A STUDY OF BIOCHEMICAL, DISPOSITIONAL AND GENETIC FACTORS UNDERLYING METIAMIDE-INDUCED AGRANULOCYTOSIS

by

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ABSTRACT

1. Whilst undergoing clinical trials, metiamide, an histamine H_2- receptor antagonist, was found to be responsible for a severe adverse reaction (drug-induced agranulocytosis) in a small proportion of treated patients. This resulted in the withdrawal of metiamide from clinical use.

2. The objectives of the work described in this thesis were to use the commercial and clinical failure of metiamide as a case-example of a drug adverse reaction, and therefore as an opportunity to evaluate the potential of retrospective biochemical, dispositional and genetic (polymorphic oxidation) studies, in drug-susceptible patients, in defining relevant aetiological factors.

3. The metabolism of orally administered [^{14}C] metiamide was studied in healthy human volunteers. Urinary metabolites included metiamide sulphoxide (10% of dose), hydroxymethyl metiamide (7%), the metiamide urea analogue (1%), metiamide N-glucuronide (24%) and metiamide (41%).

4. The possible involvement of an heritable oxidative metabolic factor underlying the occurrence of metiamide-

induced agranulocytosis was investigated by studying the influence of debrisoquine oxidation phenotype on the metabolism of metiamide in two panels of phenotyped volunteers. The debrisoquine oxidation gene locus was found to influence the quantitative urinary excretion of the above metiamide metabolites.

5. The relationships between oxidative ability, metiamide metabolism and metiamide haematotoxicity were investigated in animals in an attempt to define an animal model for this blood dyscrasia.

6. A number of host-and drug-related factors were examined in five metiamide-induced agranulocytosis and eleven control patients. It was concluded that this adverse reaction was the result of a complex interaction between a number of these factors including chemical structure, dose, duration of therapy, patient age and inherited oxidative ability of the patient.

7. These studies have highlighted the difficulties inherent in attempts at retrospective studies in patients, particularly when small numbers of patients are involved and a multi-factorial situation is under investigation.

CONTENTS

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CHAPTER ONE

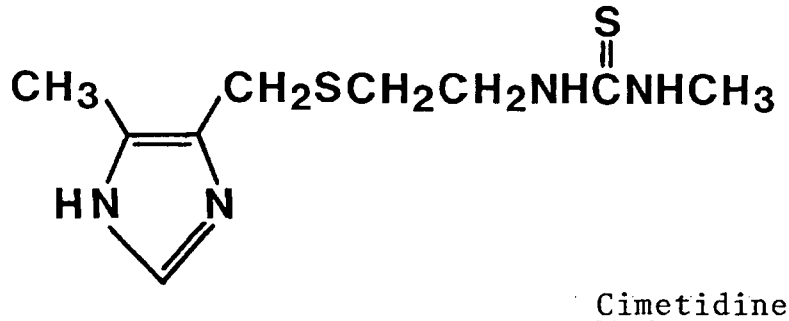
GENERAL INTRODUCTION

1.1 Introduction

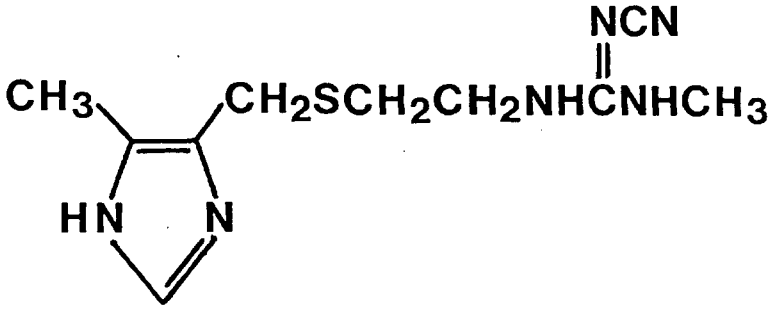
In November 1975, metiamide (Fig.1.1) was withdrawn from clinical trials due to the occurrence of a few cases of agranulocytosis associated with its use (Forest et al., 1975; Feldman & Isenberg, 1975). Metiamide, the first histamine H_2- receptor antagonist to undergo clinical trials, was the result of some ten years intensive research into the chemistry and pharmacology of histamine and its derivatives (Black et al., 1972; Brimblecombe et al.,1974). It was estimated that some twenty-three million pounds had been invested in the research programme by Smith, Kline and French Ltd. up until the time metiamide

Fig.1.1 The structures of metiamide and cimetidine

Metiamide



Cimetidine



was withdrawn. Fortunately, cimetidine (Fig.1.1), the histamine H_2-receptor antagonist in current clinical use, was already under development at that time, and although being of similar chemical structure, it proved to have none of the haematopoietic toxicity of metiamide (Burland et al., 1975; Fleischer & Samloff, 1977).

The failure of metiamide is an excellent illustration of the magnitude of the problems facing the drug industry in the development of new drugs. Vast amounts of money are invested in the research and development of new compounds, until in clinical trials an unforeseen adverse effect occurs in a small number of patients and the drug has to be withdrawn. Thus, a very large investment of time and money is put at serious risk by an 'idiosyncratic response' to the drug in some patients.

In some cases, untoward side-effects do not necessitate the withdrawal of the drug from clinical use. If the side-effects are of no serious clinical consequence, or are predictable (e.g. in a dose-dependent manner), then the drug may well be used therapeutically, so long as simple precautions are taken in prescribing and dispensing the drug.

However, the side-effect of metiamide were of the most severe type, in that they were entirely unpredictable

and involved a granulocytopenic response, an adverse response often with a high mortality. This type of adverse effect inevitably results in the prospective drug being withdrawn from clinical trials.

Dunlop (1969) has discussed the great benefits conferred on humanity by the therapeutic revolution which has occurred since the late 1930's. This revolution has been chiefly responsible for the fact that since 1930 over ten years have been added to the life expectancy of men and women in Britain. However, Inman (1970) has pointed out that all drugs with any worthwhile therapeutic activity produce adverse reactions in a proportion of patients treated with them. With new drugs being produced each year, it is increasingly important that an understanding of the underlying mechanisms involved in adverse drug reactions is gained.

Haematological reactions rank among the commoner and more serious side-effects of drugs and also among those with the highest mortality rates. Table 1.1 gives a summary of all the haematological side-effects reported to the World Health Organisation in the period 1968-73. It can be seen that granulocytopenia was the most commonly reported adverse effect, closely followed by thrombocytopenia.

Although the underlying biochemical mechanisms involved in these blood dyscrasias are fairly well understood for

Table 1.1

MAJOR HAEMATOLOGICAL REACTIONS REPORTED TO THE W.H.O. RESEARCH CENTRE FOR MONITORING OF ADVERSE DRUG REACTIONS 1968-1973.

ADVERSE REACTION	CASE REPORTS	DEATHS
APLASTIC ANAEMIA	257	123
GRANULOCYTOPENIA	1294	239
THROMBOCYTOPENIA	948	107
HAEMOLYTIC ANAEMIA	302	20
MACROCYTIC ANAEMIA (including megaloblastic anaemia)	112	5

certain drugs particularly amidopyrine and other compounds which produce allergic-type reactions, very little is known about the haematotoxic mechanisms of some other groups of drugs, such as the phenothiazines and the antithyroid drugs. It is generally believed that these agents have a direct toxic action against the bone marrow, producing a depression in the formation of mature granulocytes. However, why some individuals appear to be particularly susceptible to the toxic effects of these drugs is unknown and remains to be elucidated.

1.2 Drug-induced agranulocytosis

Werner Shultz in 1922 was the first to describe a syndrome in which the patient manifested sore throat, severe prostration and extreme reduction or complete disappearance of granulocytes from the blood, the sequelae being sepsis and death. Reports of similar conditions had been described by Brown (1902) and Turk (1907). However Shultz was the first to consider it a clinical entity. Relatively few cases were described between 1922 and 1929, but during the next five years many cases were reported.

In 1931, Kracke pointed out the correlation between the sudden appearance of many cases of agranulocytosis and the introduction of coal-tar derivatives used as drugs. The condition was especially common in countries where drugs were in widespread use, and in people who had every access

to them (pharmacists and medical staff). It was soon recognised that amidopyrine (pyramidon) was the causative factor in a number of cases of agranulocytosis. By the end of the 1930's it was known that many compounds used as drugs might under certain circumstances give rise to agranulocytosis, and that the mechanisms involved differed, depending on the offending drug.

Two main types of mechanism have come to be recognised (Girdwood, 1973), the immunological type, as in amidopyrine-induced agranulocytosis, and the bone-marrow suppression type, as occurs in chlorpromazine-induced agranulocytosis. However, the mechanisms underlying the agranulocytosis caused by some other drugs may have some of the features of both these types of mechanism.

A large number of drugs have been reported to be associated with cases of agranulocytosis. The most frequently reported drugs to the W.H.O. in the period 1968-73 are listed in Table 1.2.

Many of the compounds reported to cause agranulocytosis contain a sulphur atom in their molecular structure.

Table 1.2

DRUGS MOST FREQUENTLY REPORTED AS ASSOCIATED WITH GRANULOCYTOPENIA

CASES REPORTED TO W.H.O. RESEARCH CENTRE 1968-1973

DRUG	CASE REPORTS	DEATHS
SULPHAMETHOXAZOLE	76	7
PHENYLBUTAZONE	64	19
CHLORPROMAZINE	55	9
OXYPHENBUTAZONE	40	10
CHLORAMPHENICOL	36	11
CARBIMAZOLE	35	9
INDOMETHACIN	33	6
SALAZOSULFAPYRIDINE	30	6
CYCLOPHOSPHAMIDE*	27	4
PROCAINAMIDE	24	6
METHOTREXATE*	23	4
THIAMAZOLE	23	3
THIORIDAZINE	22	4

* These drugs associated with a dose-related predictable effect.

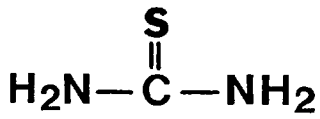
When the sulphur atom is present as part of a thiourea group, the haematotoxicity of the drug is particularly evident. Replacement of the sulphur atom by an oxygen atom or by another group appears to reduce the toxic effects of the drug on the blood. A number of sulphur-containing drugs which have been reported to have caused agranulocytosis are shown in Fig.1.2.

The antithyroid group of drugs are particularly well-known for their haematotoxic effects. They all contain the thiourea moiety in their structure, and this is the probable cause of the high incidence of agranulocytosis associated with their use. McGavack & Chevalley (1954)

Fig.1.2

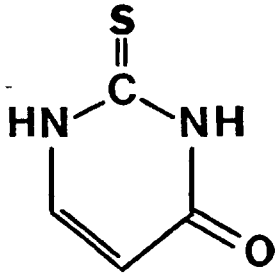
Some sulphur containing drugs known to have caused agranulocytosis

Thiourea



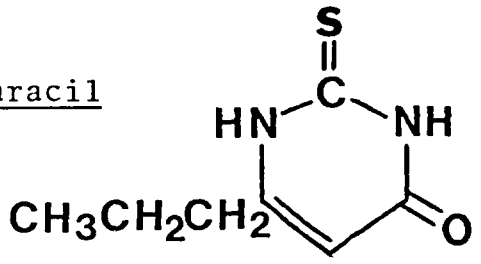
Sikkema et al. (1946)

Thiouracil



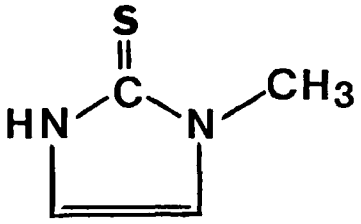
Morton (1947)

Propylthiouracil



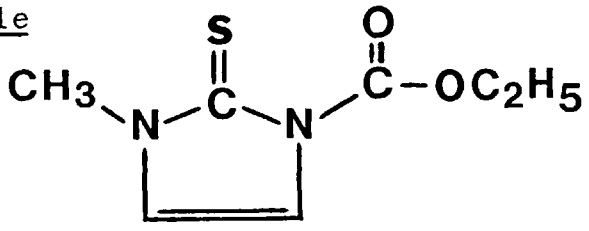
Bartels (1948)

Methimazole



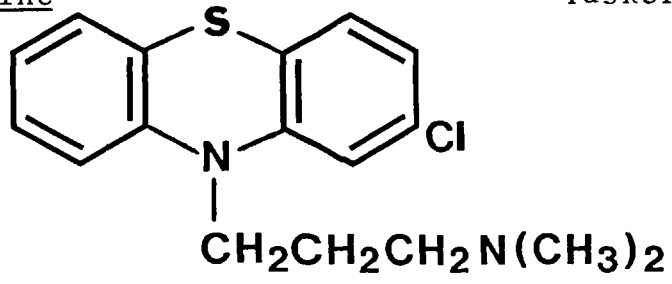
Specht & Bochme (1952)

Carbimazole



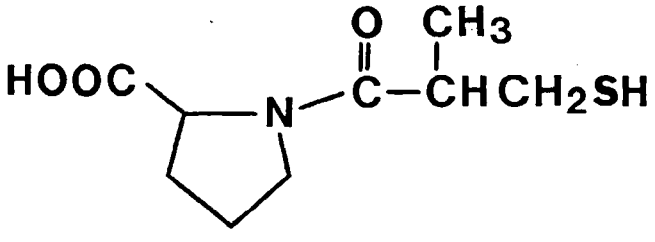
Tait (1957)

Chlorpromazine



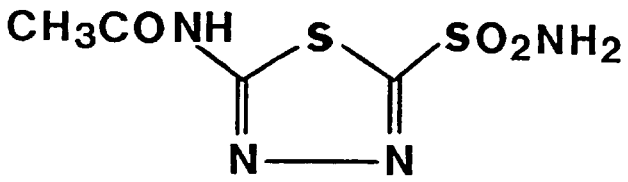
Tasker (1955)

Captopril

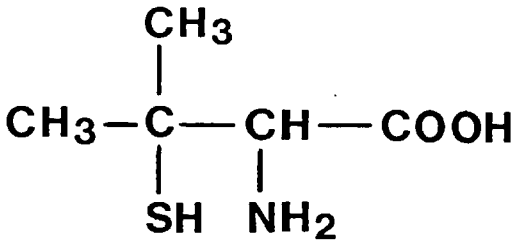


Amann et al.(1980)

Acetazolamide — Pearson et al. (1955)



Penicillamine — Corcos et al. (1964)



reviewed the literature on the haematological side-effects of the antithyroid drugs. They concluded that the bone marrow reaction to these drugs was a truly toxic, rather than allergic one, in which the nature of the drug, the size of the daily dose, the duration of administration, and a susceptible constitution all play a role.

The similarities which exist between the agranulocytosis produced by the antithyroid drugs and metiamide-induced agranulocytosis are striking, and it may well be that they share a common biochemical mechanism.

1.2.1 Polymorphonuclear Granulocytes

Blood was regarded as an homogeneous fluid until the late

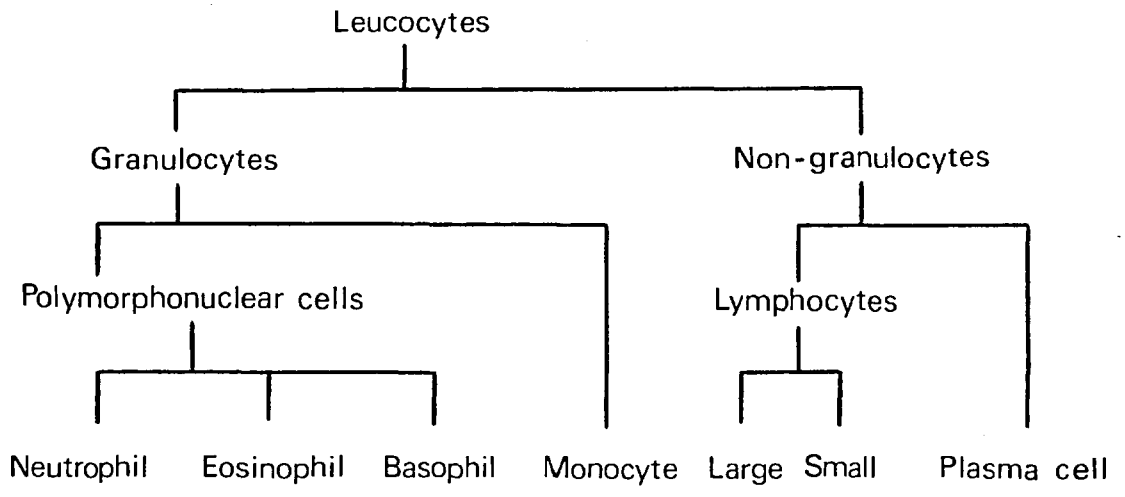
17th century when Leeuwenhock first described the red blood cell. However it was not until 1771 that the lymphocyte was first described by Hewson and not until the 19th century that the granulocyte was recognised as a distinct form of white blood cell. It is now known that the granulocytes play a very prominent role in the cellular defence system of the body, especially by phagocytosing and destroying bacteria and other foreign particles.

The classification of white blood cells into granulocytes and non-granulocytes is purely morphological but a similar classification is obtained when cell development is considered (Fig.1.3).

Total and differential white blood cell counts may vary widely in many disease states and even in healthy subjects

Fig.1.3

Classification of White blood cells



differential white blood cell counts may show wide variation (Table 1.3).

Polymorphonuclear granulocytes undergo development and maturation in the bone marrow from primitive mesenchymal cells (Fig.1.4). Development from the myeloblast stage to a mature granulocyte takes about four days to complete.

The terms 'neutropenia' and 'agranulocytosis' are used to describe conditions in which there is a reduction in the numbers of neutrophils and granulocytes, respectively, in peripheral blood.

The term 'agranulocytosis' literally means an absence of granulocytes, but in practice it is used to describe severe neutropenia. Thus, a patient with a neutrophil count of less than 5000 mm^{-3} is often arbitrarily said to have agranulocytosis (De Gruchy, 1975).

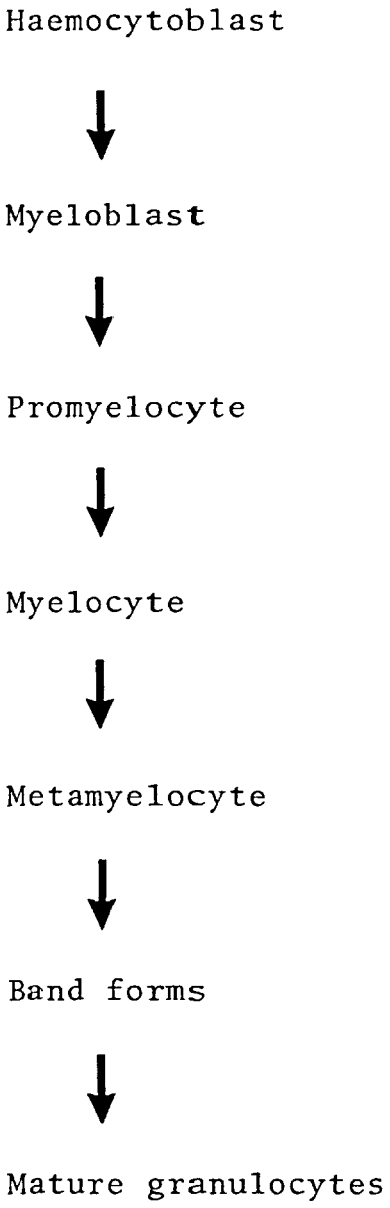
Table 1.3

Normal differential leucocyte count

Cell type	% W.B.C.	Cells (mm^{-3})
Lymphocyte	20-45	1,500-3,500
Monocyte	2-10	200-800
Neutrophil	40-75	2,500-7,500
Eosinophil	1-6	40-450
Basophil	0-1	0-100

Fig.1.4

Development of polymorphonuclear granulocytes in bone marrow



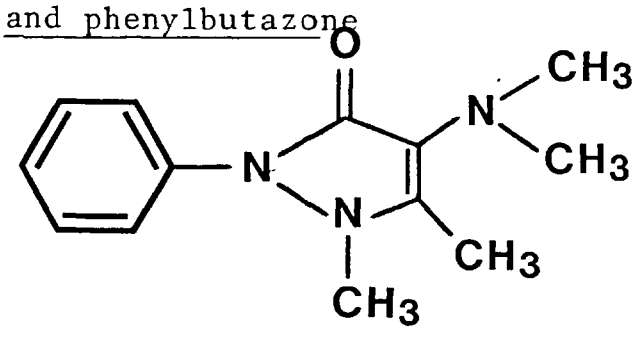
1.2.2 Immunological-type agranulocytosis

The immunological-type of agranulocytosis is typically induced by amidopyrine and related pyrazolone compounds as well as by phenylbutazone and oxyphenbutazone (Fig 1.5), which are pyrazoline derivatives. Girdwood (1971), considered the pyrazoline derivatives to be the commonest causative agents in Great Britain.

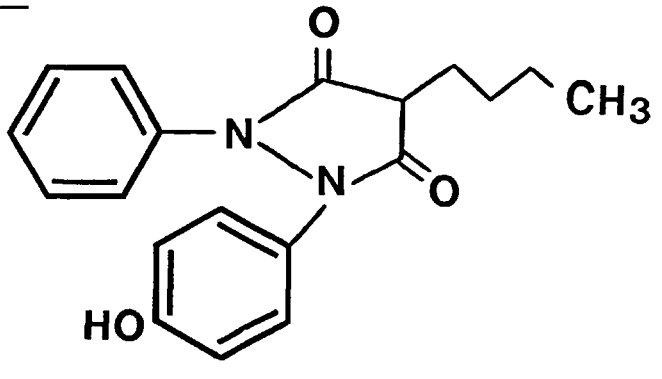
The initial features of the condition may be sudden, with high fever, chills, arthralgia and severe prostration. These symptoms may appear within six to ten days from the commencement of treatment. In the acute stage of the disease, the most dramatic haematological finding is the sudden fall in the levels of circulating leucocytes, often to below 1000 cells mm^{-3}. Granulocytes may be totally

Fig.1.5 The structures of amidopyrine, oxyphenbutazone and phenylbutazone

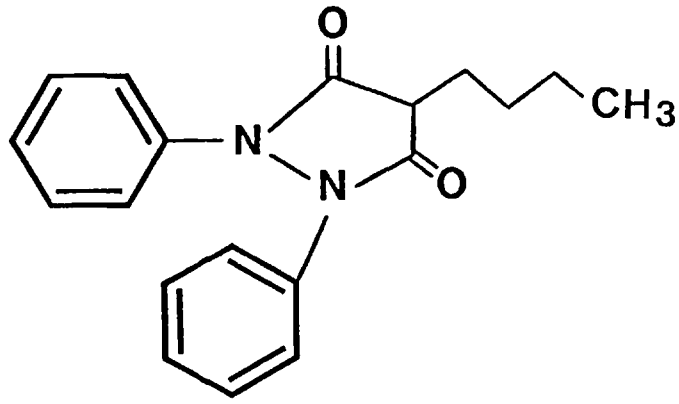
Amidopyrine



Oxyphenbutazone



Phenylbutazone



absent from the blood and those that do appear usually show toxic granulation. Studies of the bone marrow during the acute stage of the disease show a marked dimunition of mature granulocytes with a "shift to the left" of their precursors. In severe cases, there may be a complete absence from the marrow of granulopoietic stem cells.

When the peripheral leucocyte count falls below 800 cells mm^{-3} the cellular defence mechanisms of the body tend to break down leading to bacterial invasion. Ulcerations, which histologically show an absence of granulocytes, occur mainly in the oropharynx giving agranulocytic angina. They are also found on the lips, gums and tongue. Severe septic complications have become much rarer since the introduction of antibiotics and the prognosis in such conditions has much improved. Allergic rashes are uncommon in this type of agranulocytosis.

Mechanism of immunological-type agranulocytosis

Most of the work carried out on immunological-type agranulocytosis has been studies on amidopyrine-induced agranulocytosis. This drug was first introduced into clinical medicine in 1897 (see Huguley, 1964). However, it was not until nearly forty years later that it was recognised as a cause of agranulocytosis.

Hypersensitivity to amidopyrine was first described in a physician who had recovered from three previous attacks of agranulocytosis, each following a dose of amidopyrine (Madison & Squier, 1934). Ten months after the last attack he was given a test dose of 0.3g of amidopyrine by mouth. Within twelve hours his white cell count had dropped from 7800 cells mm^{-3} to 2000 cells mm^{-3}, and the granulocytes from 4750 cells mm^{-3} to 250 cells mm^{-3}. Subsequently the white blood cell count rose to normal levels. Benjamin & Biedeman (1934) and Dameshak & Colmes (1936) reported similar cases.

In the 1930's Moeschlin & Wagner (1952) and Moeschlin (1955) reported *in vivo* experiments which gave strong support to an immunologically-based theory for the blood dyscrasia. Firstly, it was shown that neutropenia developed in a normal recipient to whom plasma, from a sensitive patient, had been given three hours previously. Then,

amidopyrine was given to normal subjects in whom no haematological reaction occurred until they were given plasma from a patient who had previously suffered from amidopyrine-induced agranulocytosis. Their white blood cell counts then fell rapidly.

Moeschlin (1955) also showed that guinea-pigs developed agranulocytosis when they were given heterologous anti-leucocyte serum. He demonstrated that masses of agglutinated leucocytes could be seen in the lungs prior to their destruction. He suggested that similar sequestration and destruction of leucocytes occurred in the lungs of patients with amidopyrine-induced agranulocytosis.

Cross-reactivity between amidopyrine and related compounds has been demonstrated *in vitro* (Huguley, 1964) and the antibody involved in noramidopyrine-induced agranulocytosis characterised immunologically (Magis *et al.*, 1968). It was found to be a stable γ-globulin present in the IgM and IgG portions.

The possible immunological mechanisms for cell destruction have been considered in detail in recent years and this has led to the formation of various hypotheses. The work of Landsteiner (Landsteiner & Van der Scheer, 1936; Landsteiner & Jacobs, 1936; Landsteiner, 1947) led to the

generally accepted view that drugs, which are simple chemicals (haptens) can become immunologically active only after covalent binding to a protein carrier, thus forming an immunologically competent antigen. The antigenicity of a drug is determined by its ability to combine chemically to proteins. The specificity of the conjugated antigen (drug-protein complex), is determined mainly by the structure of the hapten and to a lesser degree, by the nature of the carrier protein.

The exact mechanism is still unclear as to how the drug-protein complex brings about cell destruction. However, it is thought that antibodies are formed against the cell-drug-protein complex. This immune complex then fixes complement and the granulocytes lyse.

2.2.3 Bone-marrow depression-type agranulocytosis

This form of agranulocytosis is commonly caused by chlorpromazine, sulphonamides and the antithyroid drugs. Unlike the first type of the disorder, the clinical onset of this form is insidious, there being no dramatic initial features. It may be only after several months treatment that a pronounced granulocytopenia becomes evident. The haematological picture may be very different

from the one which characterises the first type in that anaemia and thrombocytopenia often accompany the granulocytopenia. Sometimes the anaemia is haemolytic (Lindberg & Norden, 1961). Jaundice or minor hepatic dysfunction may be observed and skin eruptions are not uncommon.

While in the first type of agranulocytosis re-exposure to the offending agent results in an immediate relapse, patients recovering from the chlorpromazine-type agranulocytosis may tolerate small doses of the offending drug without showing signs of further haematological damage.

Mechanism of marrow depression-type agranulocytosis

A different mechanism obviously underlies this form of agranulocytosis. It is believed that the agranulocytosis occurs through the suppression of granulocyte precursor cells in the bone marrow by the offending agents. The destruction of mature granulocytes in the blood is thought not to occur, or to be of only minor importance.

Parker & Kracke (1936) found a deficiency of reduced glutathione in the blood and bone marrow of rabbits rendered agranulocytic with benzene. They suggested therefore, that a depletion of reduced glutathione by the offending agent lead directly to a granulocytopenic

state. They believed that the depletion of reduced glutathione levels, in the rabbit, was caused by an oxidative metabolite of benzene.

Pisciotta in the early 1960's studied a number of cases of agranulocytosis caused by certain phenothiazine derivatives, particularly chlorpromazine. In these cases he found no difference in either the levels of reduced glutathione or in the ability to produce reduced glutathione in the blood, from controls (Pisciotta & Daly, 1960).

In a series of experiments, in which bone marrow cells of patients recovered from chlorpromazine-induced agranulocytosis were cultured in the presence of chlorpromazine , it was found that chlorpromazine uncovered a fundamental defect in cell division involving DNA synthesis (Pisciotta, 1969, 1971).

Pisciotta suggested that these chlorpromazine-sensitive patients are normal in all respects until they encounter a drug such as chlorpromazine which inhibits cell division. Unable to compensate for the toxic effects of the drug on the granulocyte precursor cells, the patient eventually becomes agranulocythaemic.

Bock (1946) has stressed that there is no clear-cut

boundary between allergic and toxic forms of agranulocytosis. In his view, one end of the spectrum (predominently allergic) is represented by amidopyrine and related compounds, whilst at the other, or toxic end, are the arsenicals. Between these extremes are the sulphonamides, the thiourea group, gold salts and the phenothiazines.

Studies by Urban (1972) support the view that there may be an overlap of immunological and toxic factors associated with individual variation in the susceptibility of granulocytes. He transfused granulocytes from a patient who had suffered a drug-induced agranulocytosis into a healthy subject and found that they had a distinctly shorter survival time than granulocytes from a normal donor given to the same subject.

1.3 Toxicity of thiourea and thiourea-containing compounds

Thiourea and some of its derivatives have been used in the treatment of hyperthyroidism since the early 1940's. Thiourea itself, and thiouracil were the first compounds to be used clinically, but it was soon discovered that a high incidence of agranulocytosis was associated with their use (Sikkema et al., 1946; Morton, 1947; McGavack & Chevalley, 1954).

In order to reduce the haematotoxicity of these antithyroid drugs, various derivatives of thiourea have been developed for clinical use. They include methylthiouracil, propylthiouracil, methimazole , and carbimazole. However, all of these compounds have been associated with a low incidence of agranulocytosis (Rachmilewitz & Rosin, 1949; Bartels, 1948; Specht & Boehme, 1952; Tait 1957).

Thiourea and its derivatives are known to show various other toxic effects in animals. Most are hepatotoxic, and at high doses some thiourea derivatives produce pulmonary oedema and pleural effusion in susceptible animals (Hunter & Neal, 1975; Latta, 1947). Susceptibility is, at least in part, related to the concentration of glutathione in lung tissue (Mountain, 1968). Glutathione and some other thiols can protect against the lethal effects of thiourea derivatives (Baimonova & Serebrovskaya, 1973; Meyer & Karel, 1948). Thiols are also reported to decrease the binding of thiouracil metabolites to protein in vitro (Jirousek & Cunningham, 1968).

