} ratios or as K_i ratios.

In the present review, we will use binding selectivities rather than pharmacological selectivities for comparison of the various opioid peptide analogues. Even though binding data depend on the radioligands and on the particular assay procedures being used, they provide a more accurate picture of the selectivity of a ligand with regard to its initial interaction with a particular receptor type than is the case with bioassay data. The latter data are more difficult to interpret because of the receptor heterogeneity in the bioassay preparations, particularly the GPI and MVD, and because of variation in receptor reserve and perhaps also in efficacy among these preparations.

For the most selective opioid peptide analogues receptor-binding data exist and these are listed in the Tables of this review. Important analogues for which no receptor-binding data have been reported but which appear to be selective on the basis of bioassay data are mentioned in the text. Agonist potencies obtained in the GPI and MVD assay have been reported for nearly all the peptide analogues listed in *Tables 6.1, 6.2, 6.3* and *6.5*. For most of the antagonists listed in *Table 6.4*, K_e values were determined with various selective agonists in the *in vitro* bioassays.

μ-SELECTIVE AGONISTS

A number of linear analogues of enkephalin, β-casomorphin and dermorphin with selectivity for μ opioid receptors were obtained through substitution of various natural or artificial (non-proteinogenic) amino acids, amino acid deletions, end-group modification or end-group deletion. Opioid peptide dimers and cyclic opioid peptide analogues with high μ receptor preference have also been developed. Receptor binding data of the most important opioid peptide analogues with agonist properties that have resulted from these various efforts are listed in *Table 6.1*, which also shows the chemical structures of the peptides.

Linear enkephalin-related analogues

A common feature of most enkephalin analogues is a D-amino acid substituted in the 2-position of the peptide sequence which prevents attack by aminopeptidases. One of the most popular μ-selective enkephalin analogues is the pentapeptide H-Tyr-D-Ala-Gly-MePhe-Gly-ol (DAGO) (1) [37](see *Table 6.1*), which shows high preference for μ receptors over δ receptors and which, in tritiated form, is often used as a μ-selective radioligand. The Lilly compound NMe-Tyr-D-Ala-Gly-NEtCH(CH_2Ph)CH_2NMe_2 (LY164929) (2) [40] and syndyphalin, H-Tyr-D-Met(O)-Gly-MePhe-ol (3) [41,42], are two enkephalin-related tetrapeptides analogue with very high μ receptor selectivity. Both these peptides lack the 5-position residue present in the natural enkephalin sequence and contain an *N*-alkylated Phe^4 residue as a modified C-terminal group. Roques and collaborators made the interesting observation that omission of the 5-position residue in enkephalin and replacement of the aromatic side-chain in position 4 with an aliphatic one resulted in compounds with μ receptor preference [39]. Among several prepared tripeptide derivatives of this type, the analogue H-Tyr-D-Ala-Gly-$NHCH_2CH_2CHMe_2$ (TRIMU 5) (4) turned out to be the most selective one. In a direct comparison under identical binding assay conditions, TRIMU 5 showed a selectivity ratio (K_i^δ/K_i^μ = 109) comparable with that of DAGO (K_i^δ/K_i^μ = 179) but slightly lower μ receptor affinity [39]. The pentapeptide analogue H-Tyr-D-Met-Gly-Phe-Pro-NH_2 (5) [43] has often been referred to as a μ-selective ligand; however, determination of its K_i^δ/K_i^μ ratio in the receptor-binding assays indicated only moderate μ receptor preference [44]. Addition of a guanidino group at the N-terminus of the latter analogue resulted in a compound for which no receptor-binding data have been reported, but which on the basis of IC_{50} ratios determined in the GPI and MVD assays appears to be somewhat more μ-selective than the parent peptide [43]. The low K_i^δ/K_i^μ ratio of 3.4 observed with the Sandoz compound FK 33–824 (6) [26]

indicates that this analogue has only slight preference for μ receptors over δ receptors. Another well known enkephalin analogue, the Lilly compound H-Tyr-D-Ala-Gly-Phe-MeMet-NH_2 (LY 127623, metkephamid) (7) [40,50] is essentially non-selective. Analogues (5), (6) and (7) have undergone clinical trials as will be discussed below. Another enkephalin analogue of interest for potential drug development is the Hoechst compound Hoe-825, H-Tyr-D-Lys-Gly-Phe-Hctl (Hctl = homocysteine-thiolactone) (8) [46], which is quite μ-selective (P.W. Schiller et al., unpublished data).

Aside from the compounds described above, other linear enkephalin analogues with more or less pronounced preference for μ receptors have been described and it is difficult to define general rules with regard to the structural requirements for μ-selectivity. However, some of the structural modifications which seem to result in μ-selective linear enkephalin analogues can be summarized as follows:

(i) D-amino acid with relatively lipophilic side-chain in position 2 of the peptide sequence
(ii) *N*-alkylation at position 4
(iii) methylation of the N-terminal amino group
(iv) amidation, reduction to alcohol or other modifications of the C-terminal carboxyl group
(v) truncation at the C-terminus

Linear β-casomorphin-related analogues

In contrast to the δ-selective enkephalins, the heptapeptide β-casomorphin (H-Tyr-Pro-Phe-Pro-Gly-Pro-Ile-OH) (9) isolated from an enzymatic casein digest [5] shows moderate μ receptor preference (K_i^{δ}/K_i^{μ} = 8.33, *Table 6.1*). Structure-activity studies with β-casomorphin analogues revealed that, unlike in the case of enkephalin, L-configuration of the 2-position Pro residue is required for opioid activity [6]. Deletion of the C-terminal tripeptide in (9) resulted in a tetrapeptide, named morphiceptin, H-Tyr-Pro-Phe-Pro-NH_2 (10) [6], which displays considerably higher μ receptor selectivity than the parent heptapeptide. Substitution of N^{α}-methylphenylalanine (MePhe) in position 3 and inversion of the configuration at the Pro^4 residue of morphiceptin led to a compound, known as PL017 (H-Tyr-Pro-MePhe-D-Pro-NH_2 (11)) [47], with further improved μ selectivity. In a direct comparison with DAGO under identical binding assay conditions, PL017 showed about half the affinity for μ receptors but somewhat higher preference for μ receptors over δ receptors [47,49] and, thus, is one of the most selective μ agonists known to date.

Linear dermorphin-related analogues

In contrast to all mammalian opioid peptides, dermorphin (H-Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2) (12) [3] already contains a D-alanine residue in the 2-position of the peptide sequence as well as a C-terminal carboxamide group and, therefore, is relatively more stable against enzymatic degradation. The natural heptapeptide (12) shows quite high μ selectivity (*Table 6.1*) but is by no means the most selective μ ligand known, as it has been claimed in the literature [52]. The N-terminal tetrapeptide amide of dermorphin (13) is about as μ-selective as the parent heptapeptide. Two tetrapeptide analogues derived from (13), H-Tyr-D-Ala-Phe-D-Pro-NH_2 [57] and H_2N-C(NH)-Tyr-D-Ala-Phe-Gly-NH_2-adamantyl [58] are potent analgesics and appear to have high preference for μ over δ receptors, as judged by their IC_{50}(MVD)/IC_{50}(GPI) ratio; however, no binding data were reported for these two compounds. Substitution of D-methioninesulphoxide for D-Ala^2 and replacement of the free C-terminal carboxyl group with a methyl ester function in H-Tyr-D-Ala-Phe-Gly-NH_2 and its D-Ala^4 analogue resulted in compounds (14,15) that were 7-times more μ-selective than the parent tetrapeptide [51]. These two analogues are nearly as μ-selective as DAGO, but somewhat less potent. Substitution of a D-arginine residue in position 2 of N-terminal dermorphin tetrapeptide analogues resulted in a series of compounds with high analgesic potency [53]. One of these analogues, H-Tyr-D-Arg-Phe-Gly-OH (16), was recently shown to be quite μ-selective (K_i^δ/K_i^μ = 285) (P.W. Schiller, et al., unpublished results).

In a recent study it was shown that, within a series of dermorphin-(1–4) tetrapeptide analogues, gradual augmentation of the net positive charge of the peptide resulted in increasingly higher μ receptor selectivity [54]. This trend is illustrated with the analogues H-Tyr-D-Nva-Phe-Nle-NH_2 (17), H-Tyr-D-Arg-Phe-Nle-NH_2 (18) and H-Tyr-D-Arg-Phe-Lys-NH_2 (19), which carry a net positive charge of 1+, 2+ and 3+, respectively. These three analogues all show similarly high μ receptor affinity, but progressively lower δ affinity with increasing charge and, therefore, progressively higher μ selectivity. The observed decrease in δ receptor affinity with increasing positive charge is in agreement with the recent proposal that δ receptors are located in a cationic environment within the membrane from which positively charged ligands would be excluded due to an electrostatic repulsive effect [59]. Alternatively, direct electrostatic repulsion between a positive charge at or near the δ receptor-binding site itself and the positively charged peptide may be the reason for the δ affinity drop. The most selective compound was [D-Arg^2, Lys^4]dermorphin-(1–4)-amide (DALDA) (19), showing a selectivity ratio (K_i^δ/K_i^μ = 11,400) more than 10-times higher than that of DAGO (K_i^δ/K_i^μ = 1,050) and 8-times higher

Table 6.1. RECEPTOR AFFINITIES AND SELECTIVITY RATIOS OF μ-SELECTIVE OPIOID PEPTIDE ANALOGUES (AGONISTS)

Receptor affinities were obtained from binding experiments based on displacement of relatively μ- and δ-selective radioligands from rat or guinea pig brain membrane preparations or from NG108-15 cells. The various radioligands used are indicated in the footnotes. IC_{50} values were determined from log dose-displacement curves and in some cases were taken directly as measure for affinity. In most cases binding inhibition constants (K_i values) were calculated from the IC_{50} values by means of the equation of Cheng and Prusoff [36]. Receptor selectivity ratios are indicated either as $IC^{\delta}_{50}/IC^{\mu}_{50}$ ratios or as K_i^{δ}/K_i^{μ} ratios.