Biochemical mechanisms of thiourea toxicity

Smith & Williams (1961) studied the metabolism of a number of thiourea derivatives in rats and rabbits. They postulated that the toxicity of thiourea was related to its oxidative desulphuration in the body to release H_2S, which

is highly toxic, in the tissues. Thioureas with substituents on both nitrogen atoms of the thiourea group were found to be less toxic than those substituted on only one nitrogen atom. Thus, N-phenyl-N-methylthiourea was more extensively desulphurated and more toxic than the N-phenyl-N'-methylthiourea analogue, in rabbits (200 mg kg^{-1}). It was suggested that this was due to steric hindrance of the substituent groups on the nitrogen atoms not allowing a 'good fit' to the desulphurating enzyme site.

More recently, Neal and his co-workers (Hunter & Neal, 1975; Boyd & Neal, 1976) have shown that certain thiocarbamides are metabolised *in vitro* and *in vivo* to reactive intermediates which bind to the macromolecules of lung and liver.

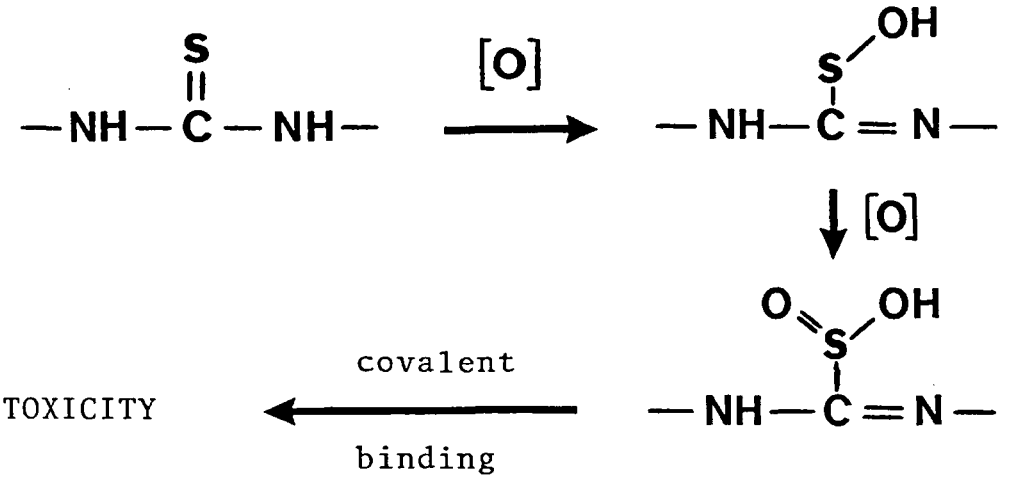
The work of Zhislin & Ovetshaya (1972) suggests that the toxicity of thiourea is the result of its metabolism to the sulphinic acid. Formamidine sulphinic acid produced pathological changes in rats similar to those observed with thiourea and the sulphinic acid was ten times more toxic than the parent compound. Oxidation of methimazole to its corresponding sulphinic acid has also been reported (Poulsen *et al.*, 1974).

Ziegler (1978) and his co-workers (Poulsen *et al.*, 1974)

have described a microsomal amine oxidase which can catalyse the rapid S-oxidation of thiocarbamides and thioureylenes to sulphenic and ultimately to the sulphinic acid analogues (Fig.1.6). However, it is unlikely that either of these metabolites would be an end product of metabolism in vivo as they are extremely reactive compounds. They appear to be rapidly reduced

Fig.1.6

Postulated metabolic activation of thiourylene sulphur and toxic consequences (after Ziegler, 1978)



back to the parent thione by glutathione, so none of these metabolites would accumulate at physiological concentrations of glutathione.

The rapid reduction of the sulphenic and sulphinic acids by glutathione thus leads to a 'futile cycle' which operates at the expense of NADPH and oxygen. Experiments carried out with hamsters (Ziegler , 1978) suggest that at toxic doses *in vivo* the cycle is terminated by the loss of glutathione. Glutathione levels fell rapidly in liver, lung and bone marrow of animals treated with methimazole and the change in glutathione concentration was both time and dose dependent. At toxic doses the rate of glutathione depletion in different tissues also correlated with the activity of amine oxidase and initial glutathione concentration.

The metabolism of propylthiouracil has been extensively studied. Lam & Lindsay (1979) demonstrated that phagocytosing polymorphonuclear neutrophils incubated with $[^{14}C]$ propylthiouracil accummulate radioactivity. A substantial amount of this radioactivity was bound to protein. Lindsay *et al*. (1978,1979) identified propylthiouracil sulphonic acid as the primary metabolite in the polymorphonuclear cells.

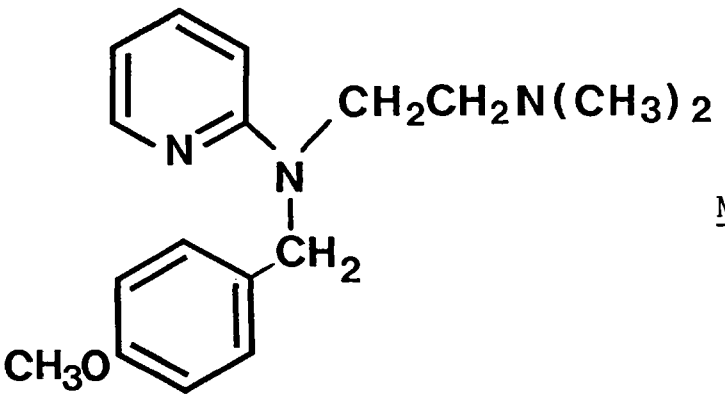
The *S*-oxidation of metiamide and cimetidine to produce their corresponding sulphoxides are catalysed by 'Ziegler's flavoprotein mono-oxygenase' enzyme with low Km values. The oxidation of the sulphur atom in the thiourea moiety of metiamide was also found to be catalysed by 'Ziegler's enzyme', but with a high Km value and it was concluded

that this reaction was probably unimportant *in vivo* (D.M.Ziegler, personal communication).

1.4 Metiamide-induced agranulocytosis

1.4.1 Development of metiamide

In 1964, Smith Kline and French Limited embarked on a research programme into the pharmacology of histamine. It was already known that histamine stimulated contractions of smooth muscle from various organs, such as gut and bronchi, and that this effect could be antagonised by low concentrations of the conventional antihistamine drugs *e.g.* mepyramine.



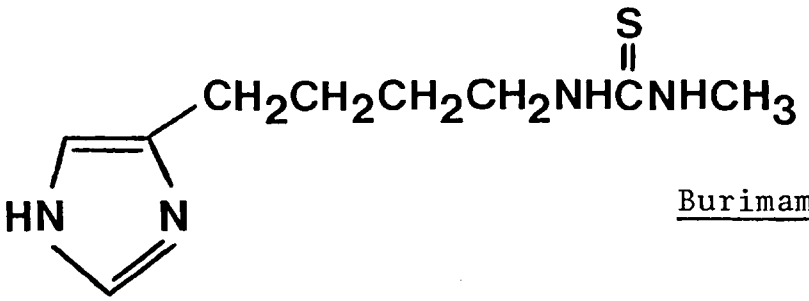
Mepyramine

The pharmacological receptors in the mepyramine-sensitive histamine responses were defined as histamine H_1-receptors (Ash & Schild, 1966).

It was known however, that histamine also stimulates

secretion of acid in the stomach, increases the heart rate and inhibits contractions in the rat uterus. These actions cannot be antagonised by mepyramine and related compounds, and were therefore thought to be mediated through a second population of histamine receptors, subsequently known as histamine H_2-receptors. The Smith, Kline & French research programme was directed towards discovering and developing specific histamine H_2-receptor antagonists.

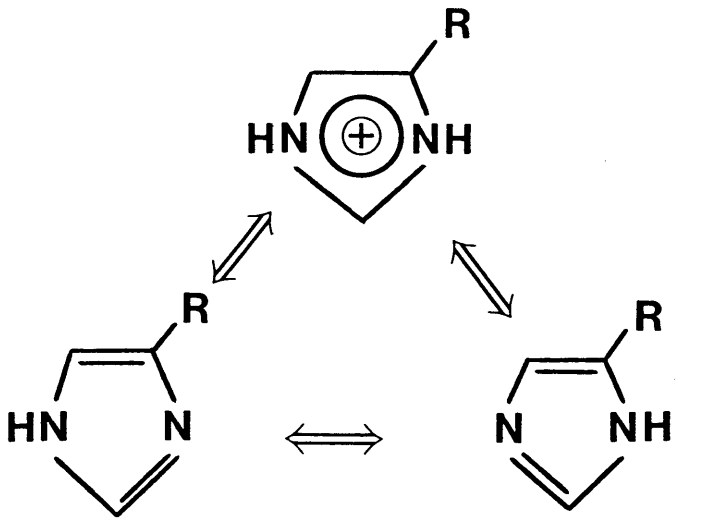
This research eventually produced burimamide.



Burimamide

Although burimamide was sufficiently selective pharmacologically, it lacked adequate oral bioavailability and so attempts continued to produce a more suitable drug. Histamine and burimamide are both substituted imidazoles, however, the predominent species of the respective imidazole rings are chemically different. If the active form of the antagonist were species A (vide infra), the form most preferred for histamine, then increasing its relative abundance might increase activity.

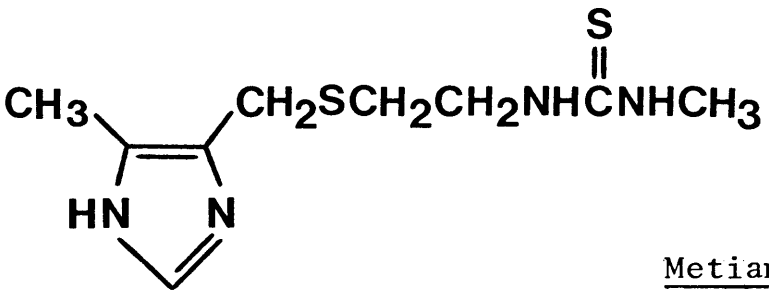
Species C



Species A

Species B

This was achieved by including an additional electronegative atom (sulphur) into the antagonist side chain to convert it into an electron withdrawing group. Since a further stabilisation of tautomer A might be obtained by incorporating an electron-releasing substituent in the 5-position of the imidazole ring, a methyl group was substituted into the ring, thus producing metiamide.



Metiamide

Metiamide was found to be pharmacologically more active than burimamide, especially after oral administration and therefore underwent extensive pharmacological, toxicological and biochemical investigations on animals and in volunteers. These investigations showed that metiamide had properties suitable for the evaluation of the therapeutic potential

of histamine H_2-receptor blockage in man, namely high specific activity, low toxicity and good oral bio-availability.

1.4.2 Toxicology of metiamide

Comprehensive toxicological investigations were carried out with metiamide by Smith, Kline and French Limited as part of the preclinical studies (Brimblecombe *et al.*, 1974).

Acute toxicity of metiamide

The LD_{50} values for the rat and the mouse were determined for both oral and intravenous routes of administration (Table 1.4).

Table 1.4 LD_{50} values for metiamide in the rat and mouse

Species	Route of administration	LD_{50} (mg kg^{-1})
Mouse	oral	2642
Mouse	i.v.	141
Rat	oral	1493
Rat	i.v.	142

Clinical and post mortem examination of the animals failed to establish the cause of death but it was thought to be due to respiratory failure. In the rat, the oral LD_{50} for metiamide is 680 times greater than the oral ED_{50} for the antagonism of histamine-induced acid secretion.

In six other species (guinea pig, hamster, baboon, rabbit, cat and marmoset) no drug-attributable deaths were observed at dose levels of up to 900 mg kg^{-1}.

However, in the dog, out of 122 dogs which received metiamide in doses of greater than 50 mg kg^{-1} 11 dogs died acutely. At autopsy they were found to have severe pulmonary oedema and 2 dogs had pleural effusions. This chronic syndrome is similar to that reported in the literature for dogs and rats dosed with arylthioureas (Latta, 1947).

Chronic toxicity of metiamide

No drug-attributable deaths were observed in rats given oral doses of up to 366 mg kg^{-1} day^{-1} metiamide for a period of 12 months.

In the dog, groups of six were administered metiamide orally at various doses for a 3 month period (Table 1.5)

Table 1.5 3 month metiamide toxicity test in the dog

Dose(mg $kg^{-1}day^{-1}$)	No.of dogs	No.dogs displaying granulocytopenia
162	6	0
81	6	2
40.5	6	0

In the group receiving 81 mg $kg^{-1}day^{-1}$ two littermates (male 7 and female 11, cited by Brimblecombe et al., 1974) had haematological changes. Male 7 had a low polymorphonuclear leucocyte count first detected on day 51 which continued to fall over the next 36 days until he had peripheral agranulocytosis and was eventually sacrificed. Female 11 had granulocytopenia on day 79 but quickly recovered when dosing with metiamide was stopped. The haematological values of the other dogs in this group were normal (Table 1.6).

In the dog 1 year study, 3 dogs developed a granulocytopenic reaction (Table 1.7).

Table 1.6

THREE-MONTH TOXICITY TEST IN DOGS

Dose 81 mg kg^{-1}.

Dog No.	Numbers of polymorphs per mm^{-3} of blood			
	Pre-dose	Day 25	Day 51	Day 79
7	6834	5720	430*	374*
8	11410	10240	9834	13398
9	6750	7020	9295	8584
10	9520	13320	8060	8500
11	7788	7080	7565	1120*
12	8550	9632	9780	9120

* Denotes granulocytopenia

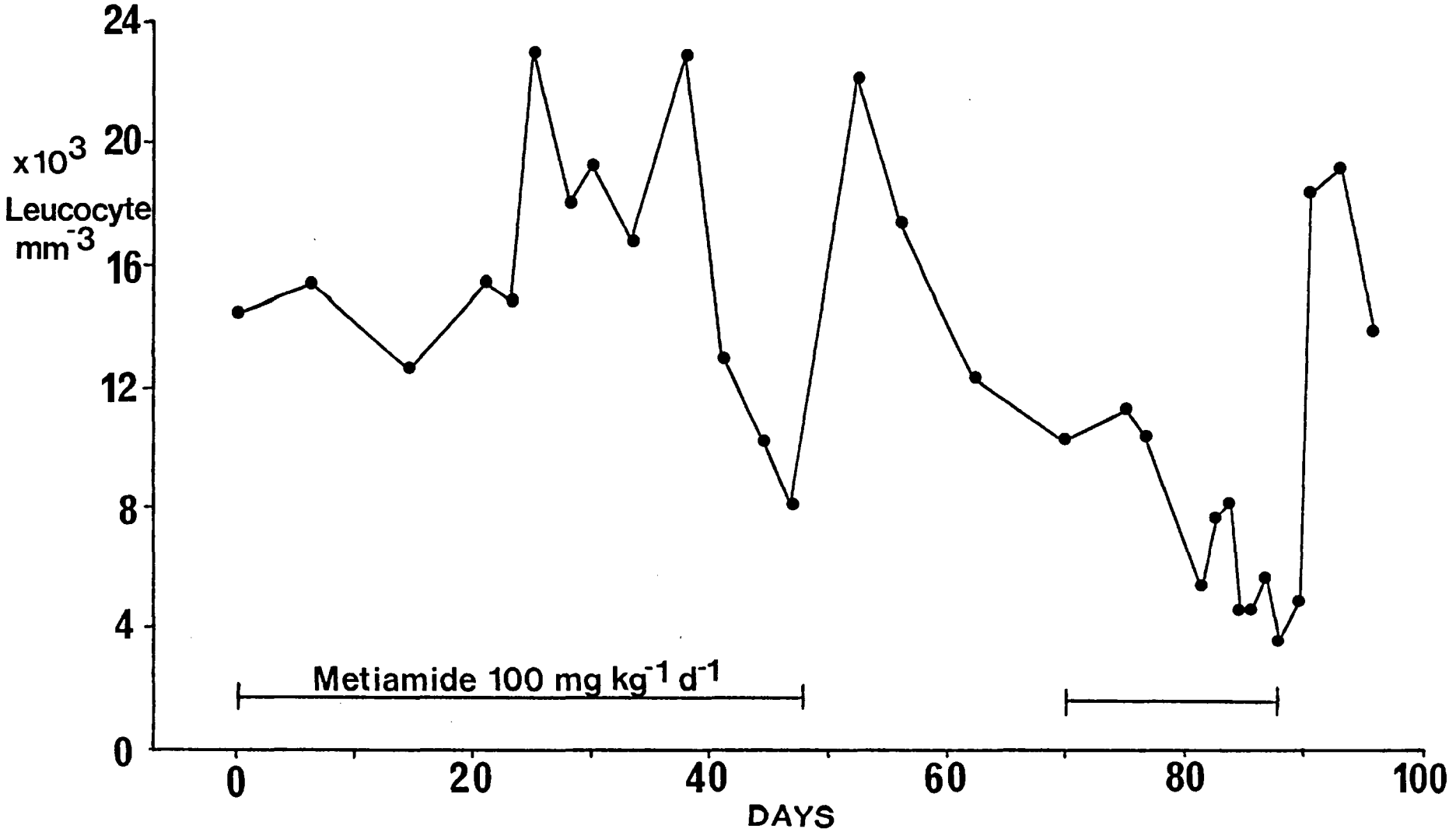
Table 1.7 One year metiamide toxicity test in the dog

Dose (mg kg^{-1} day^{-1})	No.of dogs	No. dogs displaying granulocytopenia
162	8	2
81	8	1

In one of these dogs, a female, the granulocytopenia progressed to agranulocytosis which was shown to be readily and rapidly reversible on withdrawal of metiamide treatment (Fig.1.7). Examination of the bone marrow suggested that the granulocytopenia was due to a maturation arrest in the myeloid series.

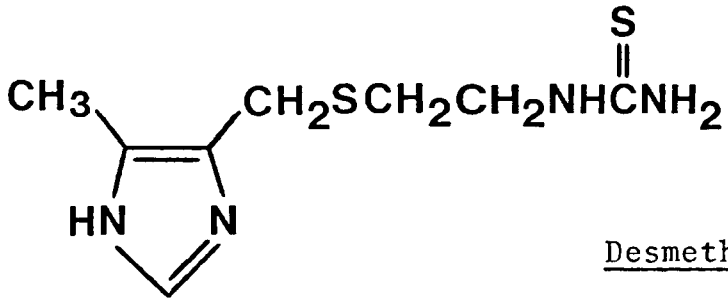
The toxicological investigations with metiamide produced some puzzling results. In dogs, the toxicity of metiamide appears to be very similar to that observed with the monoarylthioureas (Dieke & Richter, 1946) in that it produces death with pulmonary oedema

Fig.1.7 Blood leucocyte levels of one female dog during metiamide toxicity trials.



and pleural effusion, but in rats the LD_{50} of the compound is extremely high, with no apparent thiourea-like toxicity.

Structure-activity studies have indicated that greater toxicity is associated with mono-N-substituted thiourea derivatives (Dieke et al., 1947). It was considered possible that metiamides thiourea-like toxicity in some dogs was due to their ability to N-demethylate metiamide to form N-methylimidazolylmethylthioethylthiourea (desmethylmetiamide).



Desmethylmetiamide

However, up to 200 mg kg^{-1} of this compound given orally to dogs was not lethal (Spencer & Sutton, 1977).

The toxicity studies on metiamide thus showed it was of low toxicity in all species tested except the dog, in which it appeared to have a thiourea-like action in a number of 'susceptible' animals. Sensitivity to metiamide in the dog manifest itself by death from

pulmonary oedema and pleural effusions at high doses, and by the occurrence of agranulocytosis in some dogs during chronic administration. Some evidence that the susceptibility to metiamide was associated with a genetic factor was obtained when it was observed that two of the dogs which developed agranulocytosis were littermates. Whether or not this genetic factor was an inborn susceptibility of the haemopoietic tissue to metiamide, or a genetically determined variation in the metabolism of metiamide was unknown.

1.4.3 Metiamide in clinical trials

The clinical trials of metiamide were carried out over a period of about 20 months from November 1973 until August 1975. During this time some 700-800 patients received metiamide at various centres in Britain, U.S.A. and South Africa. The majority of the trials were carried out in Britain with the result that most of the reported cases of metiamide-induced agranulocytosis were from Britain. In all, 13 cases of neutropenia were reported to Smith, Kline and French Ltd. in the clinical trials, but only one patient subsequently died (Feldman & Isenberg, 1976).

None of these patients had any previous history of drug

hypersensitivity or other idiosyncratic drug response (Smith, Kline & French Ltd., personal communication). All the patients were white caucasians who had received metiamide at high dose levels for at least 3 weeks of continuous therapy. Where metiamide treatment was re-commenced after an earlier neutropenic response, the neutropenia returned only after days or even weeks of further treatment.

Blood and bone-marrow investigations

Professor Mollin of St.Bartholomew's Hospital, London, reviewed some of the bone-marrow aspiration slides and peripheral blood cell counts from patients with metiamide-induced agranulocytosis. In his view, metiamide affected more than one stem-cell line in the bone-marrow. However, since the condition was reversible in both animals and man, it was probably not affecting an original stem-cell (as does chloramphenicol, which may produce irreversible aplastic anaemia), but some other cell in the developmental line.

Data from some patients on metiamide who had been treated for four weeks or more and who had been selected because of slight falls in erythrocyte levels also showed a significant downward trend in white cell counts. It was felt that this was in agreement with the pattern of bone-

marrow toxicity seen with metiamide, as normally in the bone-marrow there is a cyclisation of all the stem lines and to find both the white and red blood cell stem lines showing a steady downward trend over one or two months would be most unlikely.

The white blood cell precursors in the bone-marrow showed obvious signs of agranulocytosis or recovering agranulocytosis. A 'shift to the left' in myelopoiesis was apparent, and there was either an absence or marked reduction in the numbers of metamyelocytes and segmented neutrophils. The appearance of the red cell precursors was variable, in some patients they were active and normal in appearance, whilst in others, erythropoiesis was macronormoblastic and some defective haemoglobinisation was noted. However, in all patients thrombopoiesis appeared normal.

Investigations on involvement of drug-hypersensitivity

A female patient was the first patient to develop a neutropenic response to metiamide in November 1973. An allergy to metiamide was suspected at this stage and so a number of investigations were performed (Table 1.8).

Table 1.8 Investigations for a possible metiamide-allergy

Test	Positive test indicates	Result
1. Lymphocyte transformation test	Delayed-type hypersensitivity or other immune reaction to metiamide	Negative
2. Total serum IgE	Increased in immediate-type hypersensitivity	Normal
3. Presence of leucocyte antibodies	Presence of antibodies against leucocyte-drug complex	Negative

These results led the investigators to exclude the possibility that the granulocytopenia was due to an allergic reaction to metiamide. They concluded that the reaction was due to bone-marrow toxicity.

Statistical analysis of blood cell counts during trials

A statistical analysis (R.Wiseman, personal communication) of the haemotological data from patients in the clinical trials of metiamide produced some interesting results. Neutrophil cell counts, based on all patients, showed a statistically significant ($2P < 0.05$) reduction for day 28 (4,090 cells mm^{-3}) as compared to day 0 (4,580 cells mm^{-3}).

These observations may reflect the specific toxic effect of metiamide on the white blood cells of all patients. Thus, given a sufficiently high dose for a long enough period of time it is possible that every patient would eventually progress to a granulocytopenic state.

However, the patients selected for the clinical trials with metiamide all had proven peptic or duodenal ulceration. It may well be that the fall in white blood cell counts was a reflection of the reduction of inflammation around the ulcer site in these patients, due to reduced acid secretion. Slight falls in the levels of white blood cells have also been observed in patients on cimetidine therapy (R.Celestin, personal communication).

Autoradiographic studies with metiamide and cimetidine

After the withdrawal of metiamide from its clinical trials some studies were performed to determine whether or not differences in the whole-body or cellular distribution of metiamide and cimetidine, could explain the observed differences in haematotoxicity between these two structurally very similar compounds (Cross, 1977).

Bone-marrow smears made onto film after the ingestion of $[^{3}H]$ metiamide, $[^{3}H]$ cimetidine or $[^{35}S]$ thiourea in the rat showed that the label from metiamide and thiourea

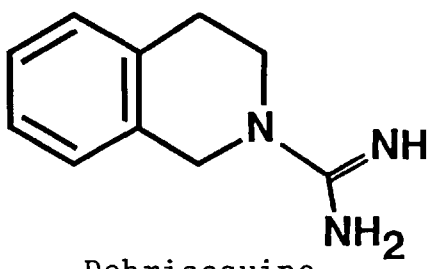
was retained in a variety of cells up to 6h after administration. [^{3}H] metiamide was retained particularly heavily in the basophils but some eosinophils neutrophils, stem cells and megakaryocytes were also labelled. Radioactivity from [^{35}S] thiourea was apparent over basophils and many granulocytes. In contrast, there was no uptake of label in bone-marrow cells after cimetidine. Bone-marrow smears from dogs showed similar patterns of retention for each of the compounds, as occurred in the rat.

These observations gave further support to the view that the haemopoetic toxicity of metiamide was not related to its histamine H_2- receptor antagonist properties, but involved the thiourea moiety in its structure. The fact that cimetidine did not appear to associate with any cells either in the bone marrow or the blood correlates well with the observed lack of haematotoxicity by cimetidine in clinical use.

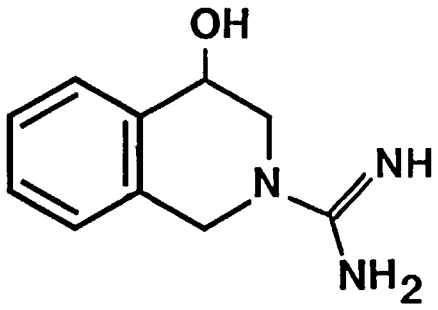
1.5 Polymorphic oxidation of drugs

It has recently been established that the ability to oxidise certain drugs is genetically controlled, and in man at least, exhibits polymorphism. This polymorphism was first uncovered with the antihypertensive drug debrisoquine, which is metabolised to its major metabolite

4-hydroxydebrisoquine by alicyclic oxidation (Idle et al., 1979).



Debrisoquine



4-Hydroxydebrisoquine

It was found that within a British population there existed a very wide range of abilities to perform this reaction. Most individuals excreted more 4-hydroxy-debrisoquine than debrisoquine in the urine, however, a few individuals were found to eliminate debrisoquine unchanged in the urine with only trace amounts of metabolite. Further research, including family studies, showed that this impaired ability to oxidise debrisoquine was under monogenic control and exhibited polymorphism (Mahgoub et al., 1977; Evans et al., 1980).

A simple test was devised to assess the extent of an individual's oxidative ability (Mahgoub et al., 1977). The subject ingests a 10 mg debrisoquine tablet and then collects all urine for the subsequent 8h. A metabolic ratio is then calculated.

$$\text{Metabolic ratio} = \frac{\%\text{ dose in urine as debrisoquine}}{\%\text{ dose in urine as 4-hydroxydebrisoquine}}$$

Thus, the lower the metabolic ratio, the higher the individual's oxidative ability.

A population study of British white caucasians showed that about 9% of the population are defective oxidisers, denoted PM's, with metabolic ratios of 12.6 and above. Individuals with ratios of less than 12.6 are relatively good oxidisers and are denoted EM's. (Evans et al., 1980).

Influence of the polymorphism on other drug oxidations

Oxidation is the commonest reaction encountered in the detoxication of drugs, however, considerable inter-individual variation occurs in the ability to carry out some of these oxidation reactions. Both environmental and genetic factors are known to contribute to this variability, but the relative importance of each factor in a large number of drug oxidations is unclear. The role played by genetic factors is particularly poorly understood.

It was therefore of some considerable interest to discover whether or not the debrisoquine polymorphism influenced the metabolic oxidation of other drugs. A number of drugs have now been investigated and some have been shown to be under the control of the same gene locus

as debrisoquine (Table 1.9).

The clinical significance of these findings has not been assessed for all of the affected drug oxidations. However, it would appear that, at least in some of these cases, the occurrence of polymorphic oxidation has important pharmacological and toxicological implications for the use of these drugs in man.

Table 1.9

Genetically controlled drug oxidations in man

Drug	Reaction affected	Reference
Debrisoquine	Alicyclic hydroxylation	Mahgoub et al. (1977)
Debrisoquine	Aromatic hydroxylation	Woolhouse et al. (1979)
Guanoxan	Aromatic hydroxylation	Sloan et al. (1978)
Phenformin	Aromatic hydroxylation	Shah et al. (1980)
Phenytoin	Aromatic hydroxylation	Idle et al. (1979)
Phenacetin	O-dealkylation	Sloan et al. (1978)
Sparteine	N-oxidation	Eichelbaum et al. (1979)

Implications of polymorphic drug oxidation

The existence within the population of large inter-individual differences in the ability to metabolise drugs via certain oxidative pathways poses some difficult problems for the correct use of therapeutic agents. Most drugs are prescribed at 'standard' doses, *i.e.* those doses which give the most benefit and least risk to the majority of patients. These 'standard' doses may however, be quite unsuitable for certain individuals whose metabolic constitution may be markedly different from the majority of the population. These individuals may have a much greater risk than others of idiosyncratic reactions to drugs.

Some information on the metabolic basis of these idiosyncratic responses to drugs is now being gained through the study of genetically-determined metabolic oxidation of drugs such as occurs with debrisoquine, phenacetin and phenformin.

In the case of debrisoquine, subjects of the PM phenotype develop postural hypotension with small oral doses (20 mg) of the drug, whereas this does not occur in the EM phenotype (Idle *et al.*, 1978). This is due to the fact that the bioavailability of debrisoquine is greater in PM subjects as a result of impaired metabolism, and

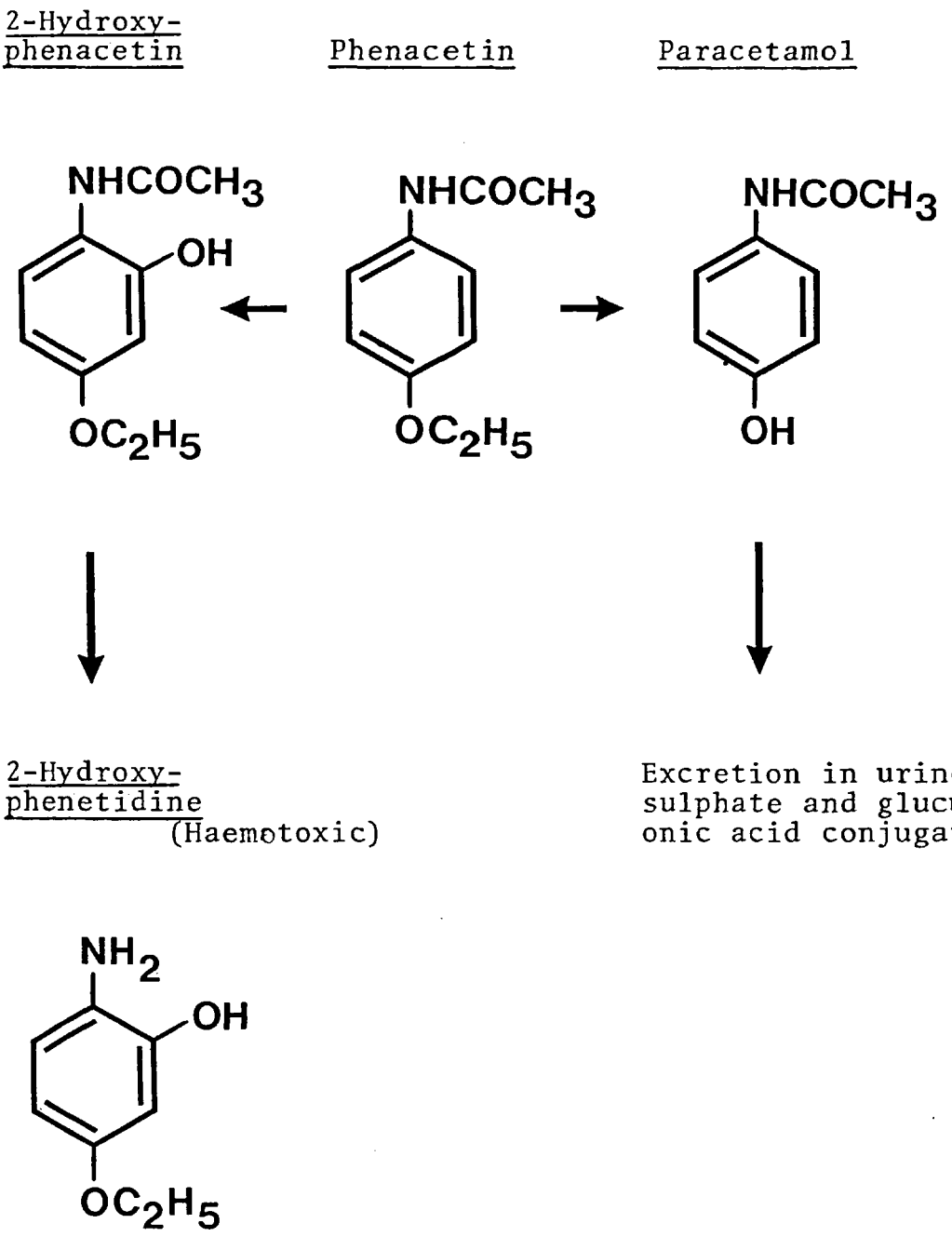
peak plasma levels may be 3-4 times higher than those found in EM individuals given an identical dose.

Other undesirable side-effects of drugs are known to be due to abnormally high levels of certain drug metabolites in the body. Phenacetin undergoes O-deethylation in the body to form paracetamol which is subsequently eliminated as its glucuronic acid and sulphate conjugates. A minor metabolic pathway also exists whereby aromatic hydroxylation of phenacetin gives 2-hydroxy-phenetidine (Fig.1.8).

The major oxidative reaction, O-deethylation is under the same genetic control as the debrisoquine hydroxylation, but the minor pathway to produce 2-hydroxy-phenetidine is not. Subjects of the PM phenotype therefore show a reduced ability to O-deethylate the drug with the result that a greater proportion of the drug is metabolised along the alternative metabolic pathways leading to the formation of 2-hydroxy-phenetidine.

Fig.1.8

Metabolism of phenacetin in man



This increased formation of 2-hydroxy-phenetidine is of considerable importance, as this metabolite is known to be haemotoxic (Jaffe, 1972). Production of MetHb in PM subjects is, in fact, several times greater than that found for EM subjects following the administration of a single 1g oral dose of phenacetin. Therefore, the subjects of PM phenotype would be more susceptible to the toxic effects of phenacetin than the EM phenotype.

These findings illustrate the important principle that when a genetically determined impairment of a particular metabolic route occurs, then the relative importance of secondary pathways may increase, with associated pharmacological and toxicological consequences.

Application of the principle to the metiamide problem

The underlying factors involved in metiamide-induced agranulocytosis are not well understood. However, several features of the dyscrasia tend to suggest that it may well embrace a genetically controlled metabolic basis. These factors are listed below:-

1. Occurrence of agranulocytosis in small numbers of susceptible individuals.

2. Apparent involvement of a genetic factor (dog litter-mates)

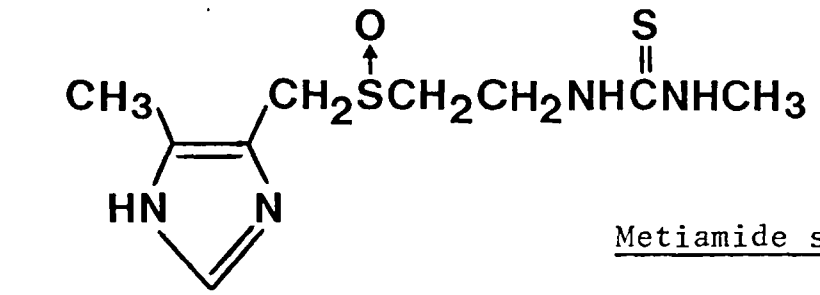
3. Metiamide undergoes oxidative metabolism in man

4. Oxidation of the thiourea group known to produce toxic metabolites

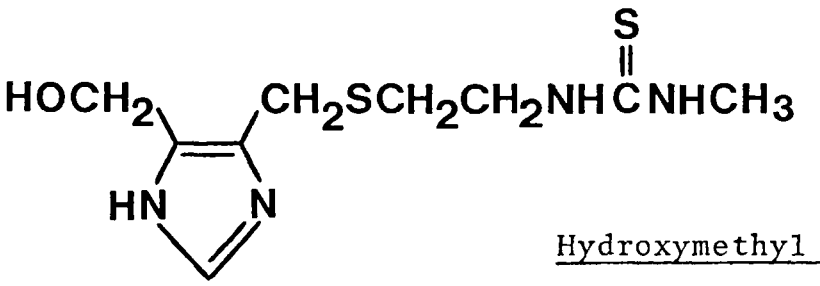
An intrinsic individual susceptibility to the haematotoxic effect is apparent as some patients received the drug for long periods at very high doses with no apparent ill-effect. This individual susceptibility appears to have a genetic basis as two of the dogs which developed agranulocytosis in the toxicity studies, were litter-mates. There has also been a report of two sisters who both progressed to agranulocytosis whilst taking carbimazole, another thiourea derivative (Hamilton & Saunders, 1977).

Metiamide is known to undergo at least three metabolic reactions involving oxidation in man (Taylor et al., 1979). The metabolites thus formed are metiamide sulphoxide, hydroxymethyl metiamide and metiamide urea (Fig.1.9).

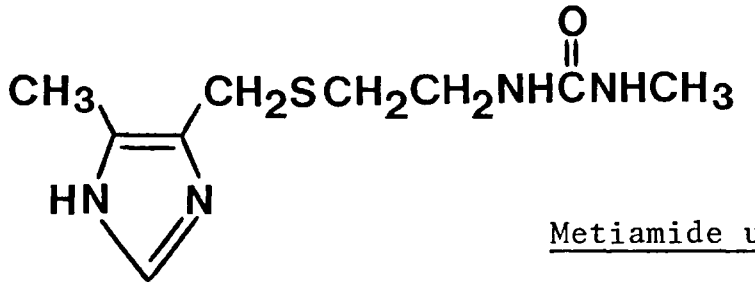
Fig. 1.9 Oxidative metabolites of metiamide in man



Metiamide sulphoxide



Hydroxymethyl metiamide



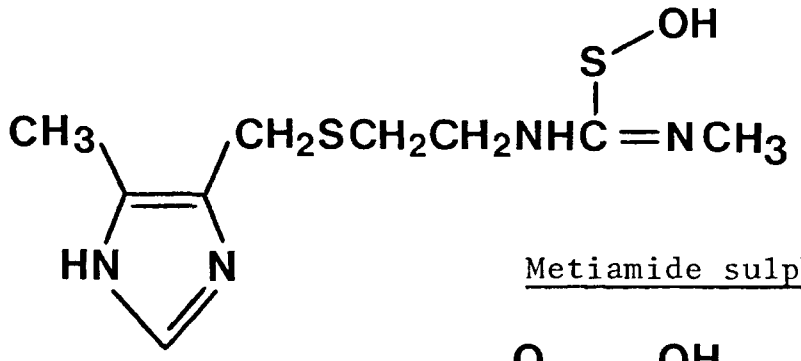
Metiamide urea

Any one, or all three of these metabolic oxidations might well be under the control of the same gene controlling other drug oxidations. Differences would therefore be expected to occur between individuals of EM and PM phenotypes with respect to the amounts of the various metabolites formed, and also in the plasma levels of unchanged metiamide. The susceptible individuals might therefore, have a much higher level of either parent drug or a metabolite in the body. These individuals would thus aggregate towards one of

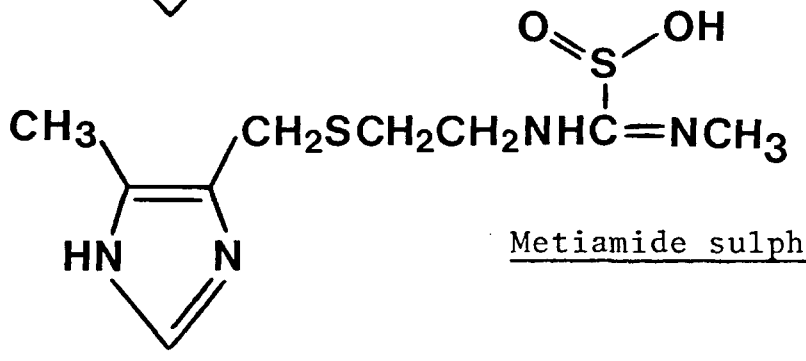
the extremes of the range of oxidative ability. They would be expected to be either very good oxidisers, and produce greater amounts of toxic oxidative metabolites, or poor metabolisers, with higher plasma levels of parent drug perhaps producing toxic metabolites by alternative pathway formation, as occurs with phenacetin.

The haemotoxic metabolites of metiamide may well be the sulphinic or sulphenic acid metabolites (Fig.1.10). However it is unknown as to whether or not these S-oxidative reactions are under the control of the debrisoquine gene.

Fig.1.10 Structures of metiamide sulphinic and sulphenic acids



Metiamide sulphenic acid



Metiamide sulphinic acid

The principle of individual susceptibility to adverse effects of certain drugs being linked to genetically-determined variant patterns of metabolism is therefore a very attractive hypothesis for the underlying mechanism of metiamide-induced agranulocytosis.

The genetically-determined oxidative ability of an individual may, however, be only one contributory factor towards a predisposition to the haemotoxic effects of metiamide. Other factors might include defects in cellular defence mechanisms against oxidative damage, particularly in the glutathione system (as occurs in drug-induced haemolytic anaemia) or a fundamental defect in cell division uncovered by metiamide (as occurs in chlorpromazine-induced agranulocytosis).

1.5.3 Scope of the present work

In order to test the applicability of the polymorphic oxidation-drug toxicity principle to the metiamide problem, a comprehensive study of the metabolism of metiamide in subjects of known oxidation phenotype would be required. Then, with a good metabolic understanding of the influence of polymorphic oxidation on the metabolism of metiamide, the patients who originally

suffered metiamide-induced agranulocytosis could be investigated with respect to their oxidative abilities. This could be determined using debrisoquine, obviating the need to rechallenge the patients with metiamide. Some understanding of the genetic and metabolic basis of metiamide-induced agranulocytosis might thus be gained.

In research of this type, into the mechanism and causes of drug toxicity, there is an obvious need for the establishment of animal models. Very few animal models of this type have been described. In the case of metiamide, the aim of producing such an animal model would be to test whether or not strain or species differences in the metabolism of metiamide could be directly related to its haematotoxicity in these species or strains. These animals might then be used as models for the human EM and PM phenotypes and provide a rapid and relatively inexpensive procedure for evaluating the potential toxicity of novel compounds; thus reducing the risk of the occurrence of 'idiosyncratic' responses to drugs.

CHAPTER TWO

THE METABOLISM OF METIAMIDE IN MAN AND THE INFLUENCE OF OXIDATION PHENOTYPE

2.1 Introduction

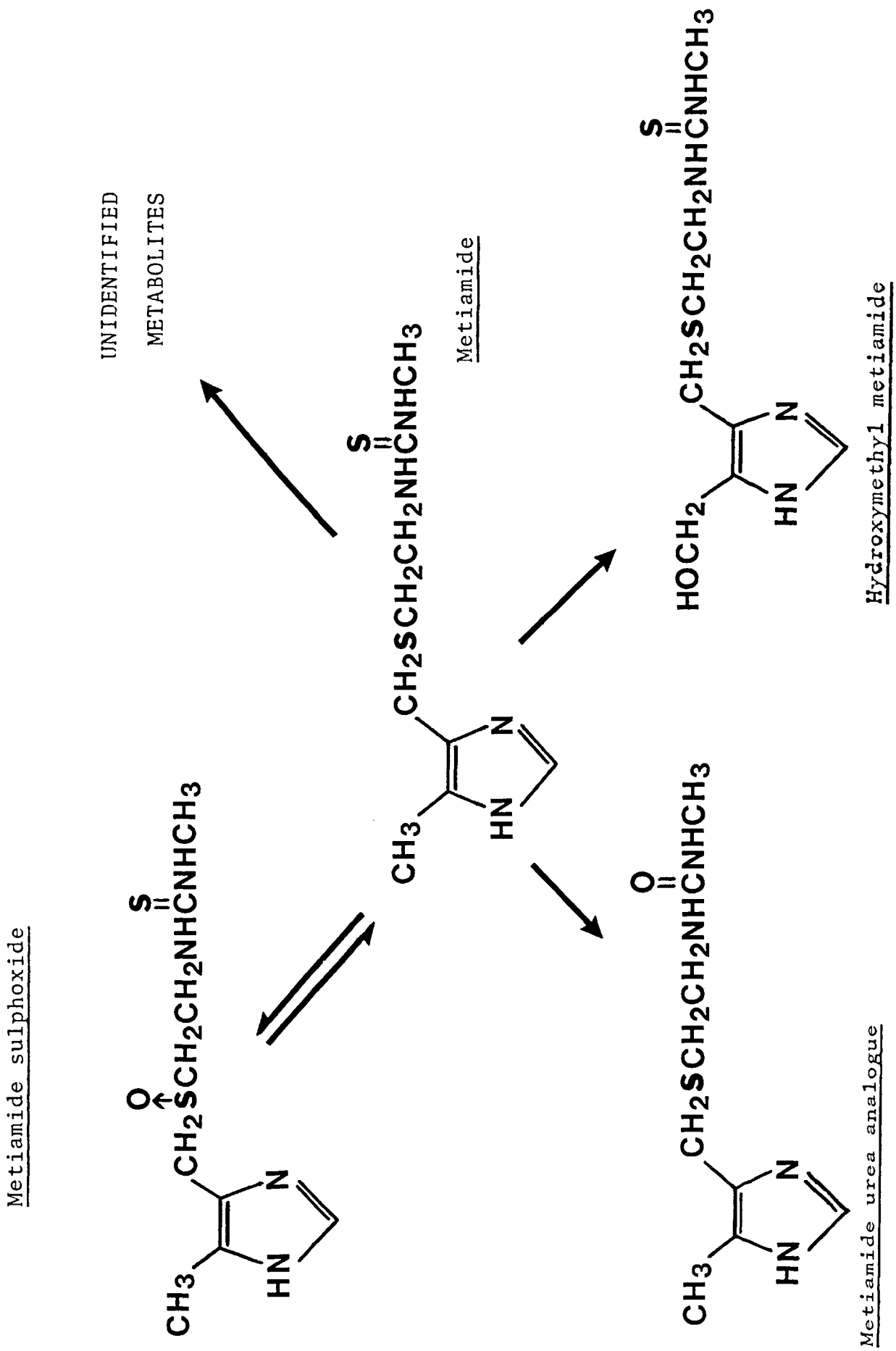
The metabolism of metiamide in rat, dog and man was investigated by Smith, Kline and French Limited as a part of the preclinical development of the drug (Taylor *et al.*, 1979).

In the rat and the dog the principal route of excretion was found to be the urine, with the major urinary component being unchanged metiamide. One major urinary metabolite was identified as metiamide sulphoxide, while smaller amounts of hydroxymethyl metiamide and the metiamide urea analogue were shown to be present by comparison with authentic standards. Some unidentified polar metabolites were observed, but were shown not to include metiamide sulphone or *N*-desmethyl metiamide by comparison with authentic samples of these compounds.

In man, over 70% of a dose of the ^{14}C-labelled compound was excreted in the urine within 48h, about 60% of which was unchanged metiamide. The remainder comprised the sulphoxide (8.9%), the hydroxymethyl (7.4%) and the urea (3.5%) analogue of metiamide. In addition a large proportion (21%) of the urinary ^{14}C consisted of unidentified polar metabolites.

Thus, the major metabolic options as reported by Taylor *et al.* (1979) are shown in Fig.2.1.

Fig.2.1
Metabolic pathways of metiamide in man



The quantitative aspects of the metabolism of metiamide in three species (rat, dog and man) as reported by Taylor et al. (1979) are shown in Table 2.1.

The aim of the investigations described in this chapter were to elucidate more fully the metabolism of metiamide in man and then to investigate whether or not it is influenced by the genetic polymorphism controlling other drug oxidations in man.

The identity of the polar metabolites which constituted the major urinary metabolites of metiamide in dog and man, were not established in the early metabolic studies reported by Taylor et al. (1979).

Table 2.1

The urinary excretion of metiamide and its metabolites in rat, dog and man

(Values are means ± S.D. for n individuals)

Compound	% radiolabel recovered in urine			
	Rat (300 mg kg^{-1})	Rat (300 mg kg^{-1})	Dog (40 mg kg^{-1})	Man (3 mg kg^{-1})
	n=6	n=6	n=4	n=1
Metiamide	61.1±7.5	83.2±5.0	52.8±8.8	58.8
Metiamide urea analogue	2.6±0.9	2.1±0.8	7.4±1.3	3.5
Hydroxymethyl metiamide	5.9±1.3	4.9±2.6	8.1±1.0	7.4

cont./

Table 2.1(cont.)

	Rat (300 mg kg^{-1})	Rat (300 mg kg^{-1})	Dog (40 mg kg^{-1})	Man (3 mg kg^{-1})
	n=6	n=6	n=4	n=1
Metiamide sulphoxide	24.1±4.5	6.3±1.5	13.3±1.6	8.9
Unidentified metabolites	6.2±3.7	2.3±1.2	21.0±4.5	20.5

Similar polar metabolites have been observed as metabolites of cimetidine in man (Griffiths et al.,1977). It was noted that greater amounts of the polar metabolites were excreted at higher dose levels of cimetidine (Griffiths et al., 1977). The polar metabolites were not fully characterised by these authors but it was suggested they might be conjugation products of cimetidine.

In view of the haemotoxicity of metiamide, the characterisation of the polar metabolites is of major importance. A metabolic study was therefore undertaken to confirm previous work on the metabolism of metiamide in man and to attempt to identify these polar metabolites.

The influence of the oxidation polymorphism on the metabolism of drugs in man may be conveniently tested by

the use of phenotyped panels (Idle et al., 1979a).

These phenotyped panels consist of individuals previously phenotyped for their ability to oxidise debrisoquine. One panel of four volunteers are of the EM phenotype (metabolic ratios 0.1-1.0) and the second panel are of the PM phenotype (metabolic ratios >12.6).

In order to test if the metabolism of metiamide was under the same genetic control as debrisoquine hydroxylation, the metabolism of metiamide would be examined in each of the two panels of volunteers and the results compared. Differences between the panels in terms of rate or amount of metabolite produced would be due to the influence of the debrisoquine polymorphism on the reaction. Other drug oxidation reactions which have been investigated in a similar manner include tolbutamide (no significant difference between phenotypes) and phenytoin (aromatic hydroxylation reaction affected; Idle et al., 1979a).

Genetically-determined variability in the metabolic handling of metiamide might help explain the occurrence of individuals susceptible to the toxic effects of metiamide in the population.

2.2 Materials and methods

Materials

Metiamide, cimetidine and their derivatives were kindly donated by Smith, Kline and French Limited, Welwyn Garden City, Herts, U.K. All these compounds were characterised by the Chemistry department at Smith, Kline and French by C,H,and N analysis and by n.m.r. analysis. The properties of these compounds are described below:-

Metiamide: N-methyl-N'-{2-[(5-methylimidazol-4-yl) methylthio]ethyl}thiourea; Molecular weight 244; Melting point 152-154°C; A white, odourless, bitter-tasting powder. Fig. 2.2, structure I.

Metiamide urea analogue: N-methyl-N'-{2[(5-methylimidazol-4-yl) methylthio]ethyl}urea; Molecular weight 228; Melting point 158-159°C; An off-white, odourless powder. Fig.2.2, structure II.

Hydroxymethyl metiamide: N-methyl-N'-[2-(4-hydroxymethyl-5-imidazolylmethylthio)-ethyl]thiourea; Molecular weight 260; Melting point 138-140°C; A white, odourless, powder. Fig 2.2, structure III.

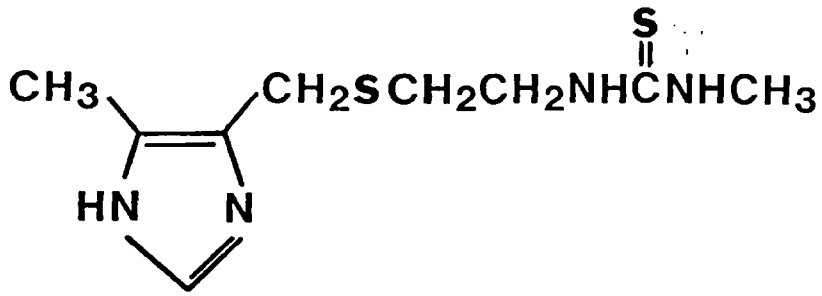
Metiamide sulphoxide: N-methyl-N'-{2-[(5-methylimidazol-4-yl) methylsulphinyl]ethyl}thiourea; Molecular weight 260; Melting point 137-140°C; An off-white, odourless, powder. Fig.2.2 structure IV.

Carboxylic acid analogue of metiamide: N-methyl-N'-[2-(4-carboxy-5-imidazolylmethylthio)-ethyl]thiourea; Molecular weight 274; Melting point not determined; An orange-red, very hygroscopic powder- Fig.2.2 structure V.

Metiamide N-hydroxy analogue: N-hydroxy-N-methyl-N'-{2-[(5-methylimidazol-4-yl)methylthio]ethyl}thiourea; Molecular weight 260; Melting point not determined; An off-white, odourless powder. Fig.2.2 structure VI.

Cimetidine: N"-cyano-N-methyl-N'-{2-[(5-methylimidazol-4-yl) methylthio]ethyl}guanidine; Molecular weight 252; Melting point 141-143°C; A white, odourless, bitter-tasting powder. Fig. 2.2 structure VII.

Fig.2.2 Structures of metiamide and related compounds



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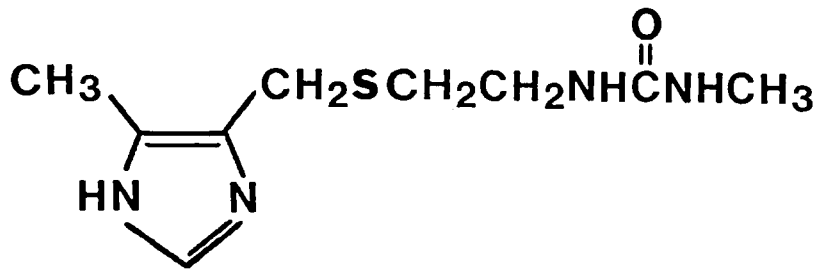

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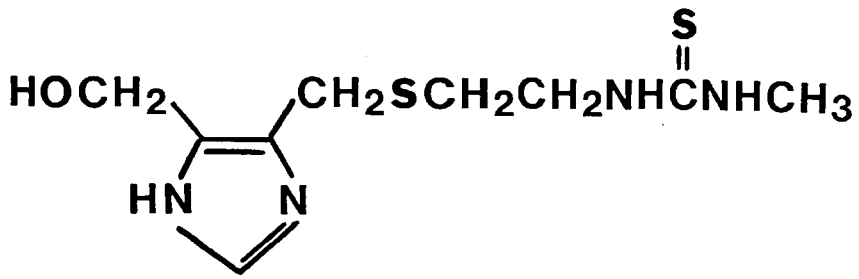

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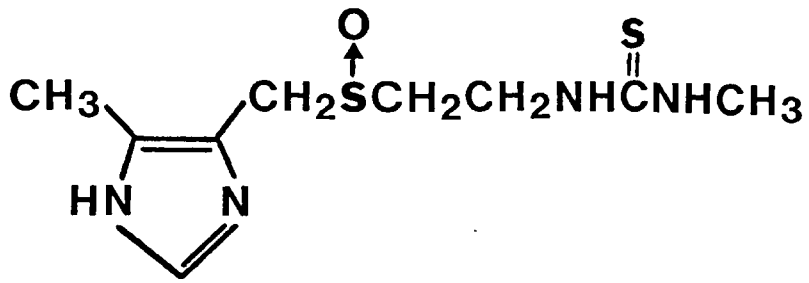

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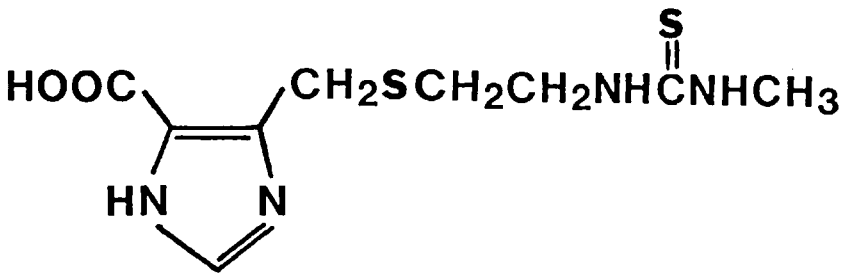

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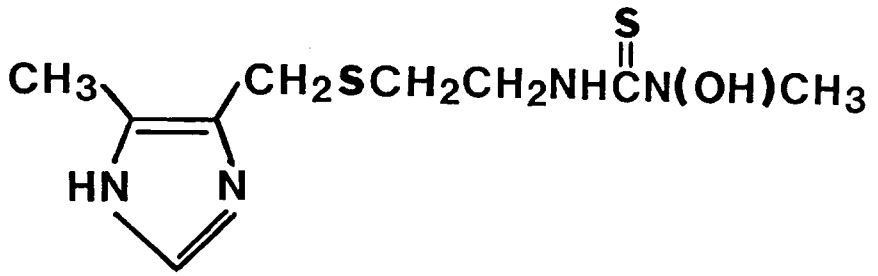

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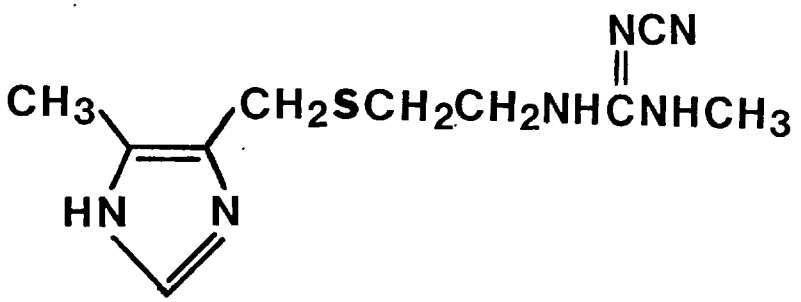
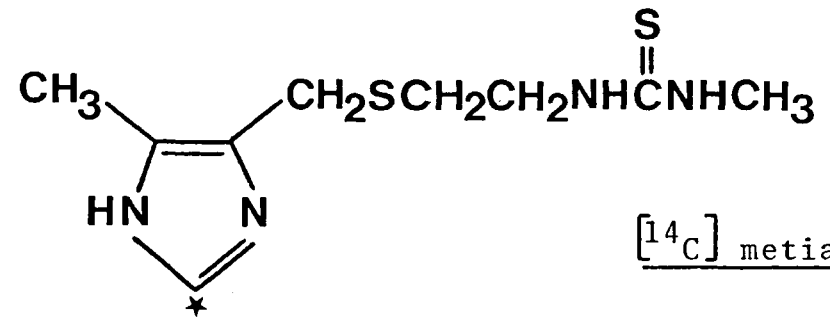


VII

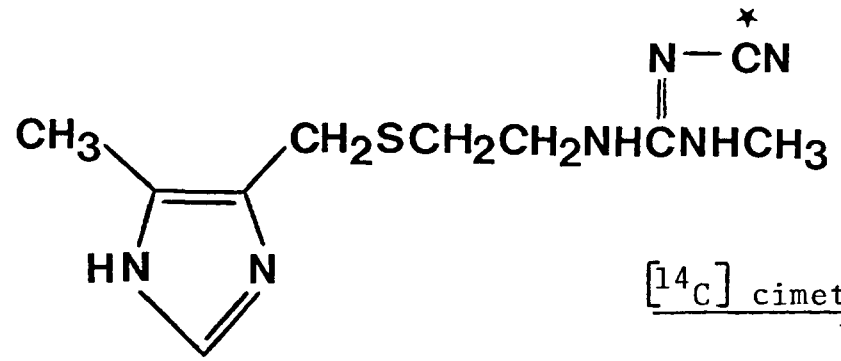
Radiochemicals

[^{14}C]metiamide (22.0μCi mg^{-1}) and [^{14}C] cimetidine (19.0 μCi mg^{-1}) were kindly donated by Smith, Kline and French Ltd. The [^{14}C] metiamide was labelled in the C-2 position in the imidazole ring and the [^{14}C]cimetidine in the cyanide group of the cyanoguanidine moiety (Fig.2.3).

Fig.2.3 Position of ^{14}C-label in [^{14}C]metiamide and [^{14}C]cimetidine.



[^{14}C] metiamide



[^{14}C] cimetidine

* Denotes position of ^{14}C-label.

The radiochemical purity of these compounds was found to be greater than 96% determined by radiochromatography and liquid scintillation counting.

The following compounds were all characterised and donated by Roche Products Ltd., U.K. Characterisation was by C,H,and N analysis and mass spectrometry.