Compound	*μ affinity*		*δ affinity*		*Selectivity ratio* $IC^{\delta}_{50}/IC^{\mu}_{50}$ *or* K_i^{δ}/K_i^{μ}	*Reference*
	IC^{μ}_{50} *[nM],*	K_i^{μ} *[nM]*	IC^{δ}_{50} *[nM],*	K_i^{δ} *[nM]*		
Linear enkephalin-related analogues						
1 H-Tyr-D-Ala-Gly-MePhe-Gly-ol		1.29[a]		120[b]	93	[37]
(DAGO)		1.22[a]		1280[h]	1050	[38]
		3.9[a]		700[h]	179	[39]
2 NMe-Tyr-D-Ala-Gly-NEt-$CH(CH_2Phe)CH_2NMe_2$ (LY164929)	0.6[d]		900[g]		1500	[40]
3 H-Tyr-D-Met(O)-Gly-MePhe-ol (Syndyphalin)	0.29[b]		1250[g]		4310	[41, 42]
4 H-Tyr-DAla-Gly-$NHCH_2CH_2CHMe_2$ (TRIMU 5)		10.0[a]		1020[h]	102	[39]
5 H-Tyr-D-Met-Gly-Phe-Pro-NH_2	1.53[c]		9.92[i]		6.48	[43, 44]
6 H-Tyr-D-Ala-Gly-MePhe-Met(O)-ol (FK33-824)		5.0[c]		17.2[i]	3.4	[26, 45]
7 H-Tyr-D-Ala-Gly-Phe-MeMet-NH_2 (Metkephamid, LY127623)	2.5[d]		4.4[g]		1.76	[40]
8 H-Tyr-D-Lys-Gly-Phe-Hctl (Hoe 825)		0.775[a]		29.2[h]	37.7	[46], Schiller et al., unpublished results
Linear β-casomorphin-related analogues						
9 H-Tyr-Pro-Phe-Pro-Gly-Pro-Ile-OH (β-casomorphin)	1800[e]		15000[j]		8.33	[5, 6]
10 H-Tyr-Pro-Phe-Pro-NH_2	19[e]		30000[j]		1580	[6]
(morphiceptin)	63[e]		30000[j]		476	[47]
	37[a]		46000[g]		1240	[48]

(continued)

Table 6.1 (continued)

Compound	μ affinity		δ affinity		Selectivity ratio	
	IC^{μ}_{50} [nM],	K_i^{μ} [nM]	IC^{δ}_{50} [nM],	K_i^{δ} [nM]	$IC^{\delta}_{50}/IC^{\mu}_{50}$ or K_i^{δ}/K_i^{μ}	Reference
11 H-Tyr-Pro-MePhe-D-Pro-NH_2 (PL017)	5.5[e]		10000[j]		1820	[47]
		2.89[a]		4250[h]	1470	[49]
Linear dermorphin-related analogues						
12 H-Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2	5.7[a]		210[g]		36.8	[3, 51]
(dermorphin)		1.05[f]		359[k]	342	[52]
		0.839[a]		160[h]	191	Schiller et al., unpublished results
13 H-Tyr-D-Ala-Phe-Gly-NH_2	36.7[a]		818[g]		22.3	[51]
		2.63[f]		1610[k]	612	[52]
14 H-Tyr-D-Met(O)-Phe-Gly-OMe	5.0[a]		800[g]		160	[51]
15 H-Tyr-D-Met(O)-Phe-D-Ala-OMe	9.0[a]		1400[g]		156	[51]
16 H-Tyr-D-Arg-Phe-Gly-OH		1.70[a]		484[h]	285	[53], Schiller, et al. unpublished results
17 H-Tyr-D-Nva-Phe-Nle-NH_2		3.53[a]		181[h]	51.3	[54]
18 H-Tyr-D-Arg-Phe-Nle-NH_2		2.91[a]		1510[h]	519	Schiller et al., unpublished results
19 H-Tyr-D-Arg-Phe-Lys-NH_2 (DALDA)		1.69[a]		19200[h]	11400	[54]
20 H-Tyr-D-Arg-Phe-NH_2		5.1[a]		455[g]	89	[55]
21 H-Lys-Tyr-D-Arg-Phe-NH_2		17.4[a]		1175[g]	68	[55]
22 H-Lys-Tyr-D-Arg-Phe-Lys-NH_2		12.3[a]		146000[h]	11900	Schiller et al., unpublished results
Dermorphin-enkephalin hybrid analogues						
23 H-Tyr-D-Ala-Phe-Phe-NH_2		1.53[a]		626[h]	409	[56]
24 H-Tyr-D-Ala-Trp-Trp-NH_2		0.833[a]		652[h]	783	[56]
25 H-Tyr-D-Ala-1-Nap-1-Nap-NH_2		2.88[a]		1180[h]	410	[56]
Opioid peptide dimers						
26 (H-Tyr-D-Ala-Gly-NH-CH_2)$_2$ (DTRE)$_2$		0.034[a]		14[g]	412	[48]
27 H-Tyr-D-Ala-Gly-NH_2 (TRE)		11[a]		112[g]	10.2	[48]
28 (H-Tyr-D-Arg-Phe-Sar-NH)$_2$	1.98[a]		370[g]		187	[65]

Cyclic analogues				
29 H-Tyr-cyclo[-D-A_2bu-Gly-Phe-Leu-]	13.8[d]	115[g]	8.33	[68]
30 H-Tyr-D-Abu-Gly-Phe-Leu-NH_2	5.61[d]	4.06[g]	0.72	[68]
31 H-Tyr-cyclo[-D-Orn-Gly-Phe-Leu-]	31.4[d]	100[g]	3.81	[69]
32 H-Tyr-cyclo[-D-Lys-Gly-Phe-Leu-]	5.58[a]	76.4[h]	13.7	[69, 72]
33 H-Tyr-cyclo[-D-Glu-Gly-gPhe-D-Leu-]	11.0[d]	389[g]	35.4	[71]
34 H-Tyr-cyclo[-D-Lys-Glyψ[CSNH]Phe-Leu-]	4.55[a]	654[h]	144	[72]
35 H-Tyr-D-Orn-Phe-Asp-NH_2	10.4[a]	2200[h]	212	[38]
36 H-Tyr-D-Asp-Phe-Orn-NH_2	9.55[a]	1320[h]	138	[73]
37 H-Tyr-D-Asp-Phe-A_2bu-NH_2	24.8[a]	4170[h]	168	[73]
38 H-Tyr-D-Cys-Phe-Cys-NH_2	11.0[a]	373[h]	33.9	[73]

[a] [^{3}H]DAGO; [b] [^{3}H]dihydromorphine; [c] [^{3}H]naltrexone; [d] [^{3}H]naloxone; [e] [^{125}I]FK33824; [f] [^{3}H]dermorphin; [g] [^{3}H]DADLE; [h] [^{3}H]DSLET; [i] [^{3}H][Leu^5]enkephalin; [j] [^{125}I]DADLE; [k] [^{3}H]DTLET.

than that of PL017 (K_i^δ/K_i^μ = 1470) in a direct comparison under identical binding assay conditions [49]. Furthermore, this compound does not significantly interact with κ receptors ($K_i^\kappa > 1$ μM). Thus, DALDA appears to be the most selective μ agonist reported to date and should be useful not only as a research tool in basic opioid pharmacology but also for studying specifically peripheral μ receptor interactions, because its very polar character should prevent it from crossing the blood-brain barrier. Among several prepared dermorphin-(1–3) analogues, H-Tyr-D-Arg-Phe-NH_2 (20) was shown to retain high μ affinity and considerable μ selectivity [55]. Interestingly, N-terminal extension of (20) with a lysine residue resulted in a compound (21) with only 3-times lower μ affinity and with only slightly reduced μ selectivity. The analogous extension at the N-terminus of DALDA led to a pentapeptide (H-Lys-Tyr-D-Arg-Phe-Lys-NH_2) (22) which was even slightly more μ-selective than the parent tetrapeptide but about 7-times less potent (P.W. Schiller, et al., unpublished results). Compound (22), carrying a net charge of 4+, is even more polar than DALDA. Finally, it should be mentioned that within a series of dermorphin-related dipeptide and tripeptide analogues those containing a homophenylalanine or a phenylpropyl moiety in the 3-position showed higher potency in the GPI assay than corresponding analogues with the 'natural' side-chain length in position 3 [60]. Receptor binding data for these compounds are not available, but one of them, H-Tyr-D-Ala-$NHCH_2CH_2$-CH_2Ph appears to be μ-selective as judged by its high GPI/MVD potency ratio of 300 [61].

Dermorphin-enkephalin hybrid analogues

In the enkephalins, the phenylalanine residue is located in the 4-position of the peptide sequence, whereas dermorphin and β-casomorphin contain that same residue in the 3-position. Therefore, the question arises whether the Phe^4 residue of the enkephalins and the Phe^3 residue of dermorphin and β-casomorphin interact with identical subsites of opioid receptors. Introduction of a nitro substituent in the *para* position of the Phe^4 aromatic ring of leu-enkephalin produced a significant potency increase in the GPI and MVD assay, whereas analogous substitution of the Phe^3 residue in dermorphin and morphiceptin resulted in a drastic potency drop in both assay systems [62]. One explanation of this interesting observation is the possibility that the Phe^3 aromatic ring of dermorphin and β-casomorphin may interact with an opioid receptor subsite different from that to which the Phe^4 aromatic ring of enkephalin binds. In view of this situation, it was of interest to prepare dermorphin-enkephalin hybrid peptides containing a Phe residue in the 3- *and* 4-position. The prototype

analogue H-Tyr-D-Ala-Phe-Phe-NH_2 (TAPP) (23) showed high μ receptor affinity and excellent μ selectivity (K_i^{δ}/K_i^{μ} = 409) (*Table 6.1*) [54,56]. Introduction of a nitro substituent in para position of the Phe aromatic rings of TAPP produced an affinity drop in position 3 and an affinity increase in position 4 both at the μ and the δ receptor [56], as it had been observed in the case of opioid peptides with a single Phe residue in either the 3 or the 4 position. This observation indicates that the two Phe residues in TAPP do indeed interact with the two distinct receptor subsites previously proposed. Unlike the polar analogue DALDA, TAPP is a relatively lipophilic molecule. In an effort to obtain even more lipophilic opioid peptide analogues, bulky hydrophobic amino acids, such as tryptophan or 1- (or 2)-naphthylalanine (Nap) were substituted in positions 3 and 4 of TAPP [56]. Two analogues of this type, H-Tyr-D-Ala-Trp-Trp-NH_2 (24) and H-Tyr-D-Ala-1-Nap-1-Nap-NH_2 (25) retained excellent μ receptor affinity and high preference for μ receptors over δ receptors [56]. Because of their high lipophilicity, the latter compounds may be able to cross the blood-brain barrier to some extent after peripheral administration.

Opioid peptide dimers

In a different approach to develop opioid receptor-selective compounds, bivalent ligands containing two opioid peptides linked via their C-terminal carboxyl group through flexible spacers of varying length have been synthesized. The rationale for the development of such ligands was based on experimental evidence that opioid receptors of the μ or the δ type might be clustered together [63] and on the expectation that at a certain spacer length bivalent ligands might be able to simultaneously occupy two receptor-binding sites. If this were the case, a bivalent ligand would exhibit considerably higher affinity than a corresponding monovalent ligand due to entropic factors. Furthermore, the average distance between two μ receptor-binding sites in such clusters might be different from that between two δ receptor-binding sites and, therefore, variation of the spacer length might result in μ- or δ-selective bivalent ligands. Since such selective bivalent ligands would permit a rough estimate of the distance between proximal receptor-binding sites, they might represent valuable tools for basic opioid receptor research but are perhaps less attractive as candidates for peptide drug development because of their increased molecular size. An example of a μ-selective bivalent opioid peptide ligand is (H-Tyr-D-Ala-Gly-NH-CH_2)$_2$ (DTRE)$_2$ (26), which showed very high μ receptor affinity and high μ selectivity [48] (*Table 6.1*), whereas the corresponding monovalent ligand H-Tyr-D-Ala-Gly-NH_2 (TRE) (27) was much less potent

and only moderately μ-selective. A direct comparison under identical binding assay conditions revealed that $(DTRE)_2$ had a K_d^{δ}/K_d^{μ} selectivity ratio about 3-times lower than that of morphiceptin. Further studies with 'handicapped' dimers, in which the Tyr^1 residue of one of the two peptide segments in $(DTRE)_2$ was replaced by D-Tyr or Phe, demonstrated that this bivalent ligand seems indeed to interact with two distinct μ receptor-binding sites [64]. However, because of the shortness of the spacer, it seems likely that this compound is able to bridge two putative subunits of the μ receptor rather than to interact with two separate receptors. The same can be said of the dermorphin-related tetrapeptide dimer $(H\text{-Tyr-D-Arg-Phe-Sar-}NH_2)_2$ (28), which in a direct comparison was shown to be slightly more μ-selective than dermorphin but not as μ-selective as DAGO [65].

Cyclic opioid peptide analogues

The natural enkephalins are very flexible molecules capable of assuming several different conformations of comparatively low energy [66]. This lack of structural rigidity might be the reason for the low receptor selectivity of most natural opioid peptides, since conformational adaptation to the different opioid receptor topographies (μ, δ, κ) is possible. Introduction of conformational constraints into these peptides has the potential to result in improved receptor selectivity. Conformational restriction can be achieved locally through N^{α} or C^{α} methylation of the peptide backbone or through substitution of amino acids with side-chain conformational constraints. Appropriate peptide cyclizations may produce a more drastic conformational restriction of the overall conformation. In the case of the enkephalins, various cyclizations via side-chains of appropriately substituted amino acid residues have been performed. For example, substitution of D-α,γ-diaminobutyric acid (A_2bu) in position 2 of the [Leu^5]enkephalin sequence and cyclization of the γ-amino group to the C-terminal carboxyl group resulted in a cyclic analogue, H-Tyr-cyclo-[-D-A_2bu-Gly-Phe-Leu-] (29, *Table 6.1*), showing high potency at the μ receptor [67] and considerable μ receptor selectivity [68]. The corresponding open-chain analogue, H-Tyr-D-Abu-Gly-Phe-Leu-NH_2 (30) (Abu = α-aminobutyric acid), was non-selective and it can thus be concluded that the μ receptor selectivity of cyclic analogue (29) is a direct consequence of the conformational restriction introduced through ring closure. Furthermore, this result indicated clearly that μ and δ receptors differ indeed from one another in their conformational requirements towards peptide ligands. Several homologues of (29) with varying side-chain length in the 2-position, for example, H-Tyr-cyclo-

[-D-Orn-Gly-Phe-Leu-] (31) and H-Tyr-cyclo[-D-Lys-Gly-Phe-Leu-] (32) also displayed high potency and μ selectivity [69].

The receptor selectivities of the cyclic enkephalin analogues described above could be further improved through peptide bond modifications. Various types of peptide bond replacement may not only affect the solubility and electronic properties of analogues but also their conformational behaviour due to subtle changes in structural flexibility or intramolecular hydrogen bonding (for a review, see [70]). For example, the reversal of two peptide bonds in the ring structure of H-Tyr-cyclo[-D-A_2bu-Gly-Phe-Leu-] (29) led to a compound, H-Tyr-cyclo[-Glu-Gly-gPhe-D-Leu-] (33) [71] which is 3-times more μ-selective than the cyclic parent peptide (gPhe denotes the gem diamino equivalent of Phe). Furthermore, replacement of the peptide bond in the 3–4 position of H-Tyr-cyclo[-D-Lys-Gly-Phe-Leu-] (32) with a thioamide moiety produced a compound, H-Tyr-cyclo[-D-Lys-Glyψ[CSNH]-Phe-Leu-] (34) showing an about 10-fold improvement in μ selectivity [72].

The side-chain-to-side-chain cyclized dermorphin tetrapeptide analogue H-Tyr-D-Orn-Phe-Asp-NH_2 (35) is the most μ-selective cyclic opioid peptide analogue with agonist properties reported to date (K_i^δ/K_i^μ = 213, *Table 6.1*) [38]. Other cyclic dermorphin tetrapeptide analogues structurally related to (35) include H-Tyr-D-Asp-Phe-Orn-NH_2 (36), H-Tyr-D-Asp-Phe-A_2bu-NH_2 (37) and H-Tyr-D-Cys-Phe-Cys-NH_2 (38), which all show pronounced μ selectivity [73]. In contrast to the structurally more flexible cyclic structures (14- to 16-membered) contained in enkephalin analogues (29) and (31)–(34), the ring structures in the cyclic dermorphin analogues (35)–(38) are quite rigid (11- to 13-membered).