Debrisoquine: 3,4-dihydro-2-(1H)-isoquinoline-carboxamidine hemi - sulphate; m.p. 274-276°C; Fig.2.4 compound I.

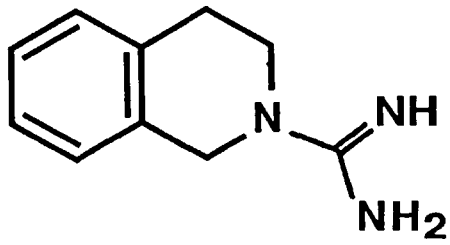
4-hydroxydebrisoquine: 4-hydroxy-3,4-dihydro-2-(1H)-isoquinoline carboxamidine sulphate; m.p. 253-254°C; Fig.2.4 compound II.

5-hydroxydebrisoquine: 5-hydroxy-3,4-dihydro-2-(1H)-isoquinoline carboxamidine sulphate; m.p. 298-300°C; Fig.2.4 compound III.

6-hydroxydebrisoquine: 6-hydroxy-3,4-dihydro-2-(1H)-isoquinoline carboxamidine sulphate; m.p. not determined; Fig.2.4 compound III.

7-hydroxydebrisoquine: 7-hydroxy-3,4-dihydro-2-(1H)-isoquinoline carboxamidine sulphate: m.p. 303°C: Fig.2.4 compound III.

Fig.2.4 Structures of debrisoquine , its metabolites and 7-methoxyguanoxan.



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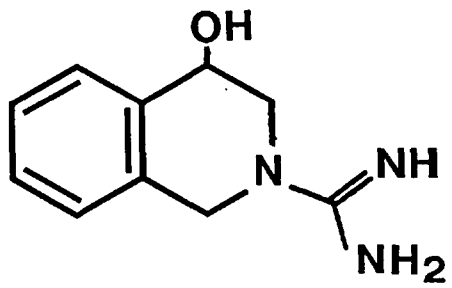

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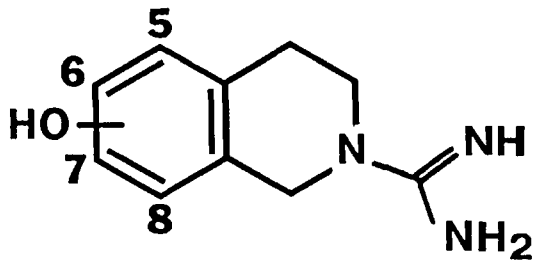

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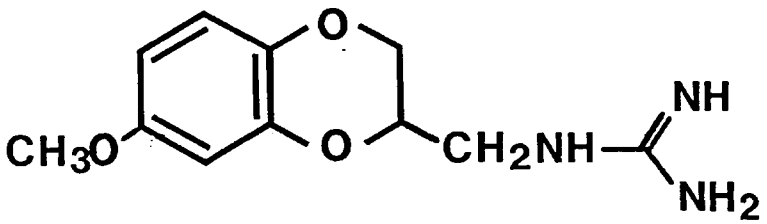


IV

8-hydroxydebrisoquine : 8-hydroxy-3,4-dihydro-2- (1H)-isoquinoline carboxamidine sulphate; m.p.302-304^{o}C; Fig.2.4 compound III.

7-methoxyguanoxan (internal standard) was characterised and donated by Pfizer Ltd., Sandwich, Kent, U.K. ; Fig.2.4 compound IV.

Volunteers

Volunteers gave their informed consent to taking part in these studies and ethical approval was given by the St.Mary's Hospital ethics committee. The volunteers were all healthy males aged between 20 and 45 years of age, with body weights of between 60 and 95 kg.

Dosing of compounds

Metiamide and cimetidine were administered as neutral solutions in water (100 ml; the pH being adjusted with dilute HCl), the beaker was then rinsed with water (100 ml) which was also drunk. Debrisoquine was given in tablet form (10 mg; Declinax, Roche Products Ltd.). In all cases, the volunteers fasted overnight,

then received the drug in the morning without taking breakfast. Food was allowed one hour after drug administration. No alcohol or medication was allowed during the period of urine collection. Urine samples were collected for the appropriate time, the volumes measured, and aliquots (20 ml) stored at -20°C until analysed.

Determination of ^{14}C in urine and plasma

Urine (0.1-1.0 ml) and plasma (0.1 ml) samples were counted in duplicate in plastic inserts within glass vials containing a triton-toluene scintillation cocktail (5 ml of the following mixture: 3.331 Triton X-100, Kock-Light Labs.Ltd., Colnbrook, Bucks; 6.661 Scintillation grade toluene , May and Baker Ltd., Dagenham; 55g PPO, Fisons Scientific Apparatus Ltd., Loughborough, Leics. ; 1g POPOP, Fisons Scientific Apparatus Ltd., Loughborough, Leics.).

The vials were then counted in a liquid scintillation spectrometer (Packard Model 3385), after sufficient time had elapsed for cooling in the spectrometer. Quench correction was performed by the external standard ratio method.

Chromatographic and radiochromatographic methods

Several chromatographic systems were used for separating metiamide and its metabolites. The R_F values of authentic samples of each metabolite were determined (Table 2.2) using silica gel (aluminium-backed silica gel 60 F_{254}, 20 x 20 cm, E.Merck,Darmstadt) or cellulose plates (plastic-backed, PEI cellulose F, 20 x 20 cm, E.Merck, Darmstadt) or paper chromatography (Whatman No 1 paper, Whatman Ltd, England).

The solvents were made up fresh and left to equilibriate for several hours before use.

The silica gel plates were used for examining the metabolic profile in urine while the unidentified polar metabolites were examined by paper chromatography and on cellulose plates. Solvent system III (ethylacetate: methanol: O.88 ammonia soln 10:1:1 by vol.) was used with the silica-gel plates as it was found to give good resolution of metiamide and its metabolites and also had good reproducibility of R_F values.

Table 2.2 Chromatographic properties of metiamide and its metabolites

Compound	R_F in system:			
	Paper chromatography	Silica gel t.l.c.		Cellulose t.l.c.
	solvent I	solvent II	solvent III	solvent IV
Metiamide	0.80	0.85	0.82	0.82
Metiamide urea analogue	0.68	0.73	0.75	0.76
Hydroxymethyl metiamide	0.67	0.78	0.67	0.72
Metiamide sulphoxide	0.60	0.76	0.62	0.70
N-hydroxy metiamide	0.76	0.75	0.66	0.83
Metiamide carboxylic acid	0.60	0.71	0.31	0.66

Solvent I butan-1-ol: glacial acetic acid: water 60:15:25 by vol.

Solvent II ethanol: water: 0.88 ammonia soln 80:15:5 by vol.

Solvent III ethyl acetate: methanol: 0.88 ammonia soln 10:1:1 by vol.

Solvent IV butan-1-ol: glacial acetic acid: water 60:25:25 by vol.

For routine examination of urinary metabolites, samples of urine (50-100μl) were banded on small (5 x 10 cm) silica-gel thin-layer plates. Samples of non-radioactive metiamide and its metabolites were then banded on top of the urine to act as cold carrier, and to aid in visualising the metabolite bands under ultra-violet light at 254 nm (Hanovia chromatolite). The plates were then developed in solvent system III.

After development the plates were thoroughly dried and then scanned on a radiochromatogram strip scanner (Packard Model 7201). The areas on the plates corresponding to metiamide, metiamide sulphoxide and hydroxymethyl metiamide were visualised under U.V. light (254 nm), and their positions marked. The plates were cut into strips, placed in vials, scintillant added (5 ml) and then counted.

Co-chromatographic determination of metiamide urea analogue in urine.

The metiamide urea analogue could not be determined by the method described above as it did not quench U.V. light and so could not be detected on the thin-layer plate by this method. It was quantified by carrying out thin-layer chromatography of urine as described

above, with authentic samples of the metabolites added to the urine to improve the separation of the bands. After development a 2 cm strip was cut from one side of the thin-layer plate and sprayed with a solution of p-dimethylaminocinnamaldehyde (1% w/v, in conc.HCl diluted 1:4 v/v with acetone just before use; BDH Labs., Poole, England). The metiamide urea analogue showed up as a pink band with this reagent. The position of the metiamide urea analogue band on the unsprayed plate was then marked, the chromatogram cut into strips and counted.

Counting efficiency of t.l.c. plates

Samples of urine (100 μl) containing metiamide and its metabolites (250-10,000 d.p.m.) were spotted onto thin-layer chromatography plates and then thoroughly dried. The plates were then cut up, counted and then compared with similar samples of urine (100 μl) pipetted directly into vials with scintillant and counted. The recovery of radioactivity from the thin-layer plates was found to be quantitative.

Spray Reagents

Paper chromatograms and thin-layer plates were sprayed

with a number of different reagents after development, in order to aid in visualising the metabolite bands in urine, and also to provide some information on the chemical properties of the metabolites.

Pauly's reagent

Reagent A was made up by dissolving sulphanilic acid (9 g) in water (900 ml) and then adding conc. HCl (90 ml). Reagent B was a solution (5% w/v) of sodium nitrate in water. One volume (5 ml) of A was mixed with one volume (5 ml) of B in a fumecupboard. After five minutes sodium carbonate (10% w/v;10 ml) was added and the chromatograms then sprayed. Compounds containing an imidazole ring showed up as red-orange spots on a yellow background.

Tollens' reagent

0.1M silver nitrite (10 ml), 10% (w/v) sodium hydroxide (5 ml) and concentrated ammonium hydroxide (sp.gravity 0.880; 10 drops) were mixed just before use. The chromatograms were then sprayed and after full colour development, washed in 5% (w/v) sodium thiosulphate solution, and then in water to prevent background colour. Intact thiourea groups showed up as brownish

spots on a pale yellow background.

Naphthoresorcinol reagent

A 0.2% (w/v) solution of naphthoresorcinol in acetone (5 ml) was mixed with a 9% (w/v) phosphoric acid solution (1 ml) in a test-tube. The plates were sprayed with this reagent and then heated at 100°C in an oven for 10 minutes. Glucuronic acid conjugates give a blue colour with this reagent.

Modified Grotes reagent

A 5% (w/v) solution of sodium nitroprusside (10 ml) and a 10% (w/v) solution of hydroxylamine hydrochloride (5 ml) were gently mixed in a 50 ml flask and allowed to stand for 2 minutes. 10% (w/v) sodium bicarbonate (10 ml) was then added. After 10 minutes bromine (0.11 ml) was added, then after a further 10 minutes 2% (w/v) phenol solution (5 ml) was added. This mixture was allowed to stand at room temperature for 12 h and was then refrigerated at 0-5°C. This stock reagent was stable for at least 75 days. It was diluted (1:20) with 0.2M-phosphate buffer (pH 6.8) before use. Intact thiourea groups showed up as blue spots on a white background with this reagent.

Hydrolysis methods

Acid hydrolysis

Urine samples (1 ml) were heated at 100°C for 1 h together with conc. HCl (0.2 ml) in a screw-capped vial. The samples were then allowed to cool before solid sodium bicarbonate was added to neutralise the acid. The samples were then used for thin-layer chromatography.

β-Glucuronidase treatment

Urine samples (0.5 ml) were adjusted to pH5 with 0.2M-acetate buffer (0.5 ml) and β-glucuronidase solution added (0.5 ml; Ketodase, Warner & Co.Ltd, Eastleigh, U.K.). The solution was incubated at 37°C for 24 h.

Methanol (2 ml) was then added and the denatured enzyme removed by centrifugation. The supernatant was used for thin-layer chromatography. Control tubes were set up using phenolphthalein-β-D-glucuronide sodium salt (Kock-Light Lab.Ltd., Colnbrook, U.K.) as substrate and saccharic acid 1,4-lactone (Sigma Chemical Co.Ltd, Poole, Dorset) as inhibitor.

Sulphatase treatment

Urine samples (1 ml) were buffered to pH 5 with 0.2M- acetate buffer and 580 units of sulphatase (Sigma Chemical Co.Ltd., Poole, Dorset) added. The mixture was incubated at 37°C for 24 hours, methanol (2 ml) was then added, the denatured enzyme removed by centrifugation and the supernatant used for thin-layer chromatography.

Nucleoside phosphorylase treatment

Urine samples (1 ml) were buffered to pH 8 with 0.2M-phosphate buffer and 12 units (0.25 ml) of the nucleoside phosphorylase enzyme (Purine nucleoside orthophosphate ribosyltransferase, Sigma Chemical Co. Ltd., Poole, Dorset) added. The incubation was carried out at 37°C for 24 h. The enzyme was removed by precipitation with acetone (2 ml) and then centrifugation. The supernatant was used for thin-layer chromatography.

Isolation of unknown metabolite for N.M.R.analysis

The bulked 0-24 h urine collection from one volunteer after receiving metiamide (50 mg, 10 μCi) was used. The pH of the urine (1.5 l) was adjusted to pH 7.1-7.2

and then passed through a moderately-basic Dowex anion exchange column (resin bed volume, 25 ml; Dowex 2X8-100, Sigma Chemical Co., Poole, Dorset, England). The column was well washed with distilled water (2l) until less than 50 c.p.m. ml^{-1} of eluent was obtained. The column was then eluted with 0.05M-HCl, again until the radioactivity in the eluent was less than 50 c.p.m. ml^{-1}.

This acid fraction was then passed through an Amberlite XAD-2 column(resin bed volume 200 ml; BDH Laboratories, Poole, England) which had been prepared by successive washings of acetone (2 bed-volumes) methanol (2 bed-volumes) and water (2 bed-volumes). The resin was well washed with distilled water (2l) before the metabolites were eluted from the column with methanol (500 ml).

The methanol fraction was reduced under vacuum at $36^{o}C$ to a small volume (1 ml) and then streaked onto thick-layer chromatography plates (Silica gel G F_{254} type 60 , E.Merck, Darmstadt). The plates were developed in solvent II (see Table 2.2).

The metabolite bands were located by radiochromatogram scanning and by examination of the plates under u.v. light (254 nm). The areas on the plates corresponding to the unidentified metabolite were removed by scraping

and then the silica gel eluted with methanol and filtered. This methanol fraction was reduced to dryness under vacuum and further dried *in vacuo* over silica gel. The sample was then ready for n.m.r. analysis.

Nuclear Magnetic Resonance (n.m.r.) spectra

A Varian Associates Model A60A analytical spectrometer attached to a C1024 computer was used to obtain the n.m.r. spectra. The samples were dissolved in deuterium oxide before examination.

Isolation of glucuronides from urine using lead acetate

Bulked 0-24 h urine from a subject after receiving metiamide (50 mg; 10μCi) was acidified to pH 4 with glacial acetic acid and then 1M-lead acetate solution (500 ml) added. After filtering off the precipitate the pH of the solution was adjusted to pH 10 with ammonia solution (specific gravity 0.88). Saturated basic lead acetate solution was then added (200 ml) and the precipitate allowed to settle.

The precipitate was collected by filtration and washed

several times with distilled water. The precipitate was then resuspended in water and H_2S bubbled through until all the lead was converted to a precipitate of lead sulphide. The clear filtrate was collected and then reduced under vacuum to produce a buff-coloured powder, which gave a strongly positive naphthoresorcinol test.

Assay for debrisoquine and 4-hydroxydebrisoquine

The sample urine (100μl) was pipetted into a screw-capped vial and 7-methoxy guanoxan (50μl) was added as internal standard (1.0μg). This mixture was buffered with 1M-$NaHCO_3$ (100μl), then benzene (1 ml) and hexafluoroacetylacetone (HFAA; Koch-Light Labs. Ltd., Colnbrook, Bucks; 50μl) added. The reaction vial was heated in an aluminium block at 100°C for 1h, then removed and allowed to cool. 3M-NaOH (5 ml) was then added to hydrolyse the excess HFAA. The sample was vortexed, centrifuged and a portion of the benzene layer (1-2μl) injected onto the g.l.c. column. The derivatisation scheme is shown in Fig.2.5.

Fig.2.5 Derivatisation scheme for debrisoquine and its metabolites with hexafluoroacetyl acetone

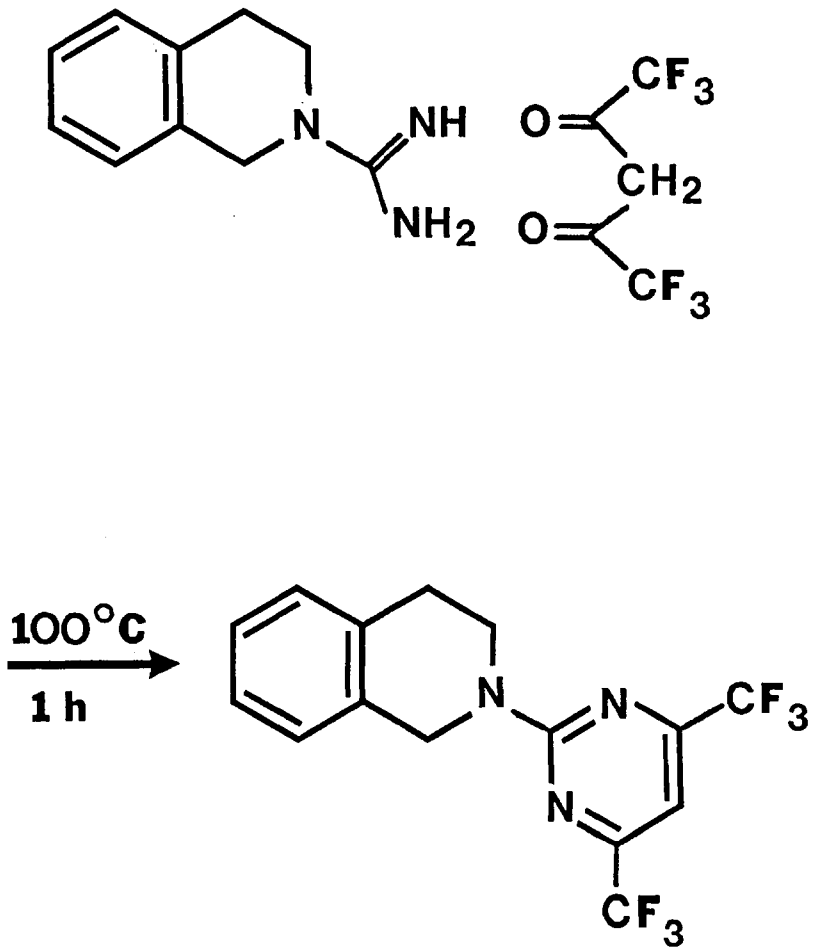
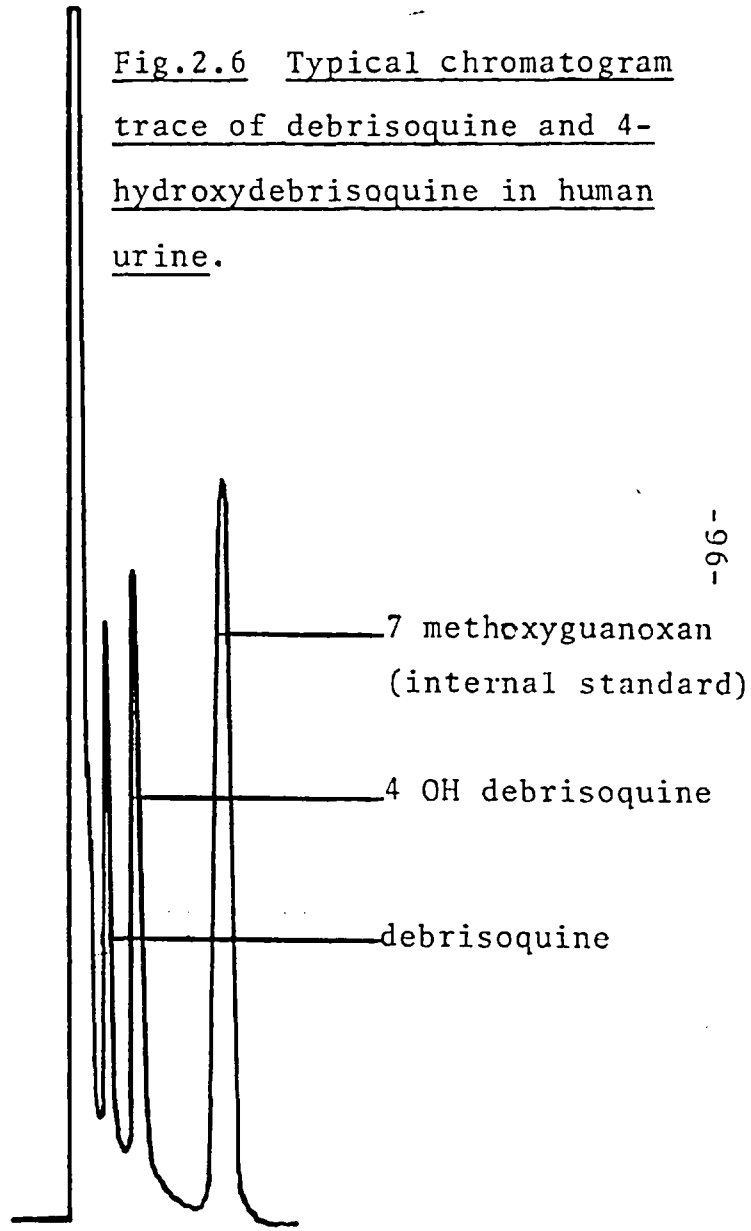


Fig.2.6 Typical chromatogram trace of debrisoquine and 4-hydroxydebrisoquine in human urine.



Gas chromatographic conditions

A Pye Unicam series 204 chromatograph with a Pye Unicam DP 101 computing integrator was used for analysis of samples.

Glass column: 3% OV-1 on Chomosorb WHP
(Phase Separations Ltd., Queensferry, Flintshire,U.K.).
Length of column 1.83 m : 3 mm internal diameter.
Carrier gas: oxygen-free nitrogen.
Flow rate: 60 ml min^{-1} Pressure: 20 psig
Column temperature $195^{o}C$
Detector temperature $235^{o}C$
A typical g.c.trace is shown in Fig.2.5.

Retention times

Compound	time (sec)
Debrisoquine	75
4-Hydroxydebrisoquine	125
7-Methoxy guanoxan	275

Calibration

Calibration curves were constructed for debrisoquine and 4-hydroxydebrisoquine by the peak area ratio method in the range 1.0-10.0μg ml^{-1}, using a Pye Unicam DP101 computing integrator. The calibration curves for debrisoquine and 4-hydroxydebrisoquine were found to

Fig.2.7. Calibration curves for debrisoquine and 4-hydroxydebrisoquine

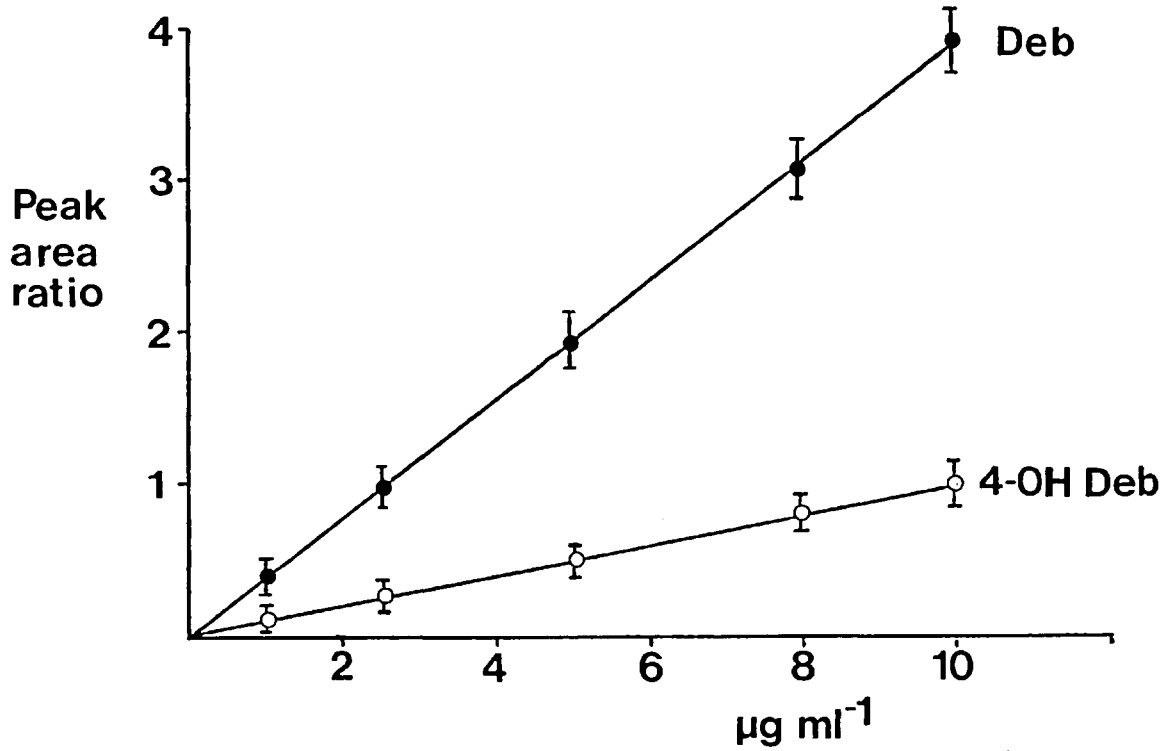
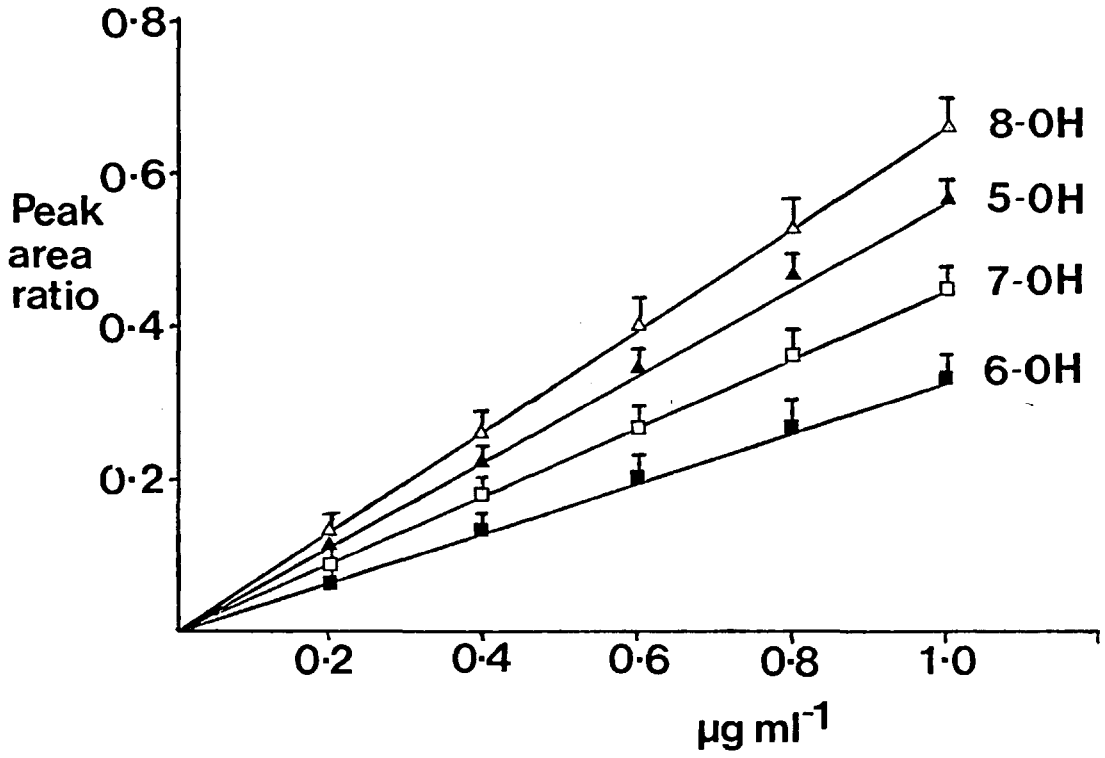


Fig.2.8. Calibration curves for the phenolic metabolites of debrisoquine



to be linear over the range 1.0-10.0μg ml^{-1} (Fig.2.7).

Coefficient of variation on the assay

The coefficient of variation for each point on the calibration curve was estimated (n=3) and never exceeded 10%.

Assay for debrisoquine phenolic metabolites

To the urine sample (1 ml) in a screw-capped vial was added aqueous 7-methoxyguanoxan (100μl; containing 2μg) and conc. HCl (200μl). The vial was heated at 100°C for 1 h in an aluminium block, then allowed to cool and the acid neutralised with solid sodium bicarbonate.

A portion of the acid-hydrolysed sample (500μl) was then added to a screw-capped vial containing 1M-$NaHCO_3$ (100μl), HFAA (100μl) and benzene (1 ml). This mixture was heated at 100°C for 1 h in a heating block, cooled and then 3M sodium hydroxide (10 ml) added. The sample was vortex-mixed and centrifuged, then a portion of the benzene layer (200μl) was transferred to a small glass reaction vial. The phenolic metabolites were

silylated by the addition of BSA:N,O-bis-(trimethysilyl) acetamide (20μl; Phase Separation Ltd., Queensferry, Flintshire, U.K.), to the reaction vial (Fig.2.9). A small portion of this sample (1-2μl) was then injected onto the g.c. column.

Gas chromatographic conditions

The g.l.c. conditions employed were the same as those for the debrisoquine assay except that the column temperature was reduced to 175^{o}C. A typical g.l.c. trace is shown in Fig.2.10.