The cyclic opioid peptide analogues described above are of great interest for conformational studies aimed at determining the bioactive conformation of opioid peptides at the μ receptor. The conformation(s) of some of them have been studied by various techniques, including theoretical studies based on the molecular mechanics or molecular dynamics approach, NMR spectroscopy, fluorescence spectroscopy, etc (for a review, see [74]). These conformational studies have not yet led to a consensus concerning a possible unique bioactive conformation at the μ receptor. An important finding of these investigations was the realization that the ring structures in the cyclic enkephalin analogues discussed above still retain some flexibility and that the various intramolecular hydrogen bonds observed are constantly formed, broken and re-formed again, as shown most convincingly in molecular dynamic studies [75]. In the case of both H-Tyr-cyclo[-D-A_2bu-Gly-Phe-Leu-] (29) and H-Tyr-cyclo[-D-Orn-Gly-Phe-Leu-] (31), the results of NMR studies [75,76] indicated the existence of

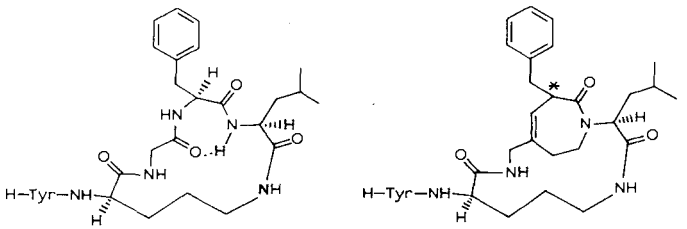

Figure 1. Structural formulas of the cyclic enkephalin analogue H-Tyr-cyclo[-D-Orn-Gly-Phe-Leu-] (left) and of a bicyclic enkephalin analogue containing a γ-turn mimic (right).

a hydrogen bond which defines a γ turn centred on Phe[4] (*Figure 6.1*). Computer simulations revealed that this hydrogen bond occurs with relatively high frequency on the picosecond time scale [75]. In a molecular mechanics study, extensive energy minimization was performed with H-Tyr-D-Orn-Phe-Asp-NH_2 (35) and with nine cyclic tetrapeptides structurally related to (35) and displaying considerable diversity in μ receptor affinity [77]. The results of this study suggested that a tilted stacking arrangement of the Tyr[1] and Phe[3] aromatic rings may represent a structural requirement for high μ receptor affinity of the examined cyclic dermorphin analogues. However, this study also revealed that the exocylic Tyr[1] residue and the Phe[3] side-chain still enjoy considerable orientational freedom. In order to obtain more definitive insight into the receptor-bound conformation of the various μ-selective cyclic opioid peptides described above, it will be necessary to develop analogues in which the Tyr residue and the Phe side-chain are also conformationally restricted.

In efforts to develop peptide-derived drugs, it would often be desirable to replace parts of the peptide backbone with other appropriate structural elements in order to eliminate susceptibility to enzymatic degradation and to reduce the relatively polar character of peptide molecules which may prevent them from being well absorbed and from crossing certain barriers, such as, for example, the blood-brain barrier. Such compounds are generally referred to as peptidomimetics. It is imperative that the spatial disposition of side-chains crucial for the interaction of the native peptide with the receptor be retained in the peptidomimetic. Whereas the various modifications of individual peptide bonds described above represent a first step in the direction of developing peptidomimetics, the substitution of structural elements mimicking larger portions of the peptide backbone has also been attempted. The latter approach is particularly applicable to peptides with a relatively well defined conformation, such as the semi-rigid cyclic analogues described above. An example is the peptidomimetic derived from the cyclic enkephalin analogue H-Tyr-

cyclo[-D-Orn-Gly-Phe-Leu-] (31) [78], shown in *Figure 6.1*. In this compound, the γ turn structure detected in the cylic parent peptide (see above) was replaced with a *trans*- olefin and the oxygen and hydrogen atoms engaged in the hydrogen bond (C = O H-N) are replaced with an ethylene moiety. Diastereomers of the bicylic compound with either *R* or *S* configuration at the chiral centre of the γ turn mimic were obtained separately. Both diastereomers were essentially inactive in the GPI and MVD assays and showed very weak affinity for μ and δ opioid receptors in the rat brain membrane binding assay. The lack of activity of these peptidomimetics can be explained in several ways. First, it is possible that the γ turn detected in the NMR and computer simulation studies of the monocyclic parent peptide may not be a structural feature of the receptor-bound conformation. Second, the Gly^3 carbonyl group, which is no longer present in the γ turn mimic, may be important for the interaction with the receptor. Third, the ethylene bridge introduced in the mimic may interfere with the receptor binding process either due to unfavourable steric interactions or due to the fact that it produces too much rigidity in the molecule. Despite the lack of activity, the preparation of this compound represents a conceptually interesting first step in the direction of developing rationally designed peptidomimetics of enkephalin and further efforts along these lines should be undertaken.

δ-SELECTIVE AGONISTS

Even though the natural enkephalins display slight preference for δ receptors over μ receptors, efforts to develop more δ-selective enkephalin analogues were not very successful for quite some time. In recent years, however, linear and cyclic opioid peptide analogues as well as opioid peptide dimers with greatly improved δ selectivity have been obtained.

Linear enkephalin-related analogues

Substitution of D-alanine in position 2 of leu-enkephalin (39) (*Table 6.2*) and inversion of the configuration in the 5-position led to a compound, H-Tyr-D-Ala-Gly-Phe-D-Leu-OH (DADLE) (40) [79], with only slightly improved δ receptor selectivity (*Table 6.2*). Nevertheless, DADLE in tritiated form has frequently been used and continues to be used as a 'δ-selective' radioligand. A slight improvement in δ selectivity was achieved through substitution of a D-serine residue in the 2-position of leu-enkephalin and extension of the peptide chain with a threonine residue at the C-terminus [82]. The resulting hexapeptide analogue, H-Tyr-D-Ser-Gly-Phe-Leu-Thr-OH (DSLET, 41) showed

quite high δ receptor affinity but somewhat reduced μ receptor affinity, presumably due to an adverse interaction of the hydroxylated side-chain of Ser^2 with a hydrophobic subsite of the μ receptor. In comparison with DSLET, an analogue containing a bulkier D-threonine residue in place of D-Ser, H-Tyr-D-Thr-Gly-Phe-Leu-Thr-OH (DTLET, 42) [84], was about 3-times more δ-selective. In an effort to further increase the bulkiness of the side-chains in the 2- and 6-position of DSLET, analogues carrying a t-butyl group on the hydroxyl function of Ser and/or Thr were recently prepared [83]. Among these compounds, H-Tyr-D-Ser(OtBu)-Gly-Phe-Leu-Thr-OH (DSTBULET, 43) and H-Tyr-D-Ser(OtBu)-Gly-Phe-Leu-Thr(OtBu)-OH (BUBU, 44) showed much improved δ selectivity with respective K_i^μ/K_i^δ values of 60.9 and 101. In another interesting development, the conformationally restricted phenylalanine analogue *E*-cyclopropylphenylalanine (∇^EPhe) was substituted in position 4 of [D-Ala^2, Leu^5]enkephalin [85]. One of the obtained isomers, H-Tyr-D-Ala-Gly-(2R, 3S)-∇^EPhe-Leu-OH (45) showed excellent δ selectivity. Analogous conformational restriction of the 1-position side-chain produced a compound, ($\pm$)∇^ETyr-D-Ala-Gly-Phe-Leu-OH, which also appears to be very δ-selective, as judged by its high IC_{50}(GPI)/IC_{50}(MVD) ratio [86].

Deltorphins

The naturally occurring deltorphins have been reported to be potent δ agonists displaying very high receptor selectivity [4]. The heptapeptide H-Tyr-D-Met-Phe-His-Leu-Met-Asp-NH_2 (46) was first isolated and characterized by Kreil et al. [87] and named deltorphin. An alternative name, dermenkephalin, was proposed by other investigators who subsequently also characterized this peptide [88]. However, the designation proposed by the original discoverers should have preference and, therefore, the peptide will be referred to as deltorphin in this review. Selectivity ratios (K_i^μ/K_i^δ) ranging from 51 to 676 have been reported for deltorphin (*Table 6.2*). In a direct comparison with the cyclic enkephalin analogue H-Tyr-D-Pen-Gly-Phe-D-Pen-OH (DPDPE) (52, see below) under identical binding assay conditions, deltorphin was found to be more δ-selective in one study [4] and less δ-selective in another [88]. Soon after the discovery of deltorphin, Erspamer and his colleagues isolated two other heptapeptides, H-Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2 ([D-Ala^2]deltorphin I) (47) and H-Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2 ([D-Ala^2]deltorphin II) (48) from frog skin extracts of *Phyllomedusa bicolor* [4]. These two peptides showed even higher δ receptor preference than deltorphin, the most selective being [D-Ala^2]deltorphin I with a reported K_i^μ/K_i^δ ratio of 21,000. Interestingly, these

δ-selective peptides contain the same N-terminal tripeptide segment, H-Tyr-D-Ala-Phe-, as the μ-selective opioid peptide dermorphin. It thus appears that the C-terminal tetrapeptide segment is of crucial importance for the δ receptor selectivity of the deltorphins. [D-Ala^2]deltorphin I has been reported to be nearly 100-times more δ-selective than DPDPE [4], primarily as a consequence of its much higher δ receptor affinity. Further binding data will have to be obtained in order to definitely establish to what extent the deltorphins are more δ-selective than other known δ agonists.

Opioid peptide dimers

On the basis of the rationale outlined above, δ-selective bivalent ligands containing two enkephalin-related peptide segments separated by an appropriate spacer were also developed. The dimeric enkephalin analogue (H-Tyr-D-Ala-Gly-Phe-Leu-NH-CH_2)$_2$ (DPE_2, 49) showed moderate preference for δ receptors over μ receptors [89] (*Table 6.2*). In an interesting experiment based on progressive receptor inactivation with an irreversible δ-selective ligand, it was demonstrated that the high δ affinity of DPE_2 was not dependent on receptor density [90]. The latter observation led to the conclusion that this bivalent ligand does not bind simultaneously to two separate δ receptors but rather may interact with two receptor-binding sites within a single receptor complex or with a single receptor-binding site and an accessory site. High δ selectivity (K_i^μ/K_i^δ = 91) was shown by a dimer containing two H-Tyr-D-Ala-Gly-Phe-NH_2 segments linked by a 12-methylene bridge (DTE_{12}, 50), whereas the monomeric tetrapeptide amide (51) was moderately μ-selective [91]. The preference of DTE_{12} for δ receptors was a consequence of poor binding to the μ receptor. This result suggests that bridging of two δ receptor-binding sites may not be the reason for the δ selectivity of this bivalent ligand. Possibly, the weak μ affinity of DTE_{12} may be due to an altered conformation resulting from an intramolecular interaction between the two peptide segments linked by the long, flexible spacer. Direct comparison showed that DTE_{12} is about 5-times more δ-selective than DTLET [91].

Cyclic analogues

Cyclic enkephalin analogues containing half-cystine residues in the 2- and 5-position (H-Tyr-D-Cys-Gly-Phe-D(or L)-Cys-X [X = NH_2 or OH]) were first synthesized by Sarantakis [92] and independently by Schiller et al. [93]. Whereas the latter analogues were shown to be relatively non-selective [93,94],

Table 6.2. RECEPTOR AFFINITIES AND SELECTIVITY RATIOS OF δ-SELECTIVE OPIOID PEPTIDE ANALOGUES (AGONISTS)

Receptorbinding affinities and selectivity ratios were determined as desorbed in the legend of *Table 6.1.*

Compound	μ affinity		δ affinity		Selectivity ratio	Reference
	IC^{μ}_{50} [nM],	K^{μ}_i [nM]	IC^{δ}_{50} [nM],	K^{δ}_i [nM]	$IC^{\mu}_{50}/IC^{\delta}_{50}$ or K^{μ}_i/K^{δ}_i	
Enkephalin-related analogues						
39 [Leu^5]enkephalin		9.43[a]		2.54[h]	3.71	[38]
40 H-Tyr-D-Ala-Gly-Phe-D-Leu-OH		7.7[a]		2.4[k]	3.20	[80]
(DADLE)		7.59[a]		1.70[g]	4.46	[81]
41 H-Tyr-D-Ser-Gly-Phe-Leu-Thr-OH (DSLET)		31.0[a]		4.80[k]	6.46	[83]
42 H-Tyr-D-Thr-Gly-Phe-Leu-Thr-OH (DTLET)		23.3[a]		1.35[k]	17.3	[83]
43 H-Tyr-D-Ser(OtBu)-Gly-Phe-Leu-Thr-OH (DSTBULET)		374[a]		6.14[k]	60.9	[83]
44 H-Tyr-D-Ser(OtBu)-Gly-Phe-Leu-Thr(OtBu)-OH (BUBO)		475[a]		4.68[k]	101	[83]
45 H-Tyr-D-Ala-Gly-(2R, 3S)-∇^{E}Phe-Leu-OH		3290[a]		13.0[g]	253	[85]
Deltorphins						
46 H-Tyr-D-Met-Phe-His-Leu-Met-Asp-NH_2		1630[a]		2.41[n]	676	[4]
		367[a]		3.15[o]	117	[88]
		267[f]		5.2[k]	51	[52]
47 H-Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2		3150[a]		0.15[n]	21000	[4]
48 H-Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2		2450[a]		0.71[n]	3450	[4]
Opioid peptide dimers						
49 (H-Tyr-D-Ala-Gly-Phe-Leu-NH-CH_2)$_2$ (DPE_2)	1.78[d]		0.27[j]		6.59	[89]
50 (H-Tyr-D-Ala-Gly-Phe-NH-$(CH_2)_6$)$_2$ (DTE_{12})	96.3[d]		1.06[g]		90.8	[91]
51 H-Tyr-D-Ala-Gly-Phe-NH_2	3.48[d]		33.2[g]		0.105	[91]

Cyclic analogues						
52 H-Tyr-D-Pen-Gly-Phe-D-Pen-OH (DPDPE)	2240[d]		16.2[g]		138	[95]
		993[a]		19.2[k]	51.7	[97]
	609[l]		5.25[o]		116	[98]
	7720[m]		6.44[o]		1200	[99]
53 H-Tyr-D-Pen-Gly-Phe-L-Pen-OH (DPLPE)	3710[d]		10.0[g]		371	[95]
		873[a]		10.9[k]	80.1	[97]
54 H-Tyr-D-Pen-Gly-p-Cl-Phe-D-Pen-OH	901[l]		1.57[o]		574	[98]
55 H-Tyr-D-Cys-Phe-D-Pen-OH	1210[m]		1.90[o]		637	[99]

Footnotes a–k are the same as in *Table 6.1*. [l] [^{3}H]CTOP; [m] [^{3}H]sufentanil; [n] [^{3}H][D-Ala^2]deltorphin I; [o] [^{3}H]DPDPE.

replacement of one or both Cys residues with a penicillamine (Pen residue) resulted in analogues with markedly improved δ selectivity. In particular, the analogues H-Tyr-D-Pen-Gly-Phe-D-Pen-OH (DPDPE, 52) and H-Tyr-D-Pen-Gly-Phe-L-Pen-OH (DPLPE, 53) showed high $IC_{50}^{\mu}/IC_{50}^{\delta}$ ratios in the receptor-binding assays [95] and became popular δ ligands. A recent study showed that the δ selectivity of these two analogues is due to the presence of the bulky *gem* dimethyl groups in the 2-position side-chain which cause more severe steric interference at the μ receptor than at the δ receptor [96]. A recent direct comparison indicated that DPDPE and DPLPE had higher δ selectivity than DTLET and were about as δ-selective as DSTBULET and BUBU [97]. However, the latter two analogues showed somewhat higher δ receptor affinity. Introduction of a chloro substituent in para position of the Phe^4 aromatic ring of DPDPE led to a compound, H-Tyr-D-Pen-Gly-pCl-Phe-D-Pen-OH (54) with 5-times higher δ selectivity than the parent peptide due to a 5-fold increase in δ receptor affinity [98]. A recently reported cyclic tetrapeptide analogue, H-Tyr-D-Cys-Phe-D-Pen-OH (55) was half as δ-selective as DPDPE, but had about 3-times higher δ affinity [99]. Several conformational studies of DPDPE have been performed, but no consensus regarding the bioactive conformation of this peptide at the δ receptor has been reached as yet.

κ-SELECTIVE AGONISTS

As the data listed in *Table 6.3* indicate, the 17-peptide dynorphin A-(H-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln-OH, 56) displays only limited selectivity for κ versus μ and δ receptors [100,101]. A similar receptor selectivity profile is shown by the C-terminally truncated peptide dynorphin A-(1–13) (57) [101,102] and, therefore, this 13-peptide has served as parent structure in efforts to develop analogues with improved κ selectivity. In comparison with dynorphin A-(1–13), the analogue [Ala^8]-dynorphin A-(1–13) (58) was about 4-times more κ-selective towards both the μ and the δ receptor, whereas the corresponding D-Ala^8 analogue (59) showed only a slight improvement in κ selectivity [102]. Slight improvements in κ versus μ and κ versus δ selectivity were also observed with the analogues [Trp^8]dynorphin A-(1–13) (60) and [D-Pro^{10}]dynorphin A-(1–13) (61) [102]. Deletion of two additional amino acid residues at the C-terminus led to a peptide, dynorphin A-(1–11) (62), which showed about 3-times higher κ selectivity towards both the μ and the δ receptor than dynorphin A [100]. A further

Table 6.3. RECEPTOR AFFINITIES AND SELECTIVITY RATIOS OF κ-SELECTIVE OPIOID PEPTIDE ANALOGUES (AGONISTS)

Receptor binding affinities and selectivity ratios were determined as described in the legend of Table 6.1.

	μ affinity	*δ affinity*	*κ affinity*	*Receptor selectivity*		
No. Compound	K_i^{μ} *(nM)*	K_i^{δ} *(nM)*	K_i^{κ} *(nM)*	K_i^{μ}/K_i^{κ}	$K_i^{\delta}/K_i^{\kappa}$	*Reference*
56 Dynorphin A	1.55[a]	6.30[h]	0.23[q]	6.74	27.4	[100]
	0.73[a]	2.38[g]	0.115[q]	6.35	20.7	[101]
	3.16[a] (IC_{50})	13.1[o] (IC_{50})	0.63[q] (IC_{50})	5.02[t]	20.8[t]	[104]
57 Dynorphin A-(1-13)	0.50[a]	4.4[h]	0.11[r]	4.55	40.0	[102]
	0.222[a]	0.485[g]	0.045[q]	4.93	10.8	[101]
58 [Ala[8]]dynorphin A-(1-13)	0.44[a]	7.4[h]	0.05[r]	16.8	148	[102]
59 [D-Ala[8]]dynorphin A-(1-13)	0.49[a]	7.8[h]	0.08[r]	6.12	97.5	[102]
60 [Trp[8]]dynorphin A-(1-13)	1.18[a]	4.6[h]	0.10[r]	11.8	46	[102]
61 [D-Pro[10]]dynorphin A-(1-13)	1.34[a]	5.8[h]	0.09[r]	14.9	64	[102]
62 Dynorphin A-(1-11)	2.71[a]	10.7[h]	0.125[q]	21.7	85.6	[100]
63 [D-Pro[10]]dynorphin A-(1-11)	2.00[a]	7.47[h]	0.032[q]	62.5	233	[100]
64 [Lys[6,7,9,15], Val[8,12,14], Ser[16,17]]dynorphin A	4.82[a] (IC_{50})	39.1[o] (IC_{50})	0.53[q] (IC_{50})	9.1[t]	73.8[t]	[104]
65 [D-Cys[8], D-Cys[13]]dynorphin A-(1-13)	0.362[p] (IC_{50})	14.3[o] (IC_{50})	0.110[s] (IC_{50})	3.29[t]	130[t]	[108]

Footnotes a–o are the same as in Tables 6.1 and 6.2. [p] [^{3}H]PLO-17; [q] [^{3}H]bremazocine; [r] [^{3}H]ethylketocyclazocine; [s] [^{3}H]U-69593; [t] IC_{50} ratio.