Retention times

Compound	time (s)
5-Hydroxydebrisoquine	620
6-Hydroxydebrisoquine	780
7-Hydroxydebrisoquine	700
8-Hydroxydebrisoquine	570
7-Methoxyguanoxan	1050

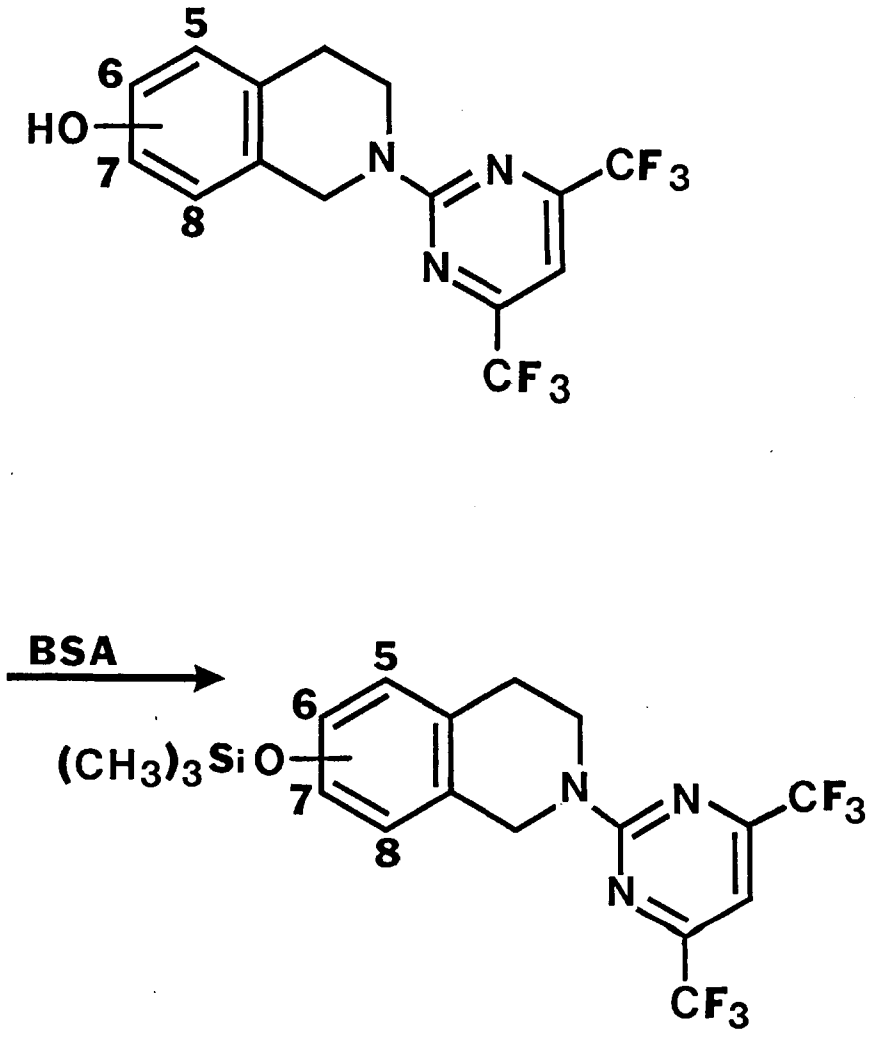


Fig.2.9 Secondary derivatisation scheme for phenolic metabolites of debrisoquine

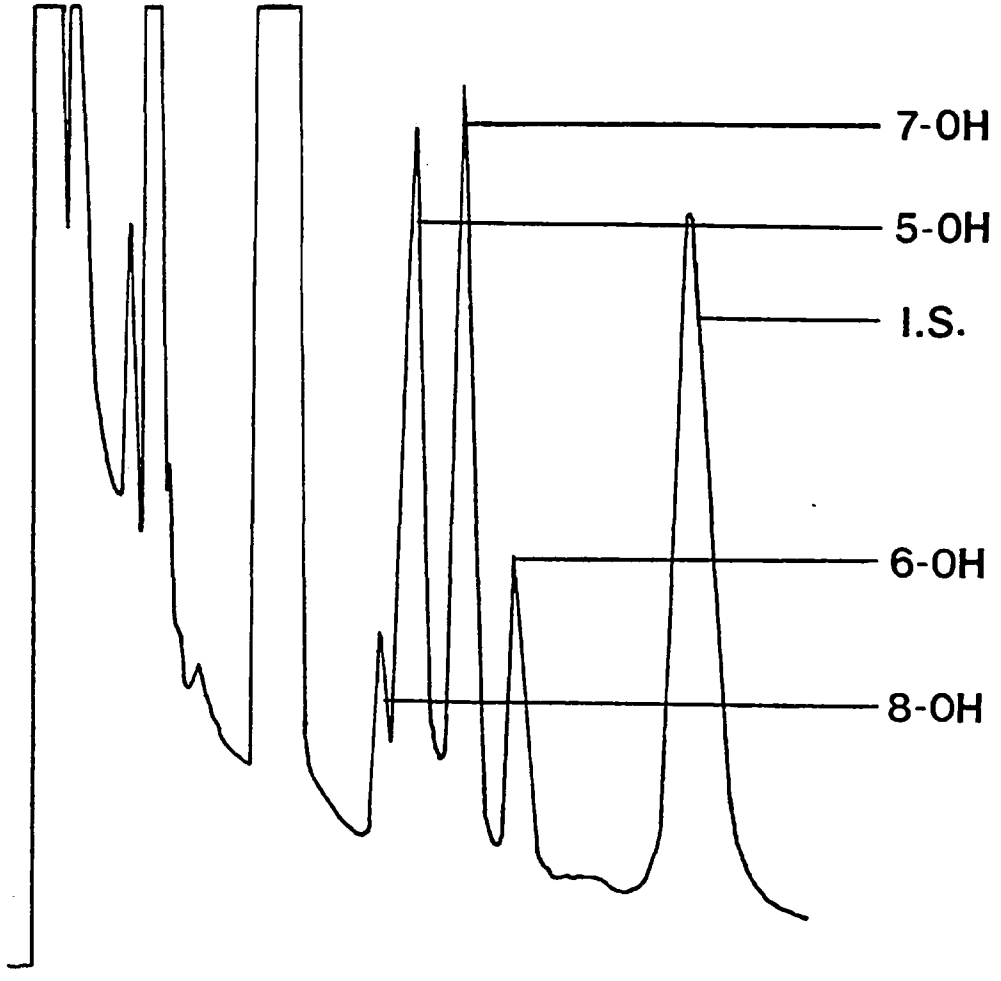


Fig.2.10. Typical chromatogram trace of phenolic metabolites of debrisoquine in human urine.

Calibration

Calibration curves were constructed for each of the phenolic metabolites, by the peak areas ratio method in the range 0.1-1.0μg/ml, using the Pye Unicam DP 101 computing integrator (Fig.2.8). Estimates of the coefficient of variation for each concentration in the calibration curve did not exceed 10% (n=3).

Phenotype allocation procedure

Subjects were phenotyped by the calculation of a metabolic ratio:-

$$\text{Metabolic ratio} = \frac{\text{\% dose in 0-8h urine as debrisoquine}}{\text{\% dose in 0-8h urine as 4-hydroxydebrisoquine}}$$

Subjects with metabolic ratios of less than 12.6 are assigned EM phenotype status, while those with ratios of greater than 12.6 are assigned the PM phenotype status as defined by Evans et al. (1980).

Only individuals with ratios of less than 1.0 were used in this study in the EM phenotype panel. These subjects were probably homozygous extensive metabolisers (genotype $D^H \times D^H$) rather than the heterozygotes (genotype $D^H \times D^L$), who probably have metabolic ratios between 1.0 and 12.6

due to the incomplete dominance (30%) of the EM over the PM trait as demonstrated by Evans et al. (1980).

Pharmacokinetic analysis of plasma data

Estimates of plasma half-life ($t_{\frac{1}{2}}$) values for metiamide were obtained by fitting the plasma concentration-time data to a two-compartment open model (Riegelman et al., 1968) using a digital computer (University of London Computing Centre; Pharmkin 3 computer programme).

Pharmacokinetic treatment of urinary excretion data

The rates of excretion of metiamide and its metabolites in urine was examined in the two phenotyped panels by the collection of serial urine samples from each volunteer.

From this data it is possible to estimate the first-order rate constants for the formation of each metabolite in the body and the first-order rate constant for the elimination of parent drug from the body by the method of Cummings et al. (1967).

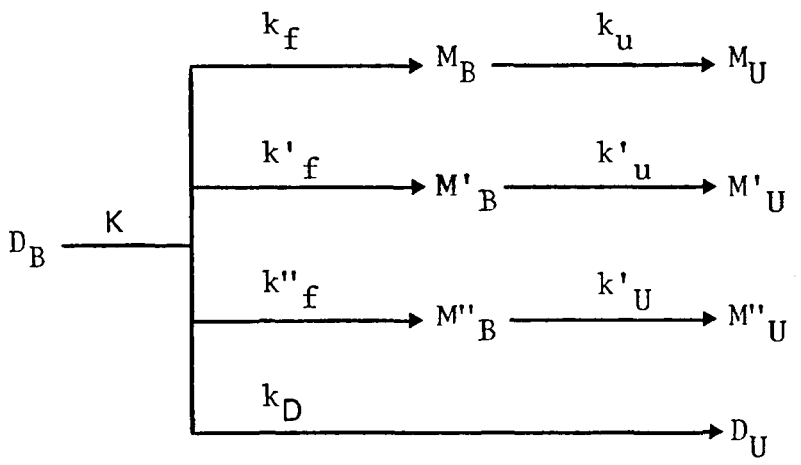
Determination of elimination rate constants for metiamide and its metabolites by the "Sigma -Minus" method

(Cummings et al.,1967)

In this method a first-order rate law is assumed for all the processes involved in the elimination of the drug and its metabolites. The drug is considered to undergo elimination by excretion of unchanged drug with the rate constant k_D, and by the simultaneous formation of a number of metabolites M, M', M"...., with the rate constants k_f, k_f', k_f''....., respectively. The overall rate constant for the elimination of drug by all routes in K where:-

$$K = k_D + k_f + k_f' + k_f'' \qquad (1)$$

The metabolites are considered to be instantaneously equilibriated in the body water and to undergo no further metabolism. They are excreted unchanged with the rate constants k_u, k_u', k_u'' ...., respectively. The elimination of the drug and the excretion of its metabolites may be represented as follows:-



The following equations for these processes are presented with respect to the unchanged drug, but they may also be applied to the metabolites when the appropriate rate constants are substituted. In these equations:-

D_0 = amount of drug in body at zero time

D_B = amount of drug in body at time t

D_U = amount of drug excreted in urine at time t

M_F = amount of metabolite formed at time t

M_B = amount of metabolite in body at time t

M_U = amount of metabolite in urine at time t

so that $D_0 = D_B + D_U + M_F$ and $M_F = M_B + M_U$

thus $$\frac{dD_B}{dt} = - KD_B \qquad (2)$$

In respect of the excretion of unchanged drug, the "Sigma –minus" method consists of plotting the amount of drug which is ultimately excreted ($D_U\infty$) minus the cumulative amount of drug(D_U) excreted in time t, against time after drug administration, where the dose is equal to D_0 ;

$$D_U\infty - D_U = \frac{k_D D_O e^{-Kt}}{K} \qquad (3)$$

so $$\ln (D_U\infty - D_U) = \frac{\ln k_D D_O}{K} - Kt \qquad (4)$$

To enable a plot of the above type it is necessary to first determine the amount of drug which is ultimately excreted ($D_U\infty$) in the unchanged form. This is achieved by a plot of the logarithm of the rate of excretion of the drug against the mid-points of the collection period. The plot generates a straight line which is described by

$$\ln \frac{d\ D_U}{dt} = \ln\ k_D\ D_O - Kt \qquad (5)$$

which has an intercept on the ordinate equal to $k_D D_O$ a slope equal to - K. ($D_U\infty$) is calculated by estimating the rate of drug elimination at the end of the last collection period and dividing by the slope of the line:

$$D_U\infty = D_U \text{ collected} + \frac{\text{Rate at time t}}{\text{Slope}}$$

Equations of the same form as (5) are also obtained for the metabolites.

The rate of formation of each metabolite may then be calculated:

$$k_f = M_U \quad x\ K$$

2.3 Results

2.3.1 The metabolism of metiamide in man

Two subjects were dosed orally with [^{14}C] metiamide dissolved in water, one (subject A) at a dose level of 50 mg (40μCi) and one (subject B) at a dose level of 2 mg (40μCi). Blood samples were collected by venepuncture or via an indwelling cannula at intervals up to 8 h post-dosing. Serial urine samples were collected during the period 0-24 h.

Plasma ^{14}C levels after [^{14}C] metiamide p.o.

The plasma data for both subjects shows (Fig.2.11) that metiamide was rapidly absorbed from the gastro-intestinal tract, peak plasma concentrations of radioactivity being reached at about 1h post-dosing. A two-compartment kinetic model fits the plasma concentration data and the values derived from such a kinetic model are shown in Table 2.3.

Fi .2.11 ^{14}C plasma levels in two subjects after oral doses of [^{14}C]metiamide

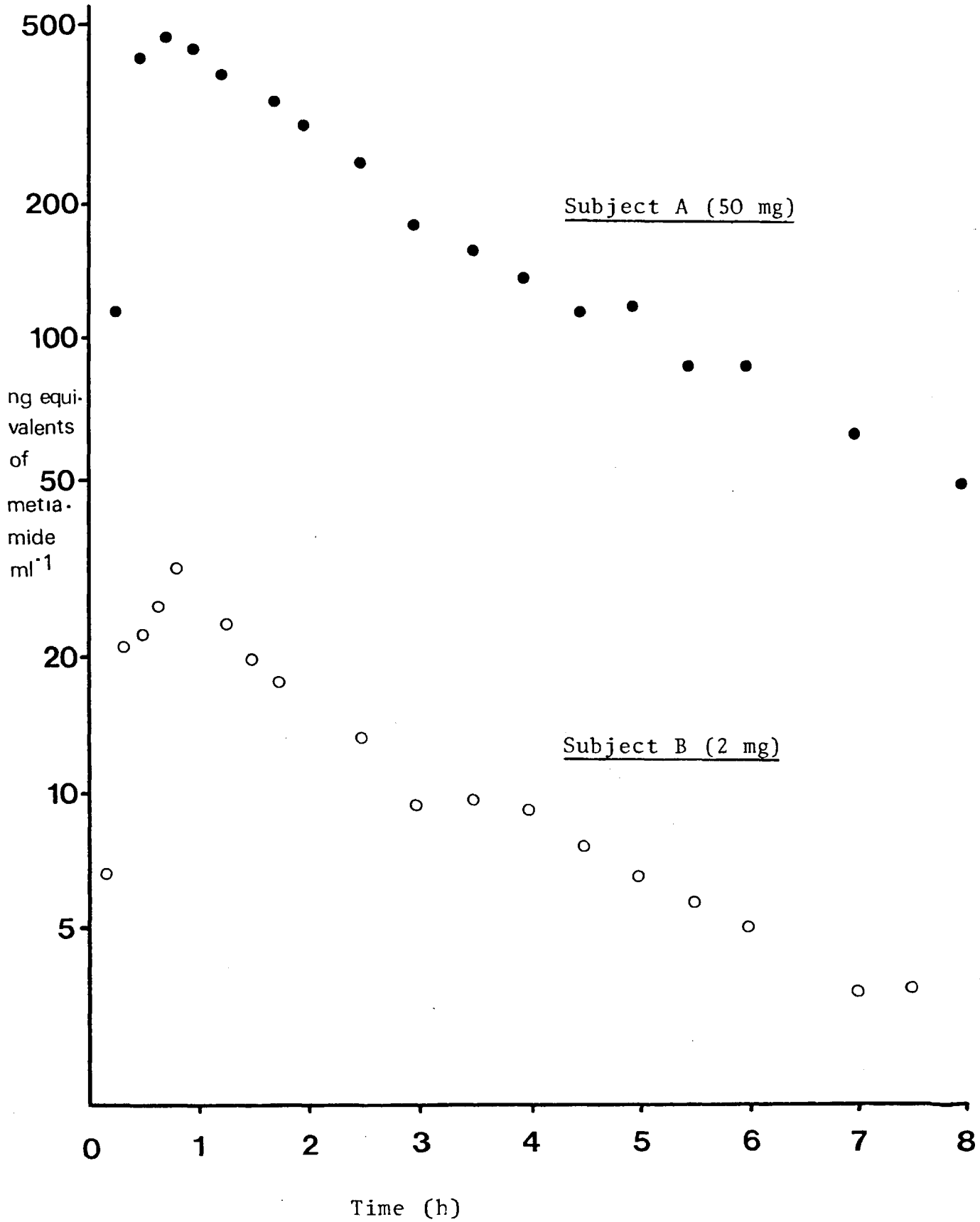


Table 2.3 Pharmacokinetic parameters derived from plasma data after [^{14}C] metiamide p.o.

Subject	Rapid disposition phase $t_{\frac{1}{2}}\alpha$ (mins)	Slow disposition phase $t_{\frac{1}{2}}\beta$ (mins)
A (50mg)	23	158
B (2mg)	28	166

Urinary excretion of ^{14}C

Urine samples were collected at hourly intervals up to eight hours post dosing and then a bulked 8-24h collection was made. Table 2.4 shows the amount of ^{14}C excreted during each time period.

Table 2.4 Urinary excretion of ^{14}C after metiamide p.o. as % of dose

Time (h)	Subject A (50 mg)	Subject B (2 mg)
0-1	21.5	13.4
1-2	18.6	6.8
2-3	15.6	3.3
3-4	8.5	7.9
4-5	5.2	3.7

	Time (h)	Subject A (50 mg)	Subject B (2 mg)
	5-6	4.1	2.2
	6-7	2.7	2.3
	7-8	1.6	1.9
	8-24	5.3	4.4
Total	0-24	83.1	50.4

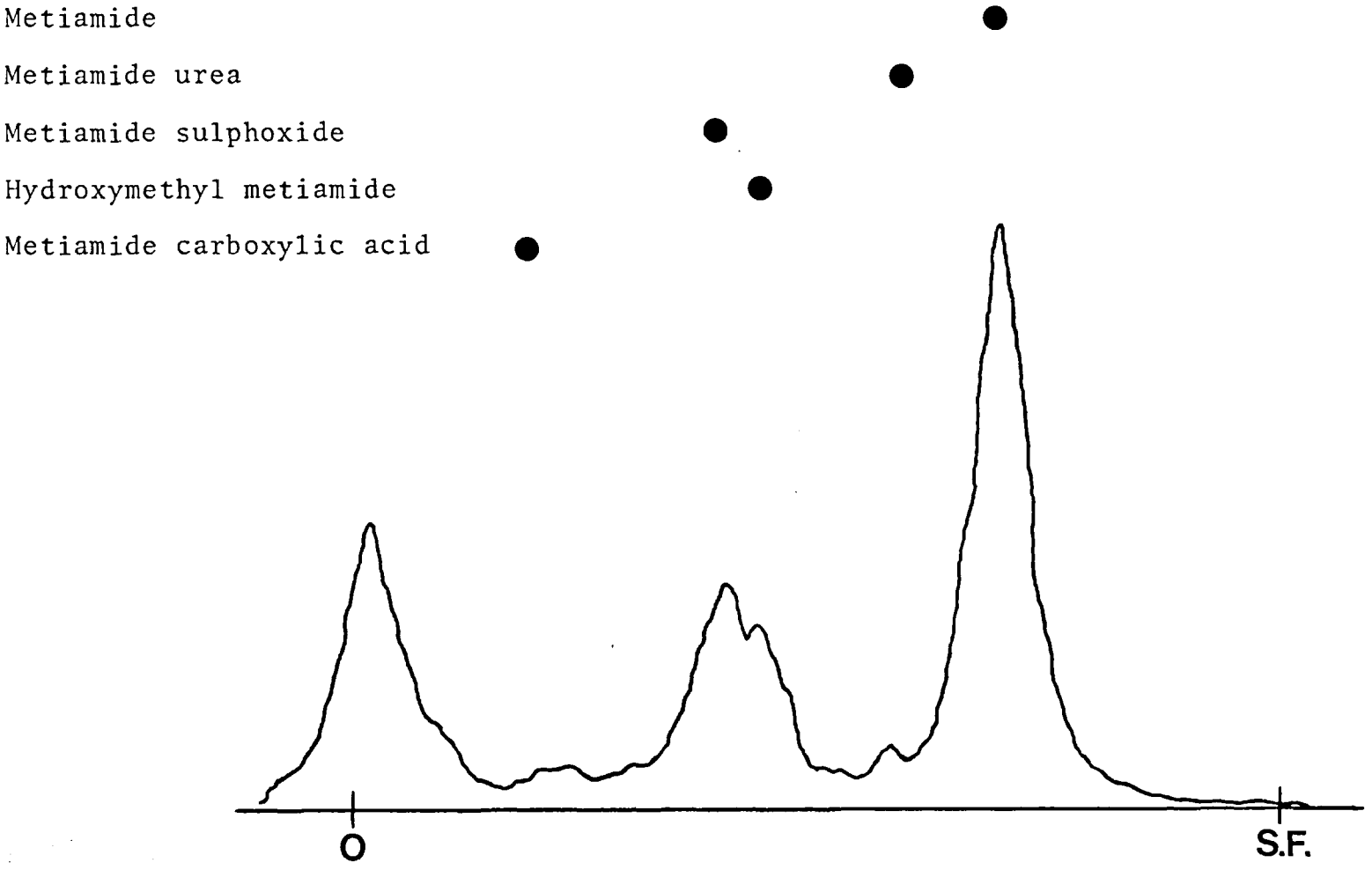
Metabolism of metiamide

The metabolic profile of the radiolabelled compounds excreted in the urine was examined. Chromatography and radiochromatogram scanning of a 0-24h urine collection (Subject A) on a silica gel TLC plate (solvent system III) revealed four major peaks (Fig.2.12). Three of these peaks were identified by their co-chromatography with unlabelled authentic standards, and proved to be metiamide , metiamide sulphoxide and hydroxymethyl metiamide. The nature of the material in the fourth major peak which remained at the origin, was unknown.

Small amounts of other metabolites, notably metiamide urea and the carboxylic acid metabolite of metiamide were observed but never in amounts greater than 5% of the recovered radiolabel.

Fig.2.12

0-24h Urine Subject A Radiochromatography on Silica gel (Solvent III) co-chromatographed with non-radioactive standards



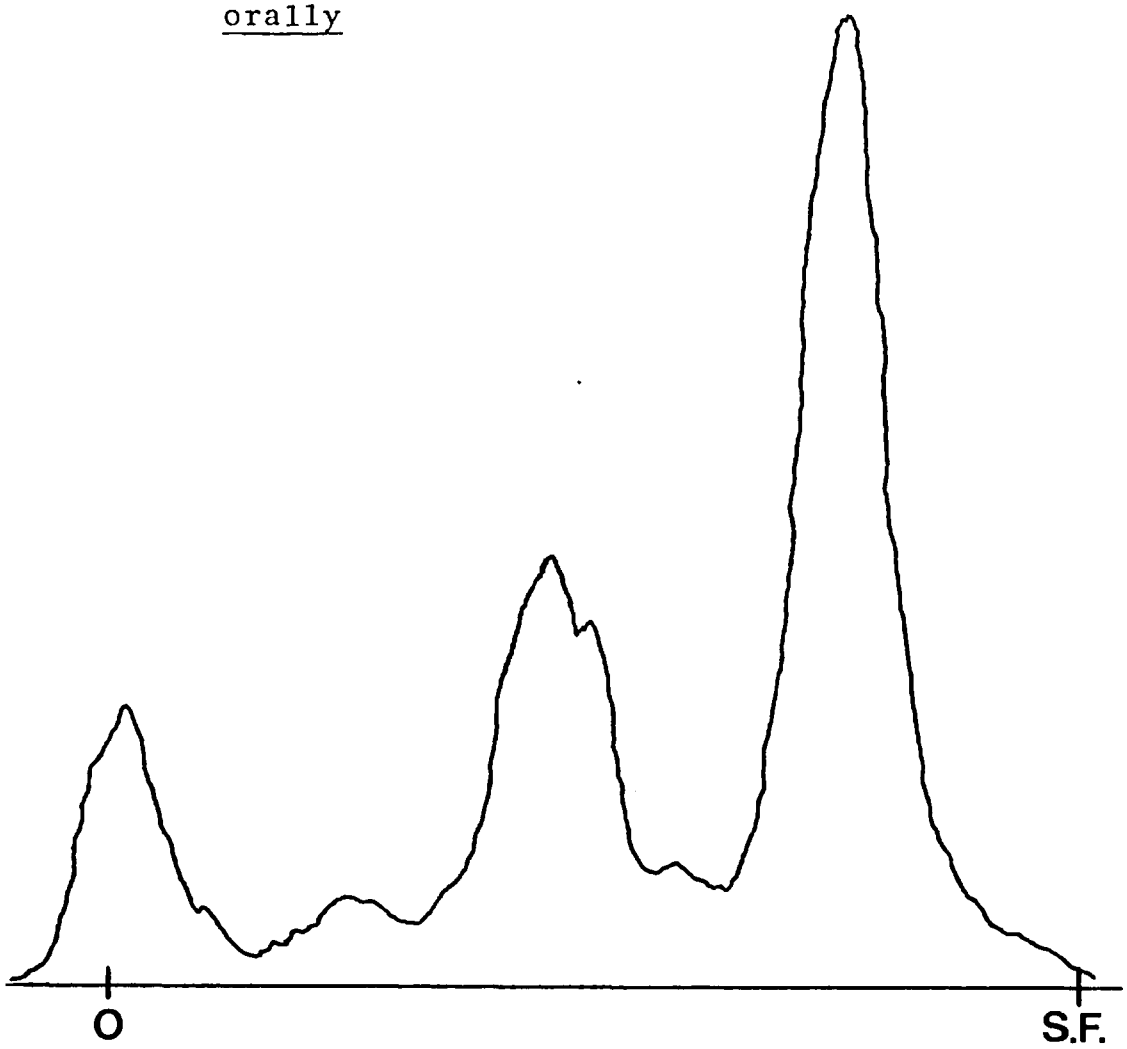
The quantitative aspects of the urinary elimination of metiamide and its metabolites is shown in Table 2.5.

Table 2.5 0-24h urinary excretion of metiamide and its metabolites after 50 mg (40μCi) of [^{14}C]-metiamide orally; (Subject A)

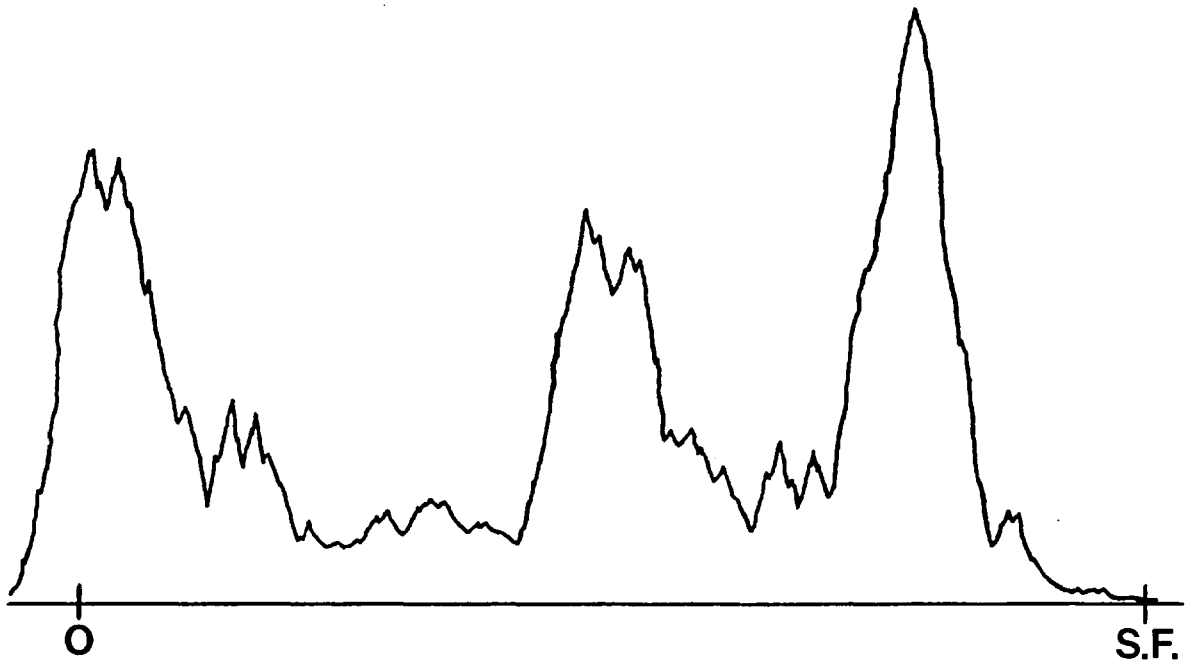
Metabolite	% Dose eliminated in urine
Metiamide	40.5
Metiamide urea	1.4
Hydroxymethyl metiamide	6.9
Metiamide sulphoxide	10.3
Unidentified metabolites	24.0

The pattern of metabolism was observed to change over the 24 hour period of urine collection. The polar unidentified metabolites were excreted in relatively minor amounts during the 0-2 hour period but in later collection periods (6-8 hour) it was the major urinary metabolite of metiamide (Fig.2.13).

Fig.2.13 0-2h urine after 50 mg (40μCi) [^{14}C] metiamide orally



6-8h urine after 50 mg (40μCi)[^{14}C]metiamide orally



2.3.1.2 Identification of Unidentified polar metabolites

Chromatographic properties

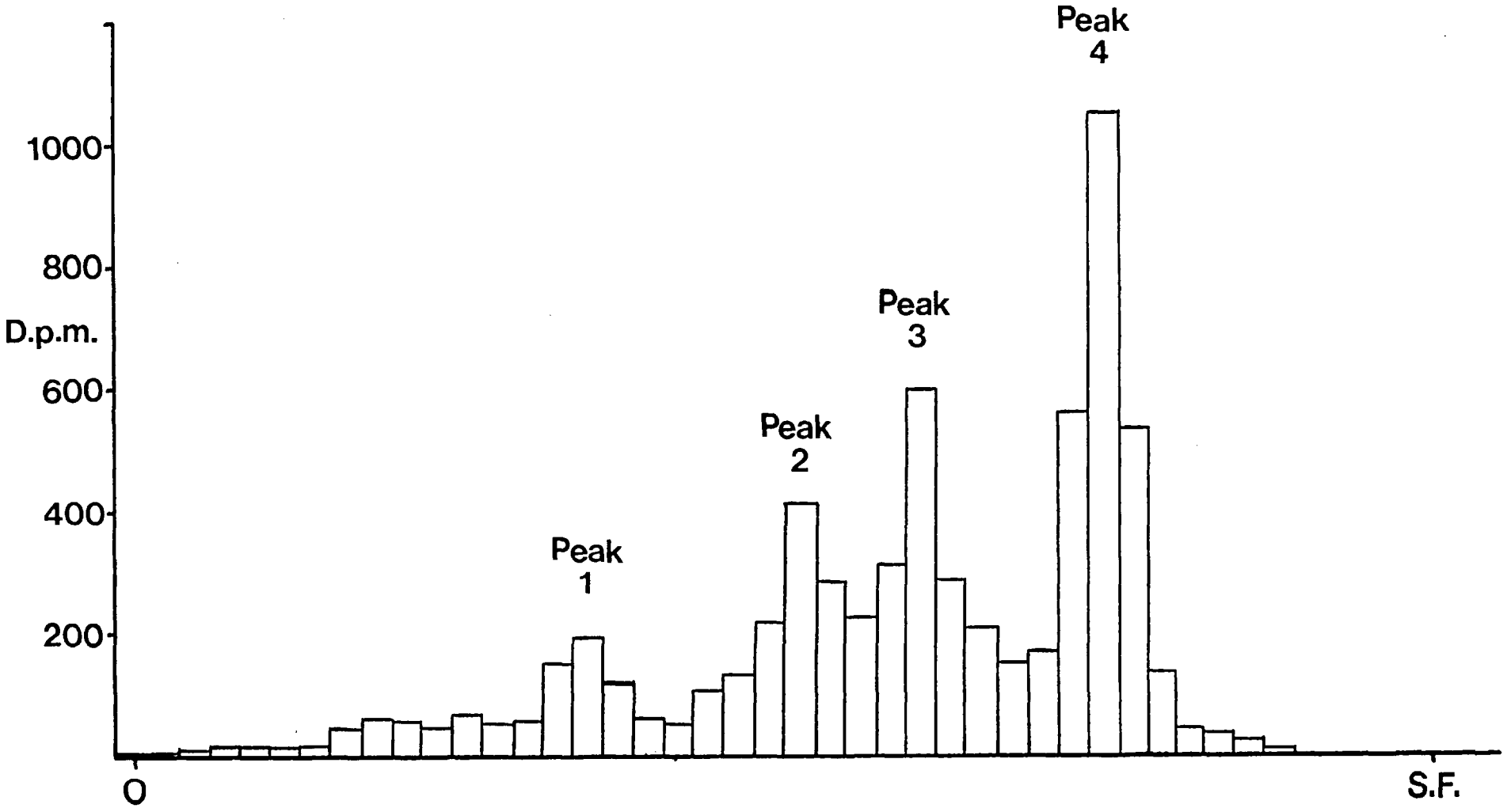
The chromatographic properties of the unidentified metabolites of metiamide were compared to those of authentic samples of metiamide and some of its oxidative metabolites in a number of different chromatographic systems (Table 2.6). The unidentified polar metabolites did not co-chromatograph with any of the authentic oxidative metabolites of metiamide.