3-fold increase in κ vs. μ and κ vs. δ selectivity was obtained upon substitution of a D-proline residue in position 10 of dynorphin A(1–11) (analogue 63) [100]. Substitution of Lys^{13} in dynorphin A-(1–13) with Arg and C-terminal extension with -Gly-NH-$(CH_2)_5$-NH_2 resulted in an analogue (DAKLI) with κ selectivity comparable to that of the native 13-peptide [103]. Derivatives of DAKLI containing various reporter groups (^{125}I-labelled Bolton-Hunter reagent, fluorescein isothiocyanate or biotin) also retained high κ receptor affinity and were about as κ-selective as the parent peptide. In an interesting recent development, the potential amphiphilic β strand region encompassing residues 7–15 in dynorphin A was replaced with a segment of alternating Lys and Val residues [104]. The resulting compound, [$Lys^{6,7,9,15}$, $Val^{8,12,14}$, $Ser^{16,17}$]dynorphin A (64), was about 2–3-times more κ-selective towards the μ and towards the δ receptor than natural dynorphin A. Several cyclic dynorphin A analogues were prepared. [D-Cys^2, Cys^5]dynorphin A-(1–13) [105] turned out to be no longer κ-selective, but δ-selective instead [106]. The cyclic lactam analogues [D-Orn^2, Asp^5]dynorphin A-(1–8), [Orn^5, Asp^8]dynorphin A-(1–13), [Orn^5, Asp^{10}]dynorphin A-(1–13) and [Orn^5, Asp^{13}]dynorphin A-(1–13) all showed preference for μ receptors over δ receptors and determination of their K_e values with naloxone as antagonist in the GPI assay revealed that these compounds did not significantly interact with κ receptors [107]. These various performed cyclizations resulted in overall folded conformations which are incompatible with the conformational requirements of the κ receptor. Another cystine-bridged analogue, [D-Cys^8, D-Cys^{13}]dynorphin A-(1–13)-amide (65, *Table 6.3*), showed quite high κ receptor affinity but low κ versus μ selectivity [108]. In conclusion, the structure–activity studies of dynorphin performed to date have led to compounds showing only a modest improvement in κ selectivity and further efforts will be required to develop highly κ-selective dynorphin analogues.

OPIOID PEPTIDE ANALOGUES WITH ANTAGONIST PROPERTIES

μ-Selective antagonists

In an interesting early development to obtain opioid antagonists through structural modification of enkephalin, derivatives of H-Tyr-D-Ala-Gly-MePhe-NH_2 containing a phenethyl group and a second bulky moiety (phenethyl, isoamyl or thiomethylpropyl) attached to the C-terminal amide nitrogen were prepared

[109]. No receptor-binding data on these compounds are available, but they were shown to be moderately potent, pure antagonists in the MVD assay with about 10-fold μ selectivity in that tissue.

Whereas the natural 14-peptide somatostatin interacts only weakly with opioid receptors, the somatostatin analogue H-D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-ol (SMS-201995, 66) is a potent opioid antagonist with high preference for μ receptors over δ receptors (*Table 6.4*) [110]. The discovery of this antagonist was serendipitous and structural relationships between SMS-201995 and the various opiates and opioid peptides are not apparent. Various structural modifications of this interesting new lead compound were subsequently performed. Substitution of Tyr in position 3 and of Pen in position 7 as well as replacement of the C-terminal alcohol group with a carboxamide function led to a compound, H-D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Pen-Thr-NH_2 (CTP, 67), which was still a potent and very selective μ antagonist but, unlike SMS-201995, had relatively weak affinity for the somatostatin receptor in rat brain homogenates [111]. Shortening of the side-chain in position 5 of CTP produced an analogue, H-D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2 (CTOP, 68) with further improved μ selectivity and with even lower affinity for the somatostatin receptor [112]. Finally, an analogue of CTOP containing the conformationally restricted phenylalanine analogue tetrahydroisoquinoline-3-carboxylic acid (Tic) in place of D-Phe[1] (H-D-Tic-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2, TCTOP, 69) showed still higher μ selectivity ($IC_{50}^{\delta}/IC_{50}^{\mu}$ = 11,400) and also bound very poorly to the somatostatin receptor [113]. Two other somatostatin analogues, H-D-Nal-Cys-Tyr-D-Trp-Lys-Val-Cys-Thr-NH_2 (70; Nal = 3-(2-naphthyl)alanine) and H-D-Nal-Cys-Tyr-D-Pal-Lys-Val-Cys-Thr-NH_2 (71; Pal = 3-(3-pyridyl)alanine) were also shown to be opioid antagonists with quite high preference for μ receptors over δ receptors [114]. However, one of these analogues (70) also showed high somatostatin activity in the assay based on inhibition of growth hormone release.

δ-Selective antagonists

A number of δ-selective antagonists have been obtained through diallylation of the α-amino group of enkephalin-related peptides. The design of these

Table 6.4. OPIOID PEPTIDE ANALOGUES WITH ANTAGONIST PROPERTIES

Receptor binding affinities and selectivity ratios were determined as described in the legend of Table 6.1.

No. Compound	μ affinity		δ affinity		κ affinity	Selectivity ratios			
	IC_{50}^{μ} (nM),	K_i^{μ} (nM)	IC_{50}^{δ} (nM),	K_i^{δ} (nM)	K_i^{κ} (nM)	$IC_{50}^{\delta}/IC_{50}^{\mu}$ or K_i^{δ}/K_i^{μ}	K_i^{μ}/K_i^{κ}	$K_i^{\delta}/K_i^{\kappa}$	Reference
μ-Selective antagonists									
66 H-D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-ol	23[b]		5000[g]			217			[110]
(SMS 201 995)		2.32[a]		16300[h]		7030	Schiller et al., unpubl. data		
67 H-D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Pen-Thr-NH_2	3.5[d]		950[g]			271			[111]
(CTP)	3.7[d]		8400[o]			2270			[112]
	3.7[l]		1150[o]			311			[113]
68 H-D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2	2.8[d]		13500[o]			4820			[112]
(CTOP)	4.3[l]		5600[o]			1300			[113]
69 H-D-Tic-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2	1.4[l]		16000[o]			11400			[113]
(TCTOP)									
70 H-D-Nal-Cys-Tyr-D-Trp-Lys-Val-Cys-Thr-NH_2	28[a]		5450[o]			195			[114]
71 H-D-Nal-Cys-Tyr-D-Pal-Lys-Val-Cys-Thr-NH_2	33[a]		> 10000[o]			> 303			[114]
δ-Selective antagonists									
72 *N,N*-diallyl-Tyr-Gly-Glyψ[CH_2S]Phe-Leu-OH		10100[a]		778[g]	> 50000[q]	0.0770			[116, 117]
(ICI 154129)									
73 *N,N*-diallyl-Tyr-Aib-Aib-Phe-Leu-OH		24700[a]		193[g]	> 69000[q]	0.00781			[117, 118]
(ICI 174864)									
κ-Selective antagonists									
74 [*N,N*-diallyl-Tyr[1], D-Pro[10]]dynorphin A-(1-11)		135[a]		354[h]	20.7[q]		6.52	17.1	[123]
75 [*N,N*-diallyl-Tyr[1], Aib[2,3], D-Pro[10]]dynorphin A-(1-11)		299[a]		530[h]	18.9[q]		15.8	28.0	[123]

Footnotes are as in previous tables.

analogues was based on analogy with the well-known *N*-allyl substituted opiate alkaloid antagonists. Whereas the *N*-monoallylated derivative of leu-enkephalin turned out be a weak mixed agonist-antagonist, *N,N*-diallylated leu-enkephalin was shown to be a moderately potent, δ-selective antagonist in the MVD assay [115]. No receptor-binding data have been reported for this compound. Replacement of the 3–4 position peptide bond in the latter derivative with a thiomethylene moiety resulted in a compound, *N,N*-diallyl-Tyr-Gly-Glyψ-[CH_2S]Phe-Leu-OH (ICI 154129) (72, *Table 6.4*), which also was a δ-selective antagonist with moderate δ receptor affinity [116,117]. In an effort to reduce the structural flexibility of *N,N*-diallylated leu-enkephalin, the Gly^2-Gly^3-dipeptide unit was replaced by either an -Aib^2-Aib^3- unit [118] or by a rigid spacer such as *p*-aminobenzoic acid (-NH-ϕ-CO-) [119]. One of these conformationally restricted analogues, *N,N*-diallyl Tyr-Aib-Aib-Phe-Leu-OH (ICI 174864) (73, *Table 6.4*) was a moderately potent δ antagonist in the MVD assay and in the receptor-binding assays showed about 4-times higher δ receptor affinity than ICI 154129 and quite high preference for δ receptors over μ receptors (K_i^μ/K_i^δ = 128). Similar δ antagonist potency and selectivity were determined for *N,N*-diallyl-Tyr-*p*-NH-ϕ-CO-Phe-Leu-OH in the MVD assay; however, no receptor-binding data were reported for the latter compound. Evaluation of a dimeric ligand, (*N,N*-diallyl-Tyr-Gly-Gly-Phe-Leu-NH-CH_2-)$_2$, in the MVD assay revealed that this compound also was a fairly potent and selective δ antagonist [120]. However, studies with related dimers that were truncated on one side indicated that this bivalent ligand did not simultaneously bind to two distinct δ receptor binding sites.

κ-Selective antagonists

Several attempts were made to develop κ-selective antagonists through structural modification of dynorphin A. [Ala^2, Trp^4]dynorphin A-(1–13) has been claimed to be a κ-selective opioid antagonist [121]; however, an accurate opioid receptor-binding selectivity profile has not been determined for this compound. The three 11-peptide analogues [D-$Trp^{2,8}$, D-Pro^{10}]-, [D-$Trp^{5,8}$, D-Pro^{10}]- and [D-$Trp^{2,4,8}$, D-Pro^{10}]dynorphin A-(1–11) showed weak antagonism against dynorphin A and low κ versus μ selectivity [122]. Finally, [*N,N*-diallyl-Tyr^1, D-Pro^{10}]dynorphin A-(1–11) (74) and [*N,N*-diallyl-Tyr^1,$Aib^{2,3}$,D-Pro^{10}]dynorphin A-(1–11) (75) were reported to act as pure but not very κ-selective opioid antagonists *in vitro* [123] (*Table 6.4*).

In conclusion, structural modification of the somatostatin analogue SMS-201995 has led to potent and highly selective μ antagonists which are superior to any of the μ-selective non-peptide opioid antagonists. On the other

hand, the best enkephalin- and dynorphin-derived δ- and κ-selective opioid antagonists are clearly less potent and less selective than the non-peptide antagonists naltrindole (δ-selective) [23] and binaltorphimine or norbinaltorphimine (κ-selective) [24].

IRREVERSIBLE OPIOID PEPTIDE LIGANDS

A number of irreversible opioid receptor ligands have been prepared as tools in either basic opioid pharmacology or in opioid receptor labelling experiments. Incorporation of both affinity and photoaffinity labels into either non-peptide opiates or opioid peptide analogues has been used to develop receptor-selective irreversible ligands. Excellent progress has been made in the development of affinity-labelled non-peptide opioids, such as β-chlornaltrexamine (β-CNA, non-selective) [124], β-funaltrexamine (β-FNA, μ-selective ?) [125], the etonitazene analogue BIT (μ-selective) [126], the oripavine derivative FAO (δ-selective) [126], and the δ-selective fentanyl derivatives FIT [126], SUPERFIT [127] and (3*S*,4*S*)-(+)-*trans*-3-methylfentanyl isothiocyanate [128].

Chemical affinity labels

An example of an enkephalin-based affinity label is the chloromethyl ketone derivative of [D-Ala^2, Leu^5]enkephalin (DALECK, 76, *Table 6.5*) [129,130]. DALECK showed moderate preference for μ receptors over δ receptors in rat brain and was used for covalent labelling of opioid receptors [130,131]. Somewhat higher μ selectivity was displayed by the more recently developed tetrapeptide derivative H-Tyr-D-Ala-Gly-MePhe-CH_2Cl (DAMK, 77), which labelled irreversibly and selectively high-affinity μ binding sites [132]. The hexapeptide analogue [D-Ala^2, Leu^5, Cys^6]enkephalin (DALCE, 78) contains a single sulphydryl group and is moderately δ-selective [133]. DALCE was shown to bind covalently to δ receptors only by forming a disulphide bond with a sulphydryl moiety present at the binding site.

Photoaffinity labels

A number of opioid peptide analogues carrying a photolabile moiety were prepared for use in receptor photoaffinity labelling experiments. It was of interest to obtain receptor-selective photoaffinity labels and, therefore, photolabile groups were mostly incorporated into known opioid peptide analogues

Table 6.5. RECEPTOR AFFINITIES AND SELECTIVITY RATIOS OF IRREVERSIBLE OPIOID PEPTIDE LIGANDS

Receptor binding affinities and selectivity ratios were determined as described in the legend of *Table 6.1.*

No. Compound	μ affinity		δ affinity		κ affinity		Selectivity ratios		Reference
	IC^{μ}_{50} [nM],	K_i^{μ} [nM]	IC^{δ}_{50} [nM],	K_i^{δ} [nM]	IC^{κ}_{50} [nM],	K_i^{κ} [nM]	$IC^{\delta}_{50}/IC^{\kappa}_{50}$ or K_i^{δ}/K_i^{μ}	$IC^{\mu}_{50}/IC^{\delta}_{50}$ or K_i^{μ}/K_i^{δ}	
Chemical affinity labels									
76 H-Tyr-D-Ala-Gly-Phe-Leu-CH_2Cl (DALECK)	3.5[a]		50[g]		> 1000[r]		14.3		[129, 130]
77 H-Tyr-D-Ala-Gly-MePhe-CH_2Cl (DAMK)	2.0[b]		80[u]				40		[132]
78 H-Tyr-D-Ala-Gly-Phe-Leu-Cys-OH (DALCE)	55[a]		4.1[o]		> 10000[q]			13.4	[133]
Photoaffinity labels									
79 H-Tyr-D-Ala-Gly-MePhe(pN_3)-Gly-ol		25.0[a]		3480[k]			136		[134]
80 H-Tyr-D-Ala-Phe-Phe(pN_3)-NH_2		1.49[a]		159[h]			107		[56]
81 H-D-Phe-Cys-Phe(pN_3)-D-Trp-Lys-Thr-Pen-Thr-NH_2		49[l]		20000[o]			408		[135]
82 H-Tyr-Pro-Phe-Bpa-NH_2	0.268[d]		3.67[g]		8.20[r]		13.7		[136]
83 H-Tyr-D-Thr-Gly-Phe(pN_3)-Leu-Thr-OH		72.2[a]		1.4[k]				51.6	[134]
84 H-Tyr-D-Pen-Gly-Phe(pN_3)-D-Pen-OH		3615[l]		33[o]				110	[135]
85 H-Tyr-D-Ala-Gly-Phe(pN_3)-Met-OH		930[b]		9.5[g]		83[r]		97.9	[137]

Footnotes a–r are as in previous Tables. [u] [^{3}H][D-Ala^2, Leu^5]enkephalin.

that display high preference for one or the other of the different receptor types. Thus, μ-selective photoaffinity labels were prepared through substitution of *p*-azidophenylalanine (Phe(pN_3)) for Phe^4 (or MePhe^4) in DAGO, TAPP and CTP or through replacement of the Pro^4 residue in morphiceptin with *p*-benzoylphenylalanine (Bpa). The resulting Phe(pN_3)-analogues, H-Tyr-D-Ala-Gly-MePhe(pN_3)-Gly-ol (79) [134], H-Tyr-D-Ala-Phe-Phe(pN_3)-NH_2 (80) [56] and H-D-Phe-Cys-Phe(pN_3)-D-Trp-Lys-Thr-Pen-Thr-NH_2 (81) [135] retained high μ selectivity (*Table 6.5*), whereas the morphiceptin analogue H-Tyr-Pro-Phe-Bpa-NH_2 (82) [136] was somewhat less selective. The *p*-azidophenylalanine analogue of CTP (81) showed highest μ receptor selectivity, but H-Tyr-D-Ala-Phe-Phe(pN_3)-NH_2 (80) was only 4-times less μ-selective and had more than 30-times higher μ receptor affinity than (81). As it has a high μ receptor affinity, the TAPP analogue may be particularly useful for μ receptor photoaffinity labelling experiments. Whereas photoaffinity labelling experiments with derivatives (80) and (81) have not yet been performed, the ability of compounds (79) and (82) to selectively label μ receptors has been demonstrated [134, 136]. Photoaffinity labels with δ receptor selectivity are the DTLET and DPDPE derivatives H-Tyr-D-Thr-Gly-Phe(pN_3)-Leu-Thr-OH (83) [134] and H-Tyr-D-Pen-Gly-Phe(pN_3)-D-Pen-OH (84) [135], as well as the enkephalin analogue H-Tyr-D-Ala-Gly-Phe(pN_3)-Met-OH (85) [137]. Radioiodination of analogue (85) at the Tyr^1 residue resulted in a loss of receptor selectivity. Selective photoaffinity labelling of δ receptors was achieved with analogue (83) [134]. Two analogues of [D-Ala^2, Leu^5]enkephalin, containing the photolabile 2-nitro-4-azidophenyl group attached to the C-terminal carboxyl group via an ethylenediamine or an ethylenediamine β-alanine spacer, were claimed to be δ-selective [138], but opioid receptor-binding selectivity profiles have not been determined for these compounds. However, subsequent determination of the IC_{50}(GPI)/IC_{50}(MVD) ratio of one of these analogues, [D-Ala^2, Leu^5]enkephalin-*N*-[(2-nitro-4-azidophenyl)amino]ethylamide, indicated that it was relatively non-selective [139]. Other opioid peptide-derived photoaffinity labels for which receptor-binding selectivity profiles have not been determined include the analogue H-Tyr-D-Ala-Gly-Phe-Leu-Lys-N^{ε}-nitroazidophenyl [140] and various enkephalin analogues containing either an *m*-azidophenylalanine residue in place of Phe^4 or an azidophenyl moiety linked to the C-terminal carboxyl group via a propylamine spacer [141].