Paper chromatography(solvent system I) of human 0-24h urine after oral administration of metiamide (50 mg, 10 μCi) revealed four major peaks, with R_F's of 0.36, 0.51, 0.62 and 0.76 (Fig.2.14). These peaks were numbered 1,2,3 and 4 respectively. Peak 4 co-chromatographed with unchanged metiamide while peak 3 co-chromatographed with authentic samples of both metiamide sulphoxide and hydroxymethyl metiamide. It was therefore considered to be a composite peak of these two metabolites.

Table 2.6 Chromatographic properties of polar metabolites of metiamide

Compound	R_F in systems: Paper Chromatography Solvent I	Silica Gel t.l.c. Solvent II	Silica Gel t.l.c. Solvent III	Cellulose t.l.c. Solvent IV
Metiamide	0.80	0.85	0.82	0.82
Metiamide urea	0.68	0.73	0.75	0.76
Hydroxymethyl metiamide	0.67	0.78	0.67	0.72
Metiamide sulphoxide	0.60	0.76	0.62	0.70
N(OH)metiamide	0.76	0.75	0.66	0.83
Carboxylic acid of metiamide	0.60	0.71	0.31	0.66
Polar 1	0.36	0.55	0.05	0.45
Polar 2	0.51	0.60	0.0	0.50

Fig.2.14 Paper chromatography of Subject A 0-24h urine after [^{14}C] metiamide (50 mg, 40μCi) orally.



Peaks 1 and 2 did not correspond to any authentic samples of metiamide metabolites. Peak 1 accounted for approximately 7% of the total ^{14}C on the chromatogram and Peak 2 about 18% of the ^{14}C.

Chemical properties of the unidentified metabolites

Samples of urine containing the unidentified metabolites were subjected to various treatments to elucidate some of their properties (Table 2.7).

Table 2.7. Properties of the unidentified metabolites

Treatment	Behaviour of unidentified metabolites
1. β-Glucuronidase treatment	No breakdown of metabolites
2. Sulphatase treatment	No breakdown of metabolites
3. Nucleoside treatment	No breakdown of metabolites
4. Acid hydrolysis	Complete breakdown of metabolites with corresponding increase in amount of metiamide on t.l.c. plates
5. Lead acetate treatment	Isolation of unidentified metabolites together with endogenous glucuronides

Spray reagents

Paper chromatograms and TLC plates were sprayed with a number of reagents to provide some information about the chemical nature of the unidentified metabolites (Table 2.8).

Table 2.8 Spray reagent tests on unidentified metabolites

Spray reagent	Positive test indicates	Polar 1	Polar 2
Pauly's reagent	Imidazole group	+	+
Grote's reagent	Thiourea group	+	+
Tollen's reagent	Thiourea group	+	+
Naphthoresorcinol test	Glucuronide conjugate	+	+
p-Dimethylamino-benzaldehyde test	Glycine conjugate	-	-

N.M.R.analysis of isolated polar metabolite (peak 2)

The sample (about 1 mg) was dissolved in D_2O and examined by 100 MH_z Fourier transform n.m.r.

Impurities

Acetate	; large	:	1.95 ppm
HOD	; large	:	4.70 ppm
Aromatic	; small	:	0.7 ppm
aliphatic	; small	:	1-2 ppm

Metabolite spectrum

Rest of the spectrum (50%) was attributable to a metiamide-related substance (Fig.2.15). Authentic samples of metiamide and N-hydroxy-metiamide were also analysed in order to compare the spectra (Figs.2.16 and 2.17).

All chemical shifts on the metabolite spectrum were

Fig.2.15 n.m.r. spectrum of unidentified metiamide metabolite

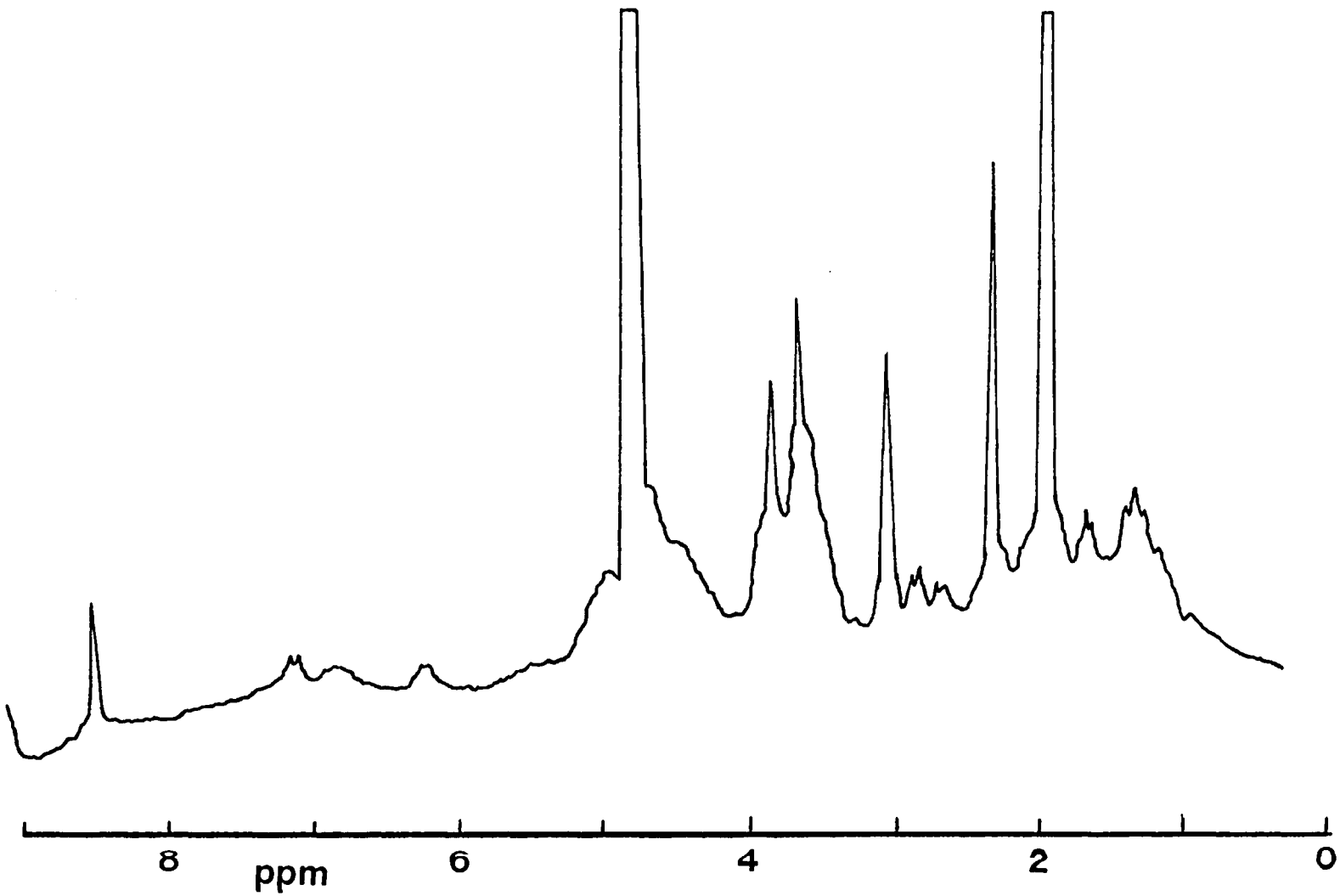


Fig.2.16 n.m.r. spectrum of metiamide

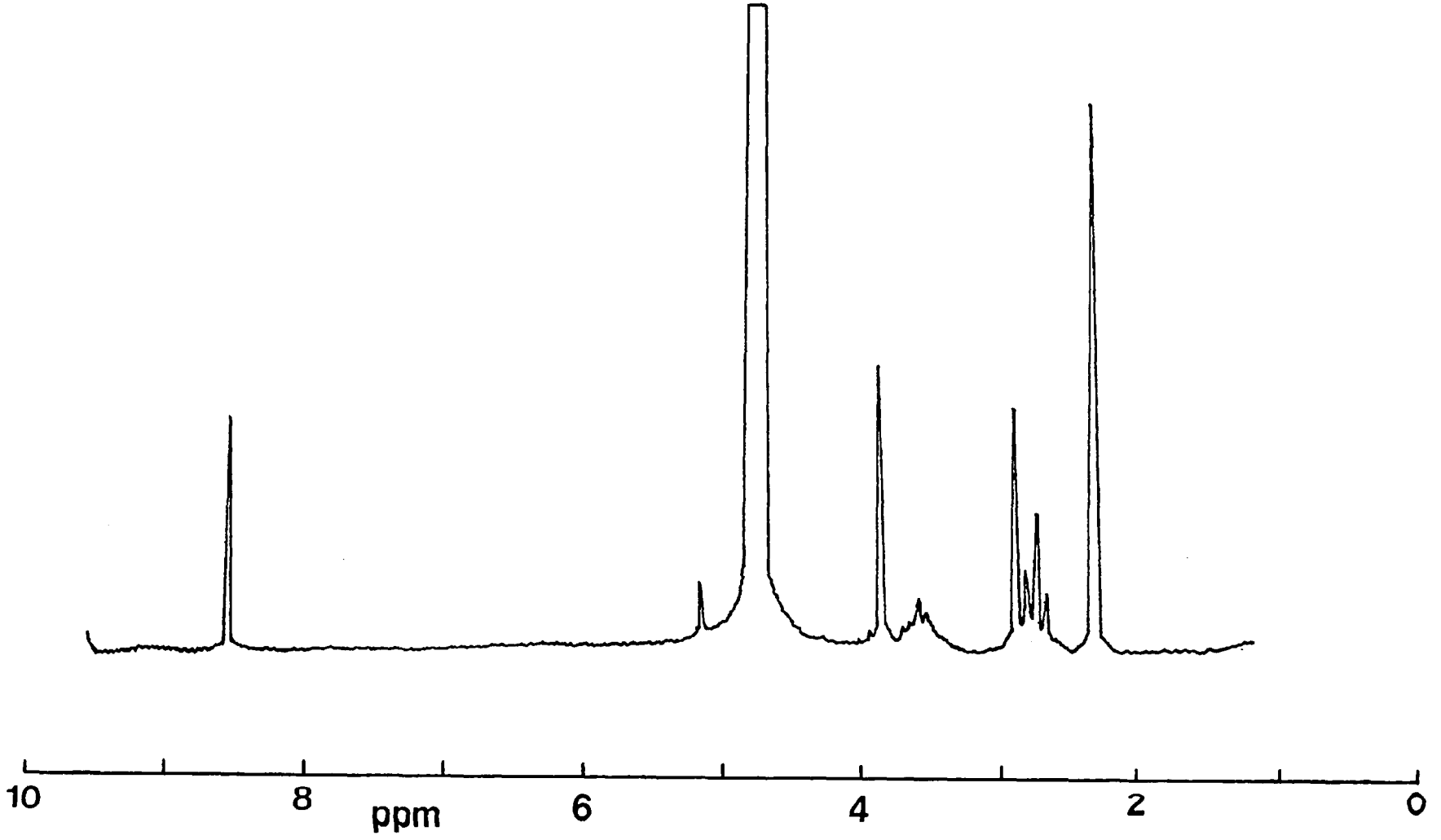
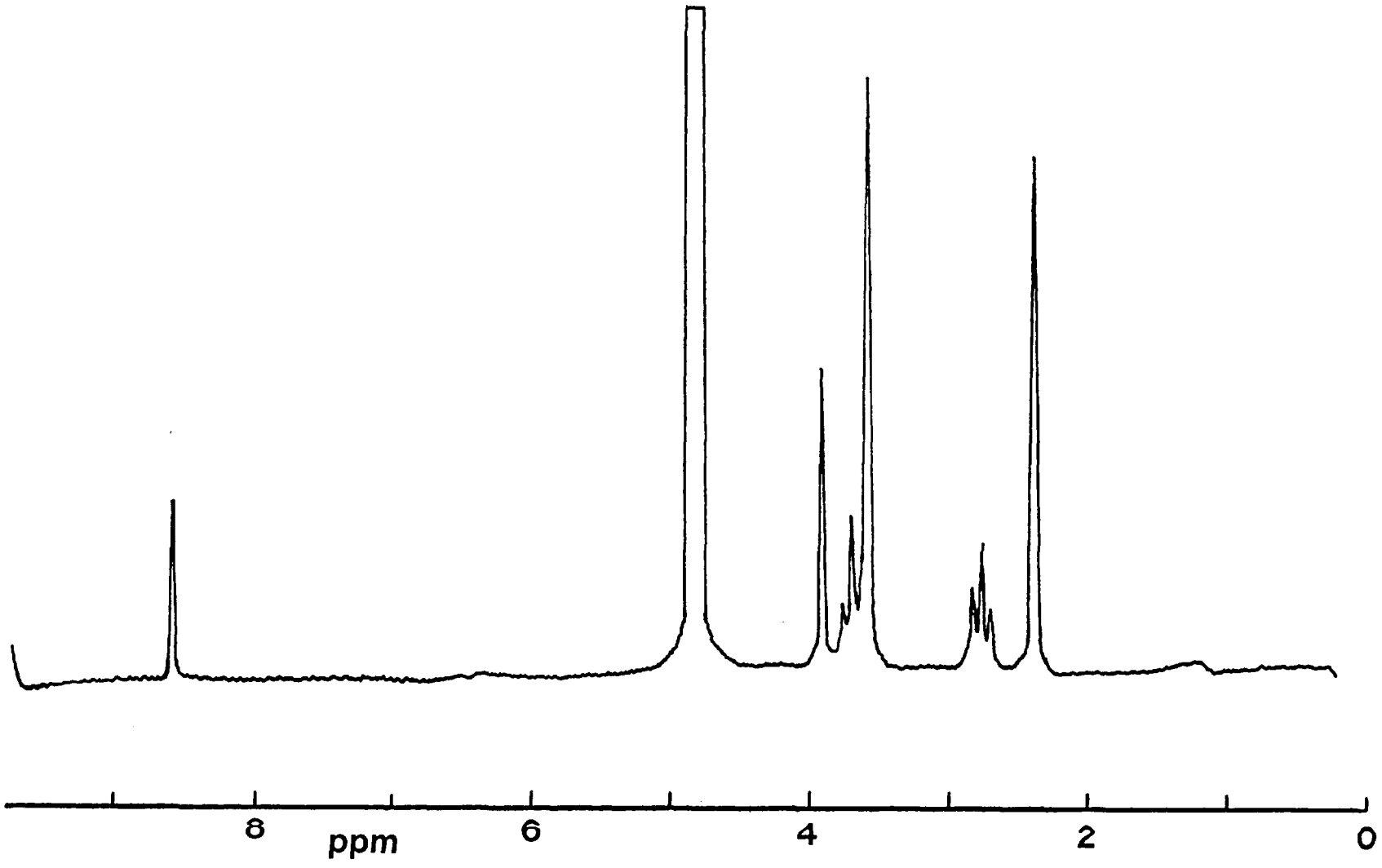


Fig.2.17 n.m.r. spectrum of N-hydroxymetiamide



within 0.01-0.02 ppm of metiamide in acid solution except for the $\underline{N}$-CH_3 protons which are deshielded (2.99 ppm) relative to metiamide $\underline{N}$-CH_3 (2.86 ppm). This would be supportive evidence for an electron withdrawing group on either of the two thiourea nitrogen atoms.

Thus, if Y = -OD (H exchanged for D in D_2O) then a similar spectrum would be expected (Fig.2.18).

However, when a sample of synthetic metiamide -$\underline{N}(OH)CH_3$ was examined under the same conditions, the $\underline{N}$-CH_3 protons were deshielded to a greater extent than either the metabolite (2.99 ppm) or metiamide (2.85 ppm).

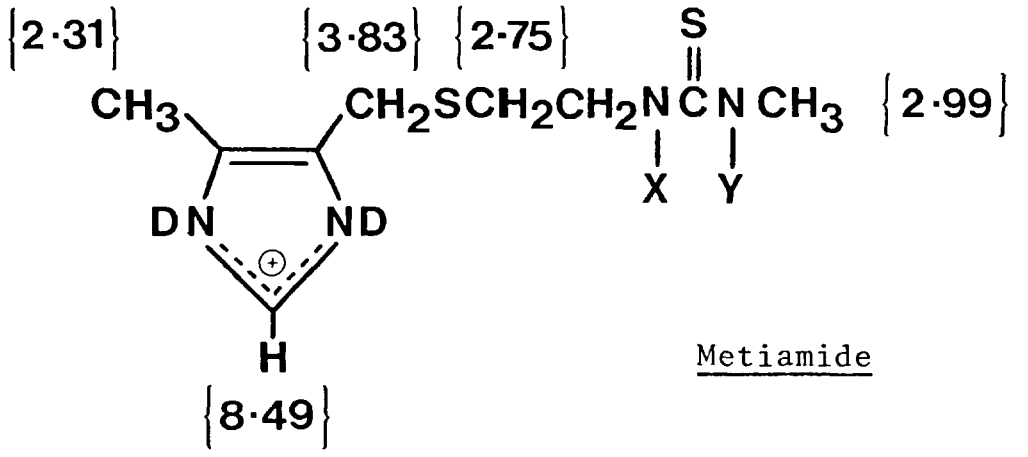
An authentic sample of the $\underline{N}(OH)CH_3$ metabolite was also examined by TLC and found to have different chromatographic properties from the polar metabolites in all the chromatographic systems.

Structure of major unidentified metiamide metabolite

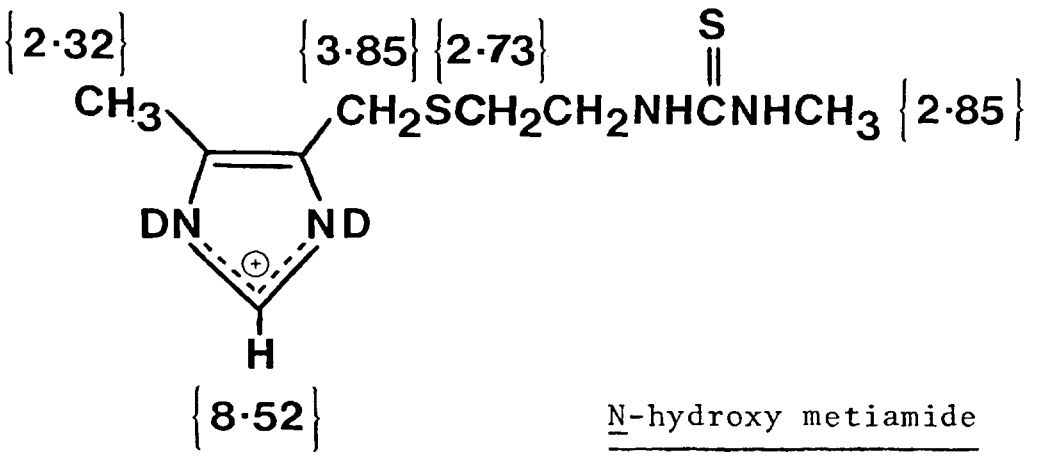
The chemical properties of the major unidentified metabolite suggest that it is a glucuronic acid

Fig.2.18 Chemical shifts in n.m.r. spectra polar metabolite compared to authentic samples of metiamide and N-hydroxy metiamide

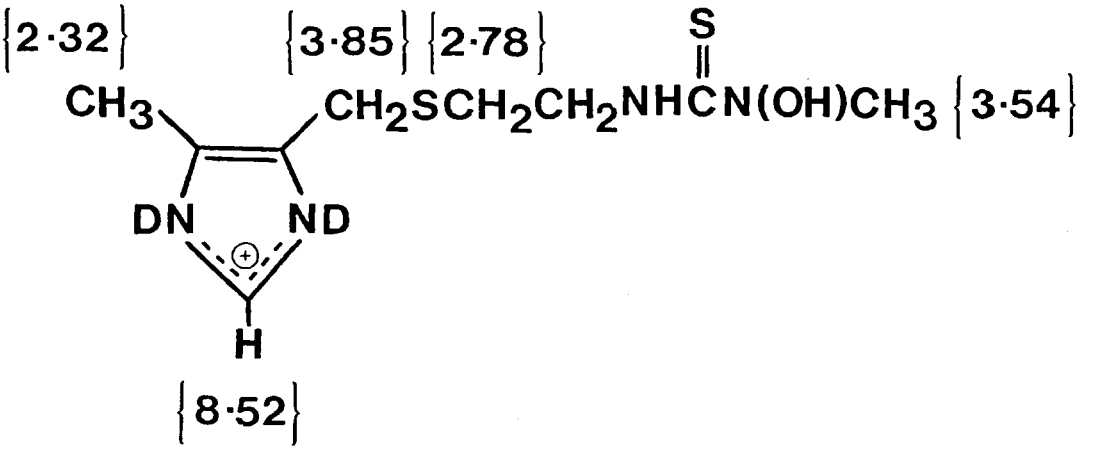
Polar metabolite 2



Metiamide



N-hydroxy metiamide



conjugate of metiamide or of a metabolite of metiamide, β-Glucuronidase-treatment of this metabolite did not, however, result in breakdown of the metabolite. This observation does not exclude the possibility that the metabolite is an N-glucuronide of metiamide since some N-glucuronides are known to be stable to β-glucuronidase-treatment (Bridges et al., 1965).

Metiamide contains four possible sites for the formation of N-glucuronides, the two nitrogen atoms in the imidazole ring, and the two nitrogen atoms in the thiourea group. The n.m.r. spectra of the unidentified metabolite would tend to suggest that the attachment of the glucuronic acid to metiamide occurs at one of the two nitrogen atoms in the thiourea group rather than at the nitrogen atoms in the imidazole ring.

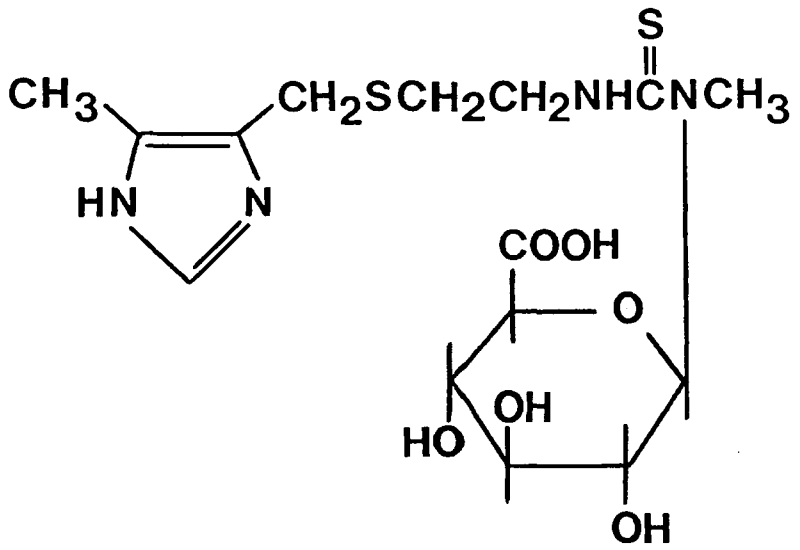
It is probable that the minor unidentified polar metabolite which had similar chromatographic and chemical properties to the major unidentified polar metabolite but could not be isolated in sufficient quantities for an n.m.r. analysis, is also an N-glucuronide of metiamide.

The putative structures for the major N-glucuronide of metiamide are shown in Fig. 2.19.

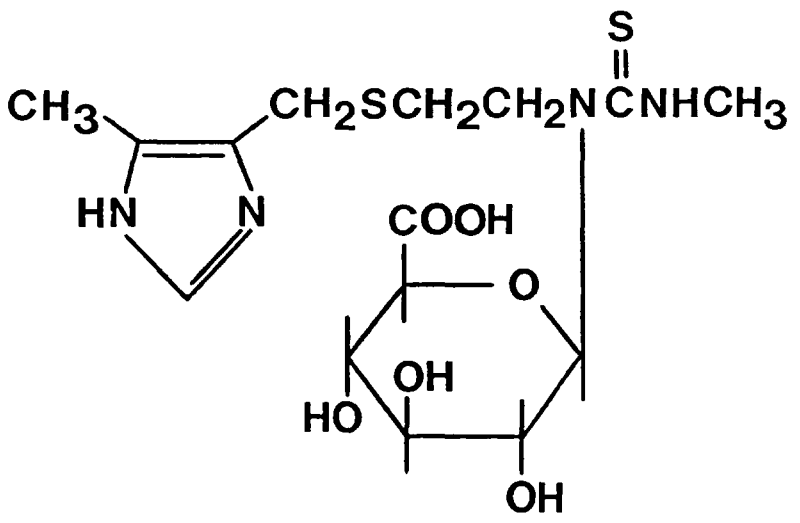
Fig.2.19

Putative structures for the major metabolite of metiamide in man

Metiamide N-glucuronide



Metiamide N'-glucuronide



2.3.2 Application of the phenotyped panel approach to the metabolism of metiamide in man

Two panels each comprising four volunteers, previously phenotyped for their ability to oxidise debrisoquine, participated in this study. One panel consisted of EM phenotype individuals and the other PM phenotype individuals (Table 2.9).

Table 2.9 Metabolic ratios of phenotyped panels

Subject No.	Metabolic ratio*	Phenotype
1	0.3	EM
2	0.7	EM
3	0.3	EM
4	0.5	EM
5	19.9	PM
6	26.4	PM
7	26.5	PM
8	24.5	PM

* Ascertained by procedure described by Mahgoub et al. (1977).

Reproducibility of phenotype assignment

Two subjects, one from each phenotype, repeated the phenotyping procedure, at an interval of several weeks, to ensure reproducibility of phenotype assignment (Table 2.10).

Table 2.10 Reproducibility of phenotype assignment

Subject No.	Metabolic ratios on 3 separate occasions			Phenotype
	1.	2.	3.	
3	0.4	0.3	0.4	EM
5	19.9	21.4	18.5	PM

The metabolism of metiamide in phenotyped panels

Each subject received 50 mg (10 μCi)[^{14}C] metiamide orally. Urine was collected during the period 0-24h postdosing.

^{14}C excretion in 0-24h urine

Recovery of ^{14}C in the 0-24h urine was significantly different ($P < 0.05$) between phenotypes, EM subjects

eliminating 77.9 ± 3.1% and PM subjects 70.1 ± 7.2% of the dose.

Elimination of metiamide and its metabolites in 0-24h urine.

The quantitative aspects of the metabolism of metiamide in the two panels are shown in Table 2.11. The values stated for metiamide N-glucuronide represent the sum of the values for the two polar metabolites, which are both glucuronic acid conjugates of metiamide.

Significant differences were observed between the phenotypes in the urinary excretion of all four of

Table 2.11 Urinary metabolites of metiamide in volunteers of known oxidation phenotype status

Subject	Phenotype*	% dose excreted as:- Metiamide	Metiamide Sulphoxide	Hydroxymethyl metiamide	Metiamide N-glucuronide	Total
1	EM	38.8	10.1	9.1	18.4	76.4
2	EM	53.3	7.3	6.0	12.6	79.2
3	EM	41.6	8.4	6.8	17.6	74.4
4	EM	47.2	6.9	4.9	22.4	81.4
5	PM	30.9	6.1	3.4	21.7	62.1
6	PM	38.8	6.4	4.9	19.7	69.8
7	PM	44.0	6.4	5.1	24.1	79.6
8	PM	30.4	5.6	5.1	27.6	68.7

+ phenotyped according to Mahgoub et al. (1977).

the metabolites examined (Table 2.12).

Table 2.12 Inter-phenotype differences in urinary excretion of metiamide metabolites

Compound	% dose in 0-24h urine		P
	EM phenotype	PM phenotype	
Metiamide	45.2±6.4	36.0±6.6	$P < 0.05$
Metiamide sulphoxide	8.2±1.4	6.1±0.4	$2P < 0.05$
Hydroxymethyl metiamide	6.7±1.8	4.6±0.8	$P < 0.05$
Metiamide N-glucuronide	17.8±4.0	23.3±3.4	$P < 0.05$

Reproducibility of results

The metabolism of metiamide in one subject (Subject 3, EM phenotype), was investigated on three separate occasions over a period of eight months to ensure that the inter-phenotype differences were reproducible (Table 2.13).

Table 2.13 Metabolism of [^{14}C] metiamide (50 mg, 10μCi) in one subject (subject 3; EM) on three separate occasions

% dose as:-	Experiment No.			Mean ± S.D.
	1.	2.	3.	
Metiamide	41.6	51.6	44.2	45.8±5.2
Metiamide sulphoxide	8.4	7.5	13.0	9.6±3.0
Hydroxymethyl metiamide	6.8	6.2	10.3	7.8± 2.2
Metiamide N-glucuronide	17.6	17.2	19.9	18.2±1.5
Total	74.4	82.5	87.4	81.4±6.6

These mean values for one subject on three separate occasions were significantly different from the mean values for the PM phenotype subjects (Table 2.14).