OPIOID PEPTIDE LIGANDS CONSIDERED FOR DRUG DEVELOPMENT

The two best known analogues that have undergone fairly extensive clinical testing so far are the Sandoz compound FK 33–824 (H-Tyr-D-Ala-Gly-MePhe-Met(O)-ol (6) [142] and the Lilly compound metkephamid (H-Tyr-D-Ala-Gly-Phe-MeMet-NH_2 (7) [143]. As discussed above, FK 33–824 shows only slight preference for μ receptors over δ receptors and metkephamid is essentially non-selective towards μ and δ receptors (*Table 6.1*). Neither of these two analogues has significant affinity for κ receptors. Both FK 33–824 and metkephamid exert a potent analgesic effect after systemic administration. However, FK 33–824 also produced a number of serious side-effects (*Table 6.6*) and is no longer pursued clinically as an analgesic candidate. Relatively fewer side-effects were observed with metkephamid. It has been claimed that metkephamid may exert its central analgesic effect through interaction with δ receptors and that it may produce less physical dependence than morphine [50]. Of particular interest was the observation that this analogue does not cross the placental barrier to a significant extent in the pregnant sheep model [143]. This finding suggested that metkephamid might be of interest for use in obstetric analgesia. However, metkephamid was finally abandoned as candidate for obstetric analgesia, because it also produced a transient hypotensive effect in the particular obstetric population that had been subjected to a clinical trial. Another enkephalin analogue, H-Tyr-D-Met-Gly-Phe-Pro-NH_2 (5), was shown to be a more potent analgesic than morphine or β-endorphin, but also produced a number of side-effects in a clinical trial [144]. The various side-effects observed with FK 33–824, metkephamid and H-Tyr-D-Met-Gly-Phe-Pro-NH_2 (5) led to a certain discouragement with regard to the potential of opioid peptide analogues as viable analgesics. It should be realized, however, that the lack of receptor selectivity of these three enkephalin analogues may be responsible for some of the observed side-effects. Progress recently made in the development of opioid peptide analogues now permits the preparation of compounds with greatly improved receptor selectivity that may have better potential for application in various types of analgesia.

In recent years increasing evidence has been obtained to indicate that opioids are able to produce peripherally mediated analgesic effects. While data on peripheral antinociceptive effects in normal non-inflamed tissue are still controversial, effects in hyperalgesic and inflammatory conditions have been well-documented [145–147]. The mechanism underlying peripherally mediated, opioid induced antinociceptive effects is not entirely clear, but they appear to be mediated by inhibition of sensory neurons which would prevent information

Table 6.6. CLINICAL TESTING OF ENKEPHALIN ANALOGUES

Compound	*Selectivity*	*Analgesia*	*Side-effects*	*Reference*
H-Tyr-D-Ala-Gly-MePhe-Met(O)-ol (FK33-824) (6)	$K_i^{\delta}/K_i^{\mu} = 3.4$	Centrally mediated (systemic administration)	Muscle heaviness Chest oppression Anxiety Bowel sound increase Chemosis Whole body flush, etc.	[26, 142]
H-Tyr-D-Ala-Gly-Phe-MeMet-NH_2 (metkephamid) (7)	non-selective	Centrally mediated (systemic administration) Does not cross placental barrier	Heavy sensation in extremities Nasal congestion Emotional detachment Conjunctival injection Hypotension, etc.	[40, 50, 143]
H-Tyr-D-Met-Gly-Phe-Pro-NH_2 (5)	$K_i^{\delta}/K_i^{\mu} = 6.48$	Centrally mediated (systemic administration)	Heaviness in the limbs Dry mouth Conjunctival injection Emotional detachment Drowsiness, etc.	[43, 144]
H-Tyr-D-Arg-Gly-Phe(pNO_2)-Pro-NH_2 (BW 443C)	somewhat μ-selective	Peripherally mediated (s.c. administration)	Nasal stuffiness Dry mouth Postural hypotension *No* central effects (sedation, mood change, resp. depression, etc.)	[148, 149, 150]

about noxious events from reaching the spinal cord. The most convincing evidence for the occurrence of peripherally mediated analgesic effects has been obtained from studies with opioid agonists and antagonists that are unable to cross the blood-brain barrier. Thus, it has been demonstrated that *N*-methylmorphine was able to produce an analgesic effect in the mouse writhing test which was antagonized with *N*-methylnalorphine [146]. Since these quaternized opiates do not have access to the brain, antinociception was induced through interaction with peripheral opioid receptors. Peripherally acting opioid agonists have potential for clinical applications, since they will not produce centrally mediated side-effects, such as dependence and respiratory depression. For example, such compounds could be used for the treatment of inflammatory painful conditions.

Recently, a relatively polar and moderately μ-selective enkephalin analogue, H-Tyr-D-Arg-Gly-Phe(pNO_2)-Pro-NH_2 (BW443C), has also been shown to produce peripherally mediated antinociception in the mouse writhing test at a dose of about 5 mg/kg (s.c.) [148]. Testing of the latter analogue in the mouse hot plate test indicated that at very high doses it may slowly penetrate into the brain [149]. Preliminary clinical studies indicated that side-effects of BW443C given by i.v. infusions (10 $\mu g\ kg^{-1}\ min^{-1}$ for 10 min) were relatively minor (see *Table 6.6*) and there was no evidence for central activity such as sedation, respiratory depression, or mood changes [150]. Subsequently, a series of polar D-Arg^2-enkephalin-derived tetrapeptide amides and tripeptide aralkylamides was prepared by the same group and many of these compounds exerted peripheral analgesic effects at low doses after s.c. administration and in some cases even after oral administration at somewhat higher doses [151]. Receptor binding selectivity profiles of these analogues have not been determined.

The polar and highly μ-selective dermorphin analogue DALDA (19) showed analgesic potency in the mouse writhing test similar to that of morphine [49]. The effect of DALDA was of somewhat shorter duration but, nevertheless, it was remarkable that this peptide analogue showed a still relatively low ED_{50} (2.80 mg/kg) when given 60 min prior to the administration of phenyl-1,4-benzoquinone. The antinociceptive effect of DALDA in the writhing assay could be antagonized with the quaternary antagonist *N*-methyllevallorphan, indicating that it is mediated via interaction with peripheral μ receptors. In the mouse hot plate test, DALDA showed no analgesic activity at low doses, but a dose of 50 mg/kg s.c. produced an analgesic effect which reached its peak 60 min after administration and persisted for four hours. DALDA at very high doses is able to accumulate slowly in the CNS, as it had also been observed with the enkephalin analogue BW443C and with other D-Arg^2-analogues [148,151]. Efforts will have to be continued to develop opioid peptide analogues with a further reduced ability to cross the blood-brain barrier.

Aside from peripherally induced analgesia, other peripheral effects of opioid peptides might be exploited for the development of potential drugs. Of particular interest are the various effects of opioid peptides in the gastrointestinal tract. Recently, the enkephalin analogue H-Tyr-D-Met(O)-Gly-Phe(pNO_2)-Pro-NH_2 (BW942C) was shown to be a safe and effective agent for controlling diarrhoea after cisplatin administration in cancer patients [152]. BW942C appears to be somewhat μ-selective [149] and displayed relatively minor side-effects. Another study performed with the μ-selective enkephalin analogue Hoe825 described above (*Table 6.1*) revealed that this compound has a powerful gut stimulating effect and may have therapeutic potential for the management of paralytic ileus or other gut dysfunctions requiring stimulation of motor activity [46,153]. In an initial clinical test with healthy volunteers, a sensation of heavy legs was the only side-effect observed with this enkephalin analogue [154].

CONCLUSION

In recent years substantial progress has been made towards the development of opioid peptide analogues showing high selectivity for μ or δ opioid receptors. Further efforts are required to obtain peptide ligands with high preference for κ receptors. Another future goal of great importance is the development of opioid peptide analogues showing selectivity for the putative μ, δ and κ receptor subtypes. Early clinical trials with relatively non-selective enkephalin analogues led to a certain disappointment because of a number of serious side-effects that were observed with these compounds. Peptide analogues with high specificity for distinct opioid receptor types or subtypes can be expected to produce fewer side-effects and should be examined in future clinical trials. Peptides offer the possibility of developing analogues that display great diversity in the relative liphophilicity-hydrophilicity of their structures, such that they may or may not cross certain barriers (such as the blood-brain barrier or the placental barrier). Therefore, analogues can be designed that may act at either central or distinct peripheral sites and that may have potential as selective therapeutic agents in various clinical situations.

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