Table 2.14 Comparison of the metabolism of [^{14}C]-metiamide (50 mg; 10μCi) between one EM phenotype on three occasions and four PM phenotype subjects

% dose as:-	EM subject	PM subjects	P
Metiamide	45.8±5.2	36.0±6.6	$P < 0.05$
Metiamide sulphoxide	9.6±3.0	6.1±0.4	$P < 0.05$
Hydroxymethyl metiamide	7.8±2.2	4.6±0.8	$P < 0.05$
Metiamide N-glucuronide	18.2±1.5	23.3±3.4	$2P < 0.05$
Total	81.4±6.6	70.1±7.2	$P < 0.05$

Metiamide urea analogue excretion

The metiamide urea analogue excretion in the 0-24h urine of the two panels of volunteers was investigated. There was no significant difference between phenotypes, the EM phenotyped subjects excreting 3.7±1.4% of the dose as the metiamide urea analogue in 0-24h urine and PM subjects 3.6±1.6 % of the dose following

an oral dose of [^{14}C] metiamide (50 mg; 10μCi).

Metabolism of metiamide at a second dose level

The metabolism of metiamide was examined in two volunteers (subject 3, EM phenotype; subject 5, PM phenotype) at a second dose level of [^{14}C] metiamide (500 mg; 10 μCi). Urine samples were collected up to 24h postdosing.

Urinary excretion of ^{14}C

The EM phenotype subject excreted 70.6% of the dose and the PM phenotype subject excreted 53.9% of the dose in the 0-24h urine.

Elimination of metiamide and its metabolites in 0-24h urine

The quantitative aspects of the metabolism of metiamide in the two subjects is shown in Table 2.15.

Table 2.15 Urinary metabolites after [^{14}C] metiamide (500 mg; 10 μCi) in one EM and one PM subject

Compound	% dose in 0-24h urine	
	EM subject	PM subject
Metiamide	37.3	23.8
Hydroxymethyl metiamide	4.6	3.3
Metiamide sulphoxide	7.1	4.6
Metiamide N-glucuronide	21.6	22.2
Total	70.6	53.9

Inter-phenotype differences in the metabolism of metiamide

The major inter-phenotype difference in the metabolism of metiamide appears to be in the relative importance of the metabolic options available for metabolism. The

EM phenotype subjects appear to have a greater capacity to form the oxidative metabolites of metiamide whereas by comparison the PM phenotype subjects have a relatively impaired ability to form oxidative metabolites and excrete greater amounts of the conjugated metabolites.

A simple metabolic ratio may be calculated from the urinary excretion data which illustrates the major inter-phenotype differences:-

$$\text{Metiamide metabolic ratio} = \frac{\%\text{ dose excreted as conjugated metabolites}}{\%\text{ dose excreted as oxidised metabolites}}$$

The metiamide metabolic ratios for each of the phenotyped panels are shown in Table 2.16.

Table 2.16 Oxidation phenotype and metiamide 'metabolic ratios'

EM phenotype		PM phenotype	
Subject	Ratio	Subject	Ratio
1	0.96	5	2.28
2	0.95	6	1.74
3	1.16	7	2.10
4	1.90	8	2.58
Mean±SD	1.24±0.45	Mean±SD	2.18±0.35

Metiamide metabolic ratios were also calculated for the 500 mg dose level in one PM phenotype subject and one EM phenotype subject (Table 2.17).

Table 2.17 Metiamide metabolic ratios for one EM phenotype subject and one PM phenotype subject (500 mg dose)

EM phenotype		PM phenotype	
Subject	Ratio	Subject	Ratio
3	1.85	5	2.81

First-order urinary elimination rate constants

The first-order urinary elimination rate constants for metiamide and its metabolites were calculated by the method of Cummings et al. (1967) vide supra (Table 2.18).

Table 2.18 First-order urinary elimination rate constants for metiamide and its metabolites in phenotyped panels

Rate constant	EM phenotype	PM phenotype	P
K	0.326±0.023	0.293±0.040	ns
k_f sulphoxide	0.027±0.005	0.018±0.003	$2P < 0.05$
k_f hydroxy-methyl	0.022±0.006	0.014±0.002	$2P < 0.05$
k_f N-glucuronide	0.061±0.017	0.069±0.006	ns
k_D	0.146±0.016	0.107±0.028	$2P < 0.05$

2.4 Discussion

The disposition and metabolism of [^{14}C] metiamide in man has been studied. Metiamide was found to be well absorbed from an oral dose with peak plasma levels occurring at about 1h postdosing. The major route of excretion was found to be the urine with 65-90% of a 50 mg (10μCi) dose of [^{14}C] metiamide being eliminated in the 0-24h urine. The major component of the urinary ^{14}C was unchanged metiamide. Other metabolites included metiamide sulphoxide, hydroxymethyl metiamide, metiamide urea and

two polar unidentified metabolites. Trace amounts of a metabolite which co-chromatographed with the carboxylic acid analogue of metiamide were also detected.

The chemical properties of the unidentified polar metabolites suggested they are glucuronic acid conjugates of metiamide. The n.m.r. spectra of the major polar metabolite isolated from urine indicated that the position of attachment of the glucuronic acid to the metiamide molecule was at one of the two nitrogen atoms in the thiourea group.

Glucuronidation has been reported to be a major pathway for the metabolism of the thioamide antithyroid drugs (Papapetrou et al., 1972; Marchant et al., 1971). The exact nature of these glucuronide conjugates has, however, been a matter of some debate. Lindsay et al. (1977) found the major metabolite of propylthiouracil in the guinea-pig was a glucuronic acid conjugate.

These authors believed that the glucuronic acid was attached to the sulphur atom in the thiourylene group rather than one of the nitrogen atoms since the conjugate could be easily hydrolysed with β-glucuronidase treatment (unlike the metiamide glucuronide conjugate).

N-glucuronides are generally not hydrolysed by β-glucuronidase treatment (Bridges et al.,1965).

The application of the phenotyped panel approach to the metabolism of metiamide showed that small yet statistically significant inter-phenotype differences occur in the metabolic handling of metiamide. The major difference between the phenotypes was in the relative importance of each metabolic pathway available to metiamide. The EM phenotype tended to metabolise metiamide via oxidative pathways to a greater extent than the PM phenotype who favoured conjugation with glucuronic acid as the major metabolic reaction. This is probably due to the PM phenotype having a lower capacity for metabolic oxidation, resulting in more of the drug being available as a substrate for conjugation.

Some evidence for this was obtained by deriving rate constants for the formation of each metabolite from the urinary excretion data (Table 2.14). The EM phenotype had significantly higher rates of formation of metiamide sulphoxide (EM: 0.027±0.005; PM: 0.018±0.003; $2P < 0.05$) and hydroxymethyl metiamide (EM: 0.022±0.006; PM 0.014±0.002; $2P < 0.05$) than the PM phenotype.

However, the rates of formation of the conjugated metabolites were not significantly different between phenotypes (EM: 0.061±0.017; PM 0.069±0.006). Thus, the inter-phenotype difference was due not to an impaired ability to perform the conjugation reaction in the EM phenotype but to a relatively impaired ability to form oxidative metabolites of metiamide in the PM phenotype.

A proportion of the unchanged metiamide excreted in the urine may represent some metiamide sulphoxide which had been reduced back to the parent drug in the body. This reaction has been shown to occur in rats (Taylor *et al.*, 1979) where some 30% of an administered dose of metiamide sulphoxide was recovered in the urine as metiamide. This might explain the higher levels of unchanged metiamide excreted by the EM phenotype, since the sulphoxide metabolite would be unavailable for conjugation and after reduction would be excreted as parent drug.

The inter-phenotype difference in urinary excretion of metabolites was most clearly expressed as metiamide 'metabolite ratios' (Table 2.16) which revealed almost a two-fold difference between phenotypes (EM :1.24±0.45; PM: 2.18±0.35). At the 500 mg dose level the metiamide

metabolic ratio increased for both the EM and PM subjects (EM : 1.85 ; PM : 2.81) due to the excretion of a greater proportion of the dose as conjugated metabolite in both phenotypes. The metabolism of metiamide would therefore appear to be dose-dependent. Griffiths et al. (1977) found a similar dose-dependency in the metabolism of cimetidine whereby a greater proportion of the drug was excreted as polar material at higher doses.

The inter-phenotype differences in the metabolism of metiamide at the 500 mg dose level corresponded to those observed at the 50 mg dose level. However, the inter-phenotype differences in terms of the actual amounts of metiamide or metabolites in the body at any given time, would be naturally much greater at higher dose levels, due to the size of the administered dose. Thus, the plasma levels of metiamide and its metabolites in patients on chronic dosing with metiamide (usually 800-1200 mg day^{-1}) would have varied widely between individuals and one would have expected these to have been influenced, among other things, by the oxidation phenotype status of the individuals concerned.

It is remarkable that interphenotype differences were observed at all with a drug such as metiamide which is

excreted largely unchanged in the urine, has several available metabolic options, and was given at such a low dose level. These factors would all tend to mask the overall influence of the oxidation polymorphism on the drugs metabolism and make it difficult to detect.

However, in clinical use, high dose-levels and chronic administration might well combine to produce a situation whereby individual's propensity to develop metiamide-induced agranulocytosis is influenced by their oxidation phenotype status. This hypothesis is tested and discussed in Chapters 3 and 4.

CHAPTER THREE

STUDIES ON THE ESTABLISHMENT OF ANIMAL MODELS FOR THE METABOLISM AND TOXICITY OF METIAMIDE IN MAN

3.1. Introduction

Over the last thirty years the use of experimental animals in investigations into the toxic properties of chemicals has increased dramatically, mainly due to the vast expansion in the number and variety of novel compounds being developed for use as drugs. The aim of toxicological studies in this context is to evaluate the safety of these prospective drugs for therapeutic use in man.

Government legislation now requires that very detailed information on the metabolism, toxicity and pharmacology of prospective drugs, in animals, be submitted by drug companies before clinical trials certificates can be awarded. These studies are very time-consuming and expensive, but, in the majority of cases, they allow overtly toxic agents to be 'weeded out' before reaching man. However, this type of toxicity testing relies heavily on the assumption that experimental animals are reliable models for man, in both their behaviour, and susceptibility to, potentially toxic chemicals. This is patently not always the case.

There have been many instances where drugs have passed all the necessary animal toxicity tests only to fail

when adverse effects are uncovered in man. Perhaps the best-known example of this was the thalidomide tragedy; the failure of metiamide was another.

It is also highly probable that some therapeutically useful drugs have never been developed for use in man due to the occurrence of toxic reactions, in test animal species, which would not have occurred in man. An obvious problem exists, therefore, in the extrapolation of toxicological information from animals to man.

The Committee on Problems of Drug Safety in the U.S.A. recognised these difficulties in 1969 when they published a report on the application of metabolic data to the evaluation of drugs (Committee on Problems of Drug Safety, 1969). However, since the publication of that report little progress appears to have been made.

Species variations in the toxicity of drugs are frequently due to differences in drug metabolism. The toxic action of a drug may be direct or may depend upon the formation of a toxic metabolite. Thus, a toxic reaction may occur either because of a failure to metabolise the drug, so that it persists in its original form, or because of the rapid and effective

production of a toxic metabolite, which may then accummulate.

Metabolic studies in experimental animals are therefore vital to ensure that both the quantitative and qualitative aspects of a drug's metabolism make those animals suitable toxicological models for man.

A further problem arises, however, when drugs are metabolised differently, either qualitatively or quantitatively, by individuals within a given species. In animals, different strains from the same species have been used to examine the variability in drug metabolism which can occur within a species, and its toxicological consequences. An example of this concerns the volatile anaesthetic methoxyfluorane. An investigation of five strains of rats revealed in one strain an unusually high rate of methoxyfluorane metabolism, yielding very high levels of free fluoride in the blood, and thus producing renal toxicity (Mazze et al., 1973).

In man, wide variations have been observed in the ability to perform some metabolic reactions, frequently with associated toxicological consequences. In some cases this variation is known to be due to the polymorphic metabolism of drugs.

The acetylation polymorphism is an example whereby an individual's genotype influences his susceptibility to the toxic effects of a number of drugs. Individuals of the slow acetylating phenotype are more likely to develop peripheral neuritis (Hughes et al., 1954) and the systemic lupus erythematosus (SLE) syndrome (Zingale et al., 1963) as toxic reactions to isoniazid than those who are rapid acetylators. However, in isoniazid-induced hepatitis, rapid acetylators constitute the susceptible phenotype (Mitchell et al., 1975).

In situations such as this, where wide variations occur in the drug-metabolising abilities within one species, it is evident that no one animal model can adequately represent every individual in that population. There is thus an urgent need for animal models in which there occurs genetically-controlled variability in drug metabolism corresponding to that found in man. The ideal animal model would include animals corresponding to human individuals of every type of metabolic status, allowing the consequences of polymorphic drug metabolism on the toxicity of the drug, to be evaluated.

Few such pharmacogenetic models have been described. The best defined model is the rabbit model for the human acetylator polymorphism (Frymoyer & Jacox, 1963), which has formed the basis for investigations into the

susceptibility of different acetylator phenotypes to various drugs which undergo acetylation in man.

More recently an animal model for the human polymorphic oxidation of drugs, such as debrisoquine, has been uncovered in two strains of rats (Al-Dabbagh *et al.*, 1981). Females of the DA strain of rat were found to have an impaired ability to 4-hydroxylate debrisoquine compared with females of the Lewis strain. Thus the DA and Lewis females appeared to be metabolic models for the human PM and EM phenotypes respectively. To test this more carefully, both strains were investigated with respect to phenacetin metabolism (Al-Dabbagh *et al.*, 1981). The *O*-deethylation of phenacetin in man is polymorphic (Sloan *et al.*, 1978) and is under the control of the same gene locus that determines the debrisoquine 4-hydroxylation polymorphism (Sloan *et al.*, 1978). The DA rats were found to be partially deficient in their capacity to effect phenacetin-*O*-deethylation compared to the Lewis rats, which was in excellent agreement with the interphenotype metabolic differences observed with phenacetin in man.

The metiamide problem would appear to be a good candidate for the application of this type of approach to the investigation of toxic reactions to drugs.

The work described in this chapter was undertaken in an attempt to answer two questions:-

1. Why was metiamide-induced haematotoxicity observed in only two species, man and dog, and not the other eight species which received metiamide during its toxicity testing?

2. Why were only certain individuals sensitive to the toxic effects of metiamide in both man and dog?

An attempt was made to answer the first question by comparing the metabolism of metiamide in two sensitive species, man and dog, with two non-sensitive species, rat and mouse. Differences in the metabolic handling of metiamide, between sensitive and non-sensitive species might well explain the species-specificity in the haematotoxicity of metiamide.

The observation that the metabolism of metiamide is polymorphic in man might well explain some of the individual susceptibility to the drug, seen in patient and dog populations. The metabolism of metiamide was studied in the rat DA/Lewis metabolic model to investigate whether or not they constituted metabolic models for the human PM and EM phenotypes respectively.

However, in order to define the polymorphic metabolism/ toxicity relationship more clearly a metabolic model had to be found in dogs. A small population of beagle dogs were therefore phenotyped with debrisoquine in order to identify individuals with high and low metabolic oxidative abilities. From the metabolic information on the agranulocytosis patients it would be expected that the dogs most closely corresponding to the EM phenotype might be more susceptible to metiamide than the control dogs which were relatively poor oxidisers. These individuals were thus dosed daily with metiamide in an attempt to reproduce the blood dyscrasia.

3.2. Materials and methods

Materials

Metiamide, its metabolites and $[^{14}C]$ metiamide were the gift of Smith, Kline and French Limited; debrisoquine and its metabolites were the gift of Roche Products Limited, U.K.; 7-methoxyguanoxan was the gift of Pfizer Limited, U.K. ; the properties of these compounds has been described vide supra.

Animals

Male and female mice (15-25g) of the C_3H strain were used from the St.Mary's Hospital Medical School colony. Male and female rats (150-200g) of the DA (Bantin and Kingman, Grimstone, Hull, U.K.) and Lewis (St.Mary's Hospital Medical School colony) were used. Both these strains were inbred. Animals were maintained on Labshure 41B diet with free access to water.

Purebred beagle dogs (9-15 kg) were obtained from E.G.Stock, Balbeggie Kennels, Fifeshire; Allen & Hanbury Limited, Ware, Herts; or E.G.Crowley, Malvern.

Dosing of compounds

Rats and mice were dosed orally, with the drug dissolved in water, using a modified spinal or venous hypodermic needle. Animals were then housed in glass metabolism cages (Jencons Scientific Limited, Hemel Hempstead, Herts.) which separated urine from faeces.

Dogs were dosed orally with debrisoquine in tablet form (10 mg Declinax, Roche Products Limited) or with metiamide made up in gelatin capsules (dose adjusted for body weight). During the debrisoquine test the

dogs were housed in large metal metabolism cages, and urine was collected for 24 h. During the chronic toxicity testing with metiamide the dogs were housed together in pens.

Storage of samples

Urine samples from all animals were immediately frozen on collection and stored at -20°C until analysis.

Analytical procedures

Analysis of urine for [^{14}C] metiamide and its metabolites was performed by thin-layer chromatography, radiochromatogram scanning, and liquid scintillation counting as previously described (vide supra)

Debrisoquine and its metabolites were determined in urine by electron capture gas chromatography as previously described (vide supra).

Analysis of dog blood samples

Samples of venous blood (5 ml) were obtained from each dog by venepuncture. These samples were then routinely analysed on a Coulter counter in the toxicology department, Smith, Kline & French Limited.

3.3. Results

3.3.1 The metabolism of metiamide in species sensitive and non-sensitive to the haematotoxic effects of metiamide.

The metabolism of metiamide was investigated in male and female mice (C_3H strain) and in male and female rats (Lewis strain). Both these species are apparently non-sensitive to the haematotoxic effects of metiamide.

The metabolic profiles observed in these two species were compared to those seen in the beagle dog (as determined by Taylor *et al.*, 1979) and in man (*vide supra*) which are both sensitive species to the haematotoxicity of metiamide.

3.3.1.1 The metabolism of metiamide in the mouse

Male and female mice (C_3H strain; 15-25 g) were dosed orally with [^{14}C]metiamide (1.4 mg; 2.0 μCi) dissolved in water (0.25 ml). Urine was collected for the period 0-48h postdosing. Urinary recovery of radio activity is shown in Table 3.1.

Fig.3.1 Radiochromatogram scans of 0-24h urine of male and female C_3H mice following [^{14}C]-metiamide (1.4 mg; 2.0 μCi).

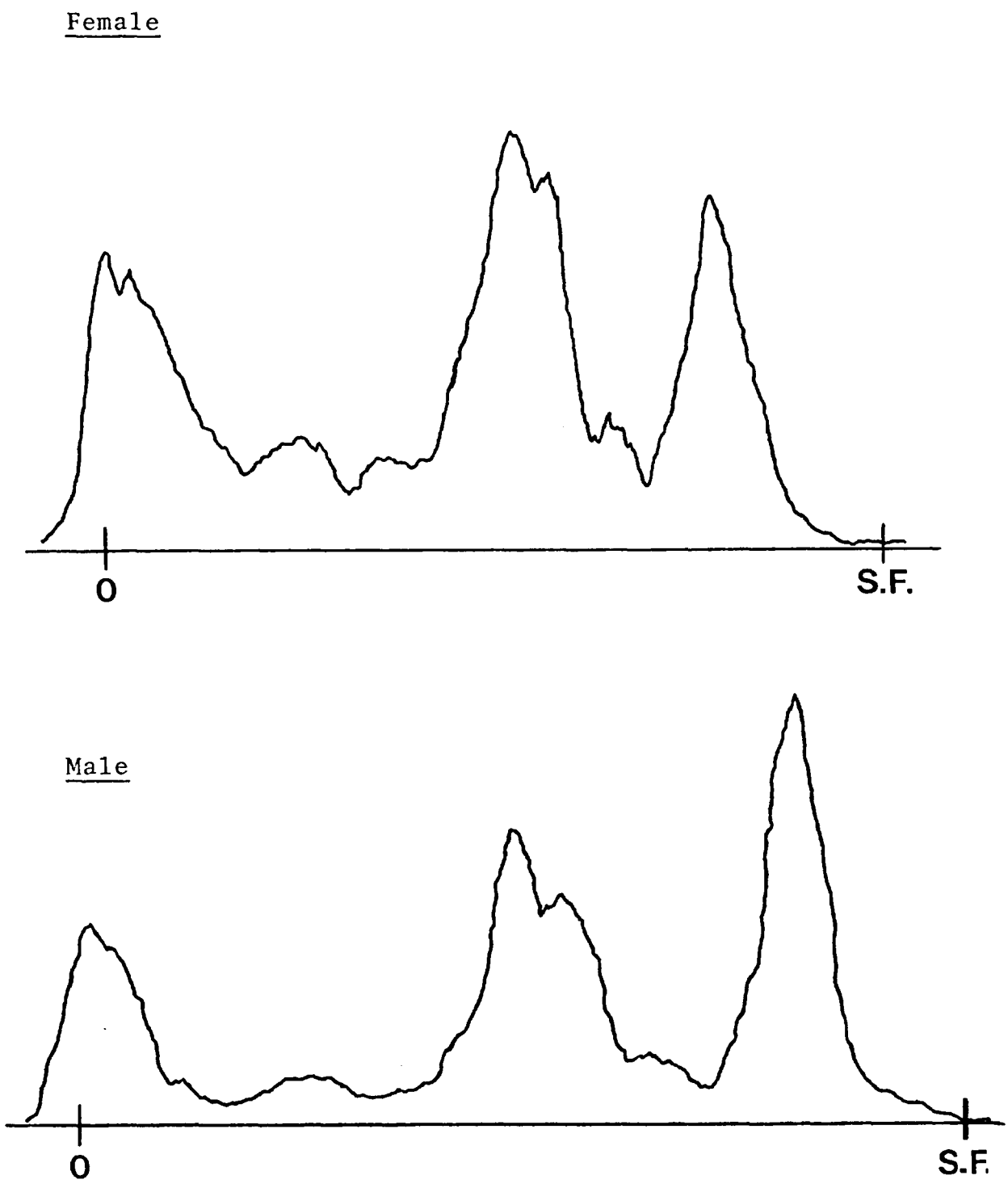


Table 3.1 Urinary recovery of ^{14}C radiolabel after [^{14}C]-metiamide (1.4 mg;2.0μCi) orally in male and female mice.

	% Dose			
	Male		Female	
Time(h)	1.	2.	1.	2.
0-24	62.1	64.8	74.2	76.2
24-48	3.6	3.6	3.2	0.6
Total	65.7	68.4	77.4	76.8

Radiochromatography of the 0-24h urine revealed four major peaks (Fig.3.1) which corresponded to metiamide, metiamide sulphoxide, hydroxymethyl metiamide and the unidentified polar material, and the unidentified polar material (silica gel plates, solvent system II).

Urinary excretion of metiamide and its metabolites

The quantitative aspects of metiamide metabolism in C_3H mice are shown in Table 3.2.

Table 3.2 Urinary metabolites of [^{14}C] metiamide (1.4 mg; 2.0μCi) in male and female C_3H expressed as % ^{14}C radiolabel recovered in 0-24h urine.

	% ^{14}C radiolabel			
	C_3H(male)		C_3H(female)	
	1.	2.	3.	4.
Metiamide	42.6	41.2	19.0	15.8
Metiamide urea	2.1	2.5	2.3	1.9
Hydroxymethyl metiamide	9.1	9.0	9.6	10.8
Metiamide sulphoxide	11.2	9.7	17.7	19.4
Metiamide N-glucuronide	23.1	23.3	24.7	28.0

A sex difference was observed in the metabolism of metiamide in C_3H mice. Females appeared to metabolise metiamide to a greater extent than the males. They excreted less unchanged metiamide (females 19.0%, 15.8% ; males 42.6%, 41.2%) and more sulphoxide (females 17.7%, 19.4%; males 11.2%,9.7%) than the males. A difference was also observed in the amounts of hydroxymethyl metiamide excreted in the urine

(females 9.6%, 10.8%; males 9.1%, 9.0%). Both male and female mice excreted large amounts of a polar metabolite in the urine and there was no apparent difference between the sexes. This polar material had identical chromatographic properties to the metiamide N-glucuronide identified in human urine.

3.3.1.2 Metiamide metabolism in the rat

Male and female Lewis rats (200-250 g) were dosed orally with [^{14}C]metiamide (14.0mg; 2.0μCi) dissolved in water (1.0 ml). The rats were placed in glass metabolism cages and urine collected for the period 0-24h.

Recovery of ^{14}C radiolabel in 0-24h urine

Table 3.3 shows the recovery of radiolabel in the 0-24h urine of the rats.

Table 3.3 Recovery of ^{14}C radiolabel in 0-24h urine of Lewis rats dosed orally with [^{14}C]metiamide (14 mg; 2.0μCi).

	Rat 1	Rat 2	Rat 3	Rat 4
% Dose in 0-24h urine	81.3	86.9	65.5	86.2

Radiochromatogram scanning of rat urine

Samples of the 0-24h rat urine (100μl) were chromatographed on silica gel plates using solvent system II (described in Chapter one). After development the plates were examined by radiochromatogram scanning (Fig.3.2).

Three major peaks were observed which corresponded to metiamide, metiamide sulphoxide and hydroxymethyl metiamide.

Urinary excretion of metiamide and its metabolites

The quantitative aspects of the urinary excretion of metiamide and its metabolites are shown in Table 3.4.

A sex difference in the metabolism of metiamide in the rat was apparent. The male rats metabolised metiamide to a greater extent than the females, excreting less unchanged metiamide and greater amounts of metiamide sulphoxide, hydroxymethyl metiamide, the metiamide urea analogue and a polar metabolite (which had identical chromatographic properties to the metiamide N-glucuronide identified in human urine).

Fig.3.2 Radiochromatogram scans of 0-24h urine from male and female Lewis rats following [^{14}C]-metiamide (14 mg; 2.0μCi) orally.

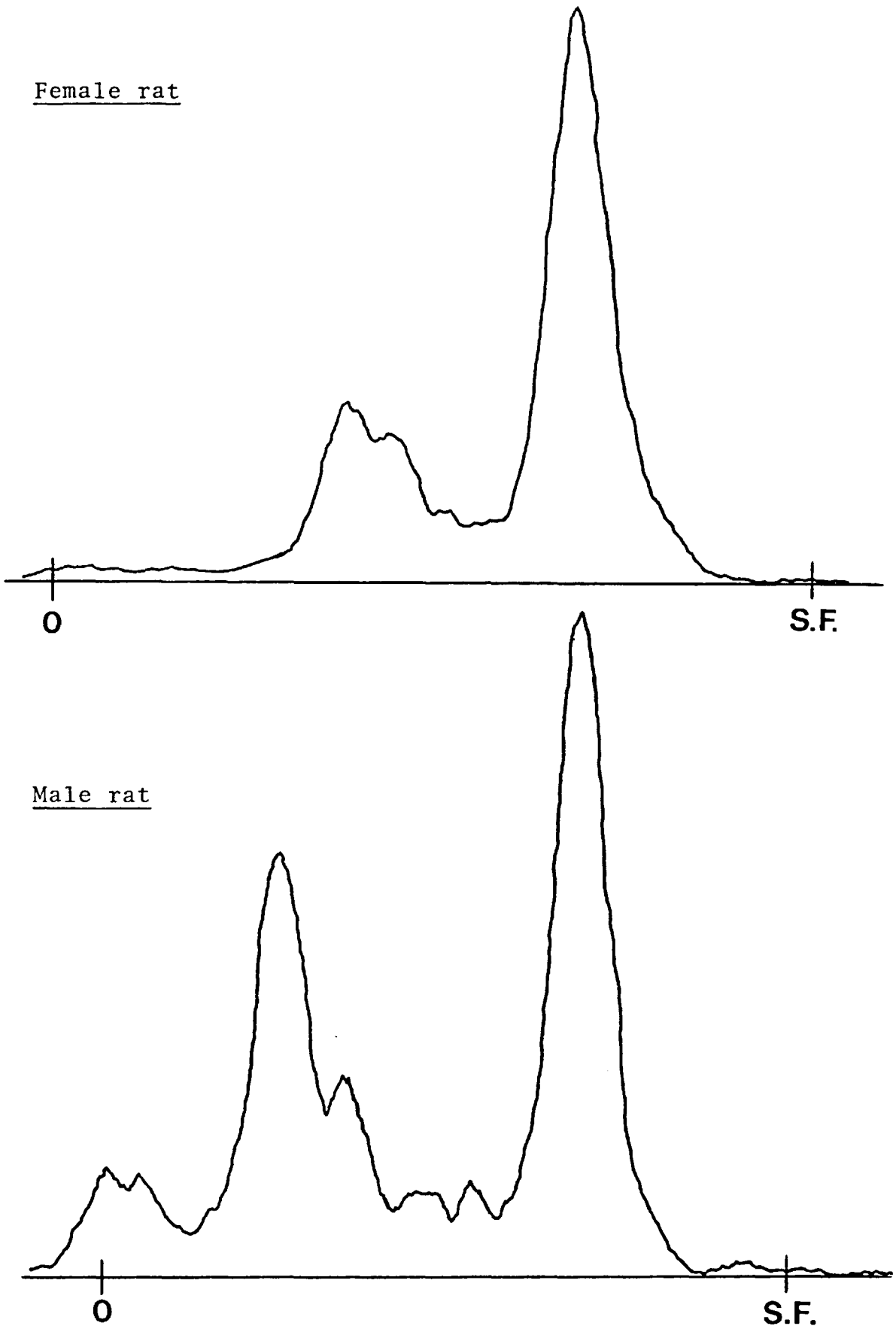


Table 3.4 Urinary metabolites of [^{14}C] metiamide in male and female Lewis rats expressed as the % of ^{14}C recovered (0-24h).

	% Urinary ^{14}C			
	Male		Female	
	1.	2.	3.	4.
Metiamide	43.5	32.3	68.9	70.6
Metiamide urea analogue	5.8	4.8	3.9	3.2
Hydroxymethyl metiamide	8.5	8.7	6.1	7.0
Metiamide sulphoxide	22.8	29.9	11.9	11.3
Metiamide N-glucuronide	19.3	24.3	9.2	7.9

Comparative metabolism of metiamide in mouse, rat, dog and man.

Table 3.5 shows a comparison of the metabolism of metiamide in the four species, mouse, rat, dog and man.

Table 3.5 Comparison of the metabolism of metiamide in four species

(% ^{14}C radiolabel in 0-24h urine)

	Mouse(70mg kg^{-1})		Rat(70mg kg^{-1})		Dog(40mg kg^{-1})	Man(0.7mg kg^{-1})	
	Male	Female	Male	Female	(n=4)	EM (n=4)	PM (n=4)
Metiamide	42.6,41.2	19.0,15.8	43.5,32.3	68.9,70.6	52.8±8.8	58.2±6.7	51.2±5.4
Metiamide urea analogue	2.1, 2.5	2.3, 1.9	5.8, 4.8	3.9, 3.2	7.4±1.3	5.7±2.2	7.0±3.3
Hydroxymethyl metiamide	9.1, 9.0	9.6,10.8	8.5, 8.7	6.1, 7.0	8.1±1.0	8.7±2.6	6.6±0.8
Metiamide sulphoxide	11.2, 9.7	17.7,19.4	22.8,29.9	11.9,11.3	13.3±1.6	10.6±1.6	8.8±0.8
Metiamide N-glucuronide	23.1,23.3	24.7,28.0	19.3,24.3	9.2, 7.9	21.0±4.5	23.0±5.0	33.4±5½3
Ratio $\frac{\text{conj.}}{\text{ox.}}$	0.36,0.37	0.51,0.58	0.24,0.32	0.10, 0.09	0.74±0.20	1.01±0.40	1.64±0.29

All four species metabolise metiamide in a broadly similar manner, excreting large amounts of metiamide unchanged in the urine together with metiamide sulphoxide, hydroxymethyl metiamide and the metiamide urea analogue. The female rat was the only group which did not also excrete large amounts of the unidentified polar material in the urine.

Comparing the mouse and rat species (non-sensitive species) with man and dog (sensitive species), no major differences in the metabolic handling of metiamide, were observed. However, the sensitive species (man and dog) did excrete a greater amount of the urinary ^{14}C as the metiamide urea analogue (man: EM phenotype, 5.7$\pm$ 2.2%; PM phenotype, 7.0$\pm$ 3.3%; dog: 7.4$\pm$ 1.3%) than the non-sensitive rat and mouse species (rat: male, 5.8%, 4.8%; female, 3.9%, 3.2%; mouse: male 2.1%, 2.5%; female, 2.3%, 1.9%). This difference is significant at the 5% level if the two groups are compared by the two sample t-test ($2P < 0.05$).

No consistent difference was observed between the two groups in the ratio of conjugated metabolites over oxidative metabolites of metiamide.

3.3.2 The metabolism of metiamide in the rat DA/Lewis metabolic model for human EM and PM phenotypes.

Establishment of the rat strain model

The DA/Lewis rat strain model for the human EM and PM phenotypes has been described by Al-Dabbagh *et al*. (1981). The DA strain females have a relative inability to perform metabolic oxidation of debrisoquine and phenacetin compared to the Lewis strain females. The two strains thus resemble the human PM and EM phenotypes respectively.

The metabolism of metiamide was therefore investigated in the rat model to determine whether or not the inter-phenotype differences observed in man could be reproduced in an animal model.

Debrisoquine metabolism in DA and Lewis female rats

Three DA and three Lewis female rats (150-200 g) were dosed orally with debrisoquine (1.28 mg debrisoquine sulphate, equivalent to 1.0 mg free base) dissolved in water. The rats were placed in glass metabolism cages and urine collected for 24h. The urines were then analysed for debrisoquine and 4-hydroxy-debrisoquine as previously described.

Urinary excretion of debrisoquine and 4-hydroxy-debrisoquine

The excretion of debrisoquine and 4-hydroxy-debrisoquine in the 0-24h rat urine is shown in Table 3.6.

The DA rats excreted small amounts of 4-hydroxy-debrisoquine but large amounts of unchanged debrisoquine and all had metabolic ratios of greater than 4. The Lewis rats, however, excreted little unchanged debrisoquine but large amounts of 4-hydroxy-debrisoquine giving them metabolic ratios of 0.3.

The rats were therefore considered suitable for use as metabolic models for the human PM and EM phenotypes.

The metabolism of metiamide in the rat DA/Lewis metabolic model.

Each rat was dosed orally with [^{14}C] metiamide (14 mg; 2.5 μCi) and then placed in glass metabolism cages for the collection of 0-24h urine.

Table 3.6 0-24h urinary excretion of debrisoquine and 4-hydroxydebrisoquine in DA and Lewis female rats.

Rat	% Dose in 0-24h urine			Metabolic ratio
	Deb	4OH Deb	Total	
DA 1.	53	10	63	5.3
DA 2.	31	7	38	4.5
DA 3.	34	8	42	4.3
Lewis 1.	11	44	55	0.3
Lewis 2.	17	55	72	0.3
Lewis 3.	10	32	42	0.3

Urinary excretion of ^{14}C

Total recovery of ^{14}C in the 0-24h urine was very similar in the two strains: DA, 73.9± 7.4% ; Lewis 75.9± 14.6%.

Radiochromatogram scanning of 0-24h urine

Radiochromatogram scanning of 0-24h urine from the two strains revealed the presence of three major peaks corresponding to metiamide, metiamide sulphoxide and hydroxymethyl metiamide. The DA strain also excreted some unidentified metabolites which remained near the origin (Fig.3.3) and had properties identical to metiamide N-glucuronide.

Urinary excretion of metiamide and its metabolites

Table 3.7 shows the metabolic profiles for metiamide and its metabolites in 0-24h urine.

Fig.3.3 Radiochromatogram scans of 0-24h rat urines after [^{14}C] metiamide (14 mg; 2.5 μCi).

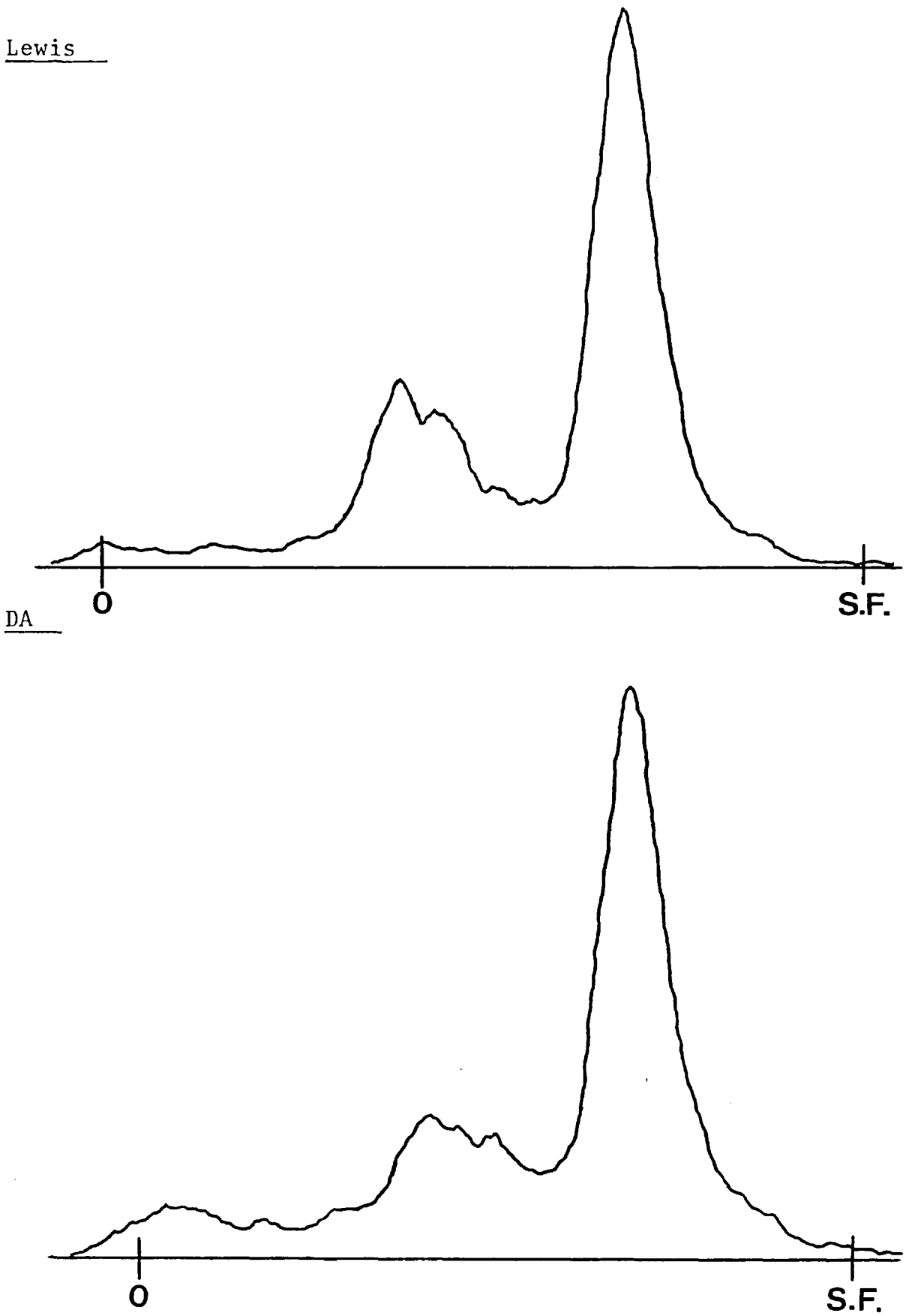


Table 3.7 Urinary excretion of metiamide and its metabolites in DA and Lewis rats.

	% Dose		
	DA(n=3)	Lewis(n=3)	2P<
Metiamide	44.9±3.3	53.0±11.2	ns
Metiamide urea	2.8±0.4	2.7± 0.1	ns
Hydroxymethyl metiamide	5.3±0.9	5.0± 1.4	ns
Metiamide sulphoxide	8.0±1.8	8.8± 1.3	ns
Metiamide N-glucuronide	12.9±3.2	6.5± 0.5	0.05

No significant differences were observed between the two rat strains in terms of urinary excretion of metiamide, metiamide urea, hydroxymethyl metiamide or metiamide sulphoxide. However the DA strain did excrete significantly greater amounts of the metiamide

N - glucuronide than did the Lewis rats (DA: 12.9±3.2%; Lewis: 6.5± 05%; $2P < 0.05$). This correlates well with the observed metabolic differences in the handling of metiamide between human EM and PM phenotypes (PM: 23.3±3.4; EM: 17.8± 4.0% dose; $P < 0.05$).

3.3.3 Studies on the formation of an animal model for the occurrence of metiamide-induced agranulocytosis in man.

The aim of these experiments was to produce an animal model for the occurrence of metiamide-induced agranulocytosis in man, using the beagle dog. The problem was approached by applying the hypothesis that the individual susceptibility to the haemotoxic effects of metiamide is influenced among other things, by an individual's metabolic oxidative ability, and that this ability might be determined by examining the oxidative metabolism of drugs such as debrisoquine in each individual.

The metabolism of debrisoquine was therefore examined in a small population of beagle dogs in order to identify individuals with exceptionally high and low oxidative abilities. These dogs would then form the basis of the metabolic model in which the haematotoxic

effects of metiamide could be investigated.

Debrisoquine metabolism in a small population of beagle dogs.

Twenty adult beagle dogs (8-15 kg) were dosed orally with debrisoquine (10 mg) in tablet form. The dogs were then placed in large metal metabolism cages and urine collected for 24h. The urine was then analysed for debrisoquine and its metabolites as previously described.

The urinary excretion of debrisoquine and its metabolites.

The urinary excretion of debrisoquine and its metabolites is shown in Table 3.8.

Metabolic ratios were calculated from the data:-

$$\text{Metabolic ratio} = \frac{\%\text{ dose excreted as debrisoquine in 0-24h urine}}{\%\text{ dose excreted as oxidative metabolites in 0-24h urine}}$$

The metabolic ratios for the dog population are shown in Table 3.9.

A frequency distribution histogram of these ratios reveals a three to four fold variability in metabolic ratio (Fig.3.4).

Table 3.8 Urinary excretion of debrisoquine and its metabolites in the dog.

	% Dose in urine 0-24h						
Dog	Deb+	4OH*	5OH*	6OH*	7OH*	8OH*	Total
73	31.3	8.6	0.4	1.0	6.8	1.2	49.3
74	21.4	8.0	0.4	0.3	3.5	1.4	35.0
75	16.1	9.5	nd	0.1	6.2	0.3	32.2
76	22.6	11.2	0.7	0.3	7.3	2.0	44.1
77	1.5	0.7	nd	0.2	4.3	0.5	7.5 (incomplete urine collection)
78	7.3	3.0	nd	2.0	4.8	1.0	18.1
79	32.0	9.5	nd	0.3	2.2	0.3	44.3
80	20.6	9.1	nd	1.7	10.2	1.4	43.0
81	24.1	9.1	nd	1.0	10.4	1.4	46.0
82	22.2	8.9	nd	1.6	9.7	1.7	44.1
83	16.1	11.4	nd	2.1	10.3	1.4	41.3
84	18.0	5.6	0.4	0.3	4.1	0.8	29.2
473	20.0	7.1	nd	0.1	5.7	0.6	33.5
852	29.5	6.0	nd	nd	1.7	0.2	37.4

cont./

Table 3.8(cont.)

Dog	Deb+	4OH*	5OH*	6OH*	7OH*	8OH*	Total
910	26.7	11.3	nd	0.7	4.1	0.1	42.9
930	22.0	6.9	0.6	1.2	11.0	1.3	43.0
215	25.6	7.4	0.1	2.4	7.3	1.0	43.8
1682	18.5	5.4	nd	1.1	6.0	1.7	32.7
171	30.5	8.8	0.1	3.3	8.1	2.2	53.0
640	21.3	12.7	0.7	0.4	19.9	1.9	56.9

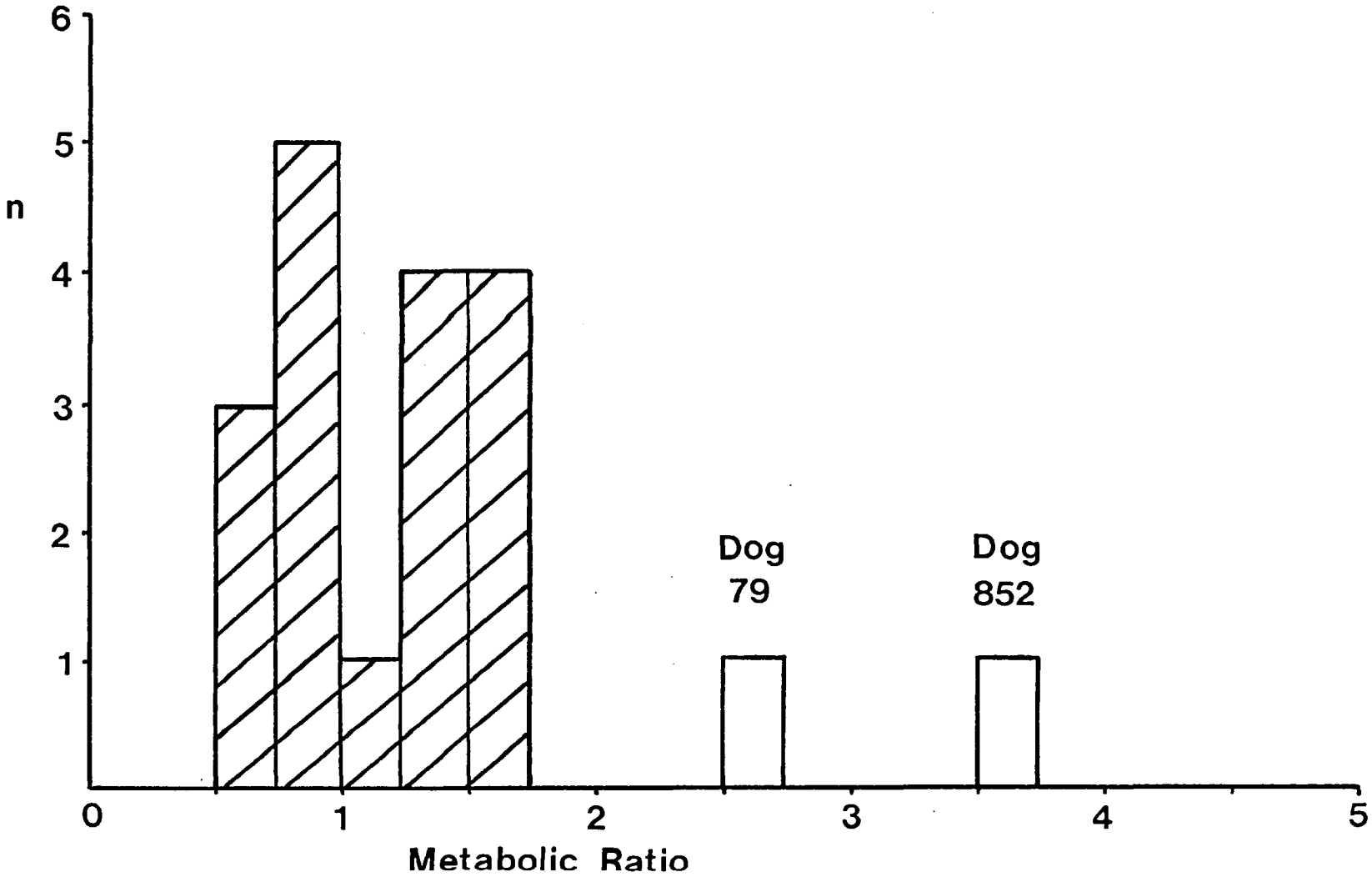
+ Deb = debrisoquine

* OH = hydroxy-debrisoquine

Table 3.9 Metabolic ratios of beagle dog population

Dog No	M.R.	Dog No.	M.R.
73	1.7	83	0.6
74	1.6	84	1.6
75	1.0	473	1.5
76	1.1	852	3.7
77	0.3	910	1.6
78	0.7	930	1.0
79	2.6	215	1.4
80	0.9	1682	1.3
81	1.1	171	1.4
82	1.0	640	0.6

Fig.3.4 Metabolic ratios of beagle dog population



Chronic dosing of dogs with metiamide

The two dogs with the highest metabolic ratios (dog 79 , ratio 2.6 and dog 852 , ratio 3.7) had significantly higher metabolic ratios compared to the rest of the dog population (dogs 79 and 852, mean ratio 3.15 $\pm$ 0.78; rest of dog population, mean ratio 1.33 $\pm$ 0.75; $2P < 0.05$). These two dogs (79 and 852) were therefore selected as the poor oxidiser models and dogs 78 (metabolic ratio, 0.7) and dog 640 (metabolic ratio, 0.6) selected as the good oxidiser models.

These four dogs were dosed with 150 mg kg^{-1} day^{-1} metiamide in gelatin capsules. This dose was selected to give the lowest probability of any dog dying acutely from the toxic effects of metiamide on the lungs but a good probability of observing the haematotoxic effects of metiamide within the six week period of the test.

Samples of venous blood were taken from each of the dogs every seven days during the test by venepuncture. These blood samples were then analysed on a Coulter counter for various blood parameters including total white blood cell count. The dogs were also weighed at weekly intervals and the doses of metiamide then adjusted for the changes in body weight.

Dog body weights during test

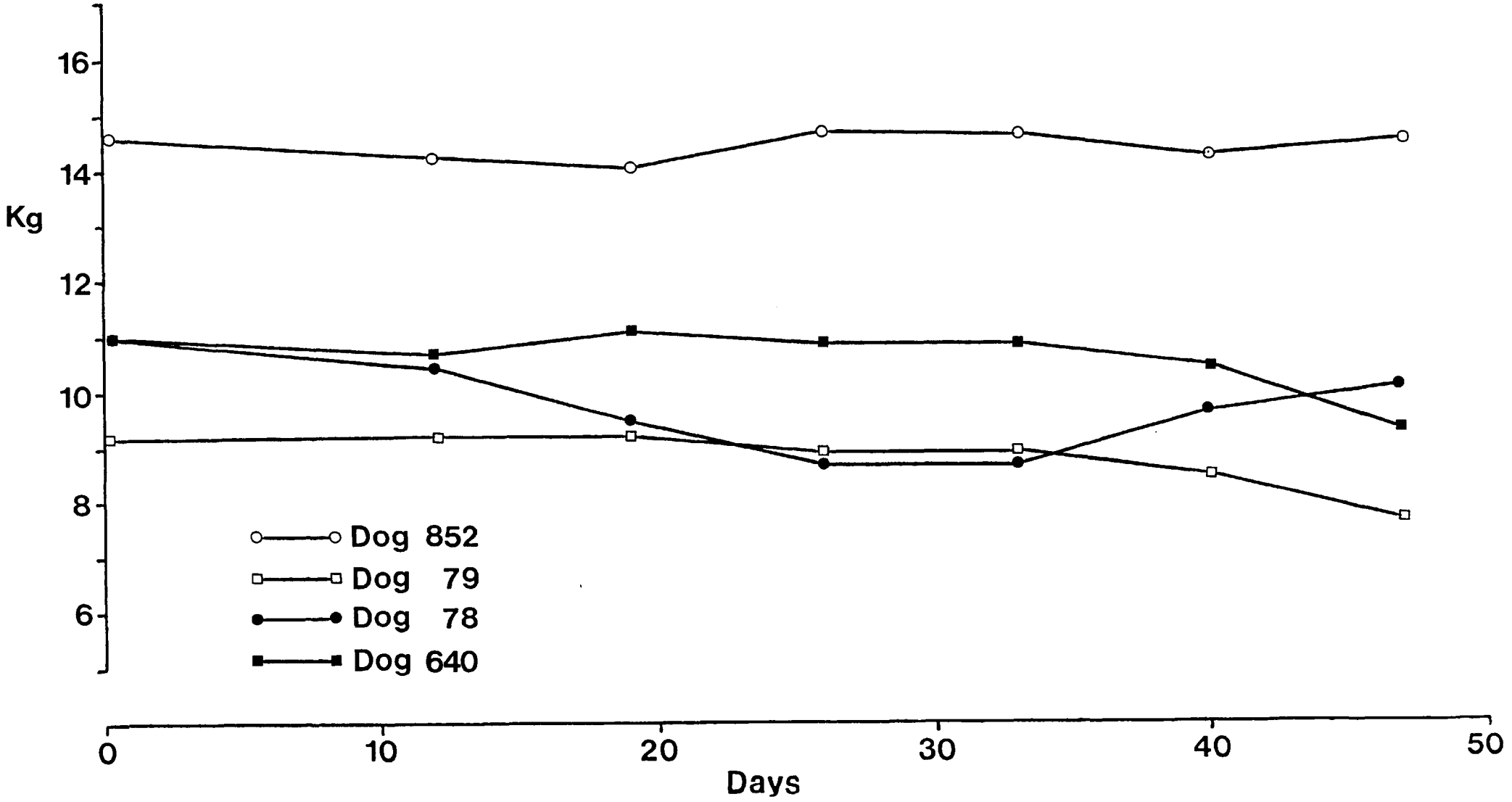
Some weight loss was observed in three of the four dogs (Nos. 78, 79, 640) during the period of the test (Fig.3.5). One dog (No.78) lost over 2 kg in 21 days and its general poor condition was such that the dose of metiamide for this dog was reduced to 100 mg kg^{-1} day^{-1} from day 22 until the end of the test. Its body weight then slowly increased until the end of the test.

White blood cell counts during test

The white blood cell counts during the period of the test may be seen in Fig.3.6. Dog No.79 had a relative leucocytosis during the test which was at a maximum on day 35. This was thought to be due to a mild infection.

One dog (No.78, good oxidiser model) had a mild leucopenic reaction, the white cell counts falling from 10,500 cells mm^{-3} to 7,200 cells mm^{-3} on day 21 of the test. This dog was in a generally poor condition at this stage having lost some 2 kg in weight, and so the dose of metiamide was reduced to 100 mg kg^{-1} day^{-1} on day 22. The dog's white blood cell count and body weight then both returned to pre-test levels showing the fall in white cell counts and

Fig.3.5 Dog weights during metiamide toxicity test



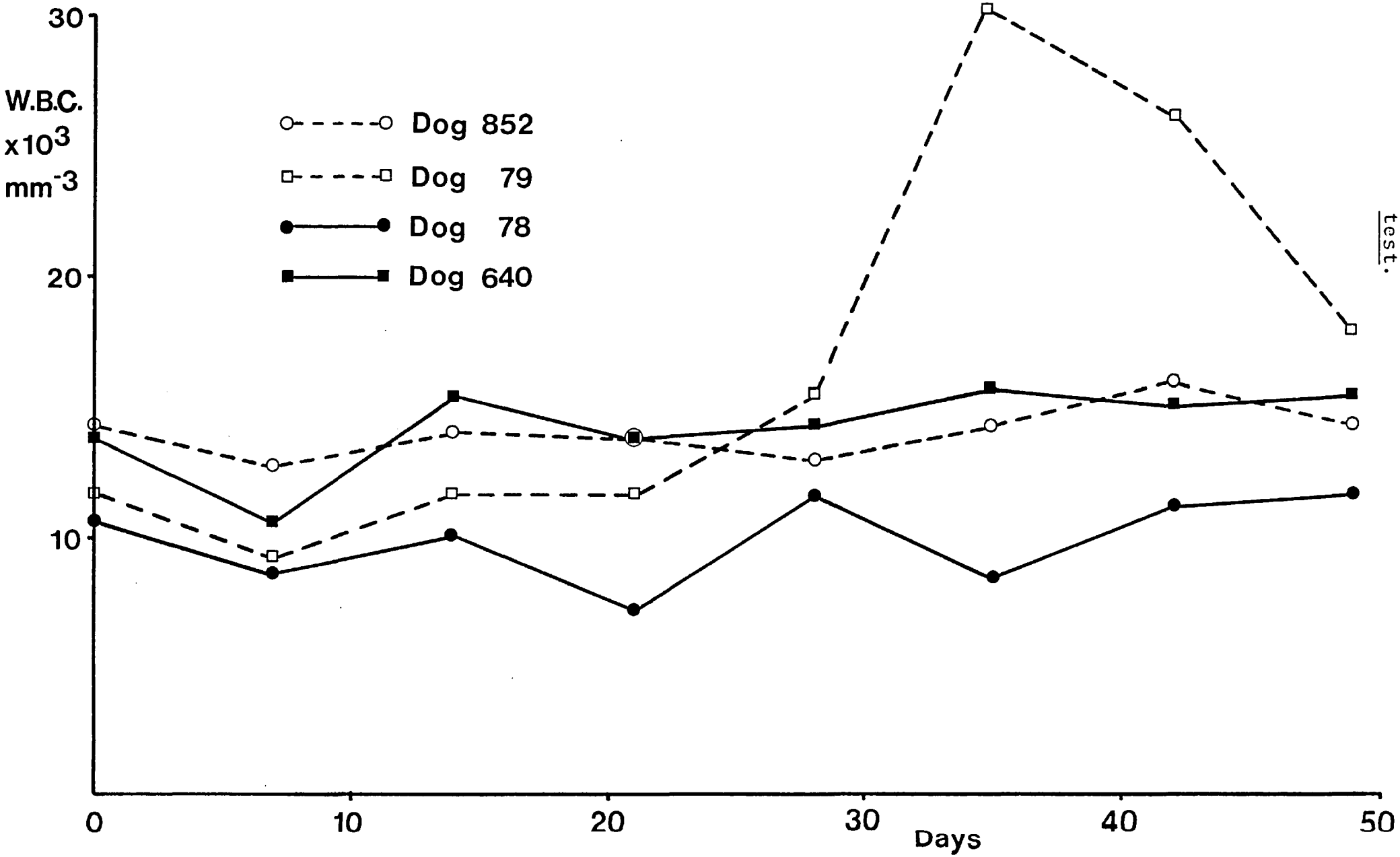


Fig.3.6 Dog white blood cell counts during toxicity test.

body weight were drug-related events.

In the other dogs metiamide had no apparent effect on the white blood cell counts.

3.4. Discussion

The work described in this chapter was undertaken to investigate whether or not metabolic differences in the handling of metiamide could account for the inter- and intra-species differences observed in the susceptibility to the haematotoxic effects of metiamide.

Comparison of sensitive (man and dog) and non-sensitive (mouse and rat) species revealed no major inter-species differences in the metabolic handling of metiamide. However, a small difference was observed between the two groups in the amounts of the metiamide urea analogue excreted in the urine. The sensitive species (man and dog) excreting a greater proportion of the urinary ^{14}C as the metiamide urea analogue than the non-sensitive (mouse and rat) species (man and dog: $6.8 \pm 2.1\%$ of urinary ^{14}C; mouse and rat $3.4 \pm 1.5\%$ of urinary ^{14}C; $2P < 0.05$).

These observations may reflect the extent to which the toxic sulphenic and sulphinic acid metabolites of metiamide

are found in these species, since desulphuration of the thiourea moiety to produce the urea analogue is thought to be indicative of the amount of oxidation occurring at this site in the molecule.

Previous investigations into the metabolic basis of species differences in the toxicity of certain compounds have often revealed large inter-species differences in the metabolism of these compounds. However, the haematotoxicity of metiamide is obviously not a simple toxic effect. It results from a multifactorial situation in which dose, duration of administration and a susceptible constitution all play a part. In such a situation, where toxicity results only after weeks or months of dosing with large amounts of drug, small differences in the metabolic handling of metiamide could produce a cumulative effect, eventually resulting in toxicity.

Application of the DA/Lewis rat metabolic model to the metabolism of metiamide did not reveal significant differences between strains in the excretion of any of the oxidative metabolites of metiamide. However a significant difference in the amount of metiamide N-glucuronide excreted by the two strains was observed which correlated with the differences seen between the

human EM and PM phenotypes (vide supra). The influence of the oxidation polymorphism on the metabolism of certain drugs, where there occurs several competing metabolic options available is particularly difficult to discern. Where the parent drug is also excreted largely unchanged in the urine with only small amounts of metabolites, the likelihood of observing inter-phenotype differences will be reduced still further. It is remarkable that with a drug such as metiamide any inter-phenotype differences have been observed at all.

The rat DA/Lewis metabolic model is probably not as sensitive a method as the human phenotyped panel approach for detecting the influence of oxidation polymorphism on certain metabolic reactions where only small differences are expected between the phenotype groups. This may be due to the fact that the range of oxidative abilities in the rat model is over about a ten-fold range (metabolic ratios, 0.3 - 4.0) whereas in the human phenotyped panels a range of at least one hundred-fold is seen (metabolic ratios, 0.2 - 20.0).

The influence of the oxidation polymorphism may be further obscured in the rat model by the reduction of metiamide sulphoxide formed in the body, back to metiamide. This reaction has been shown to occur in rats

(Taylor et al., 1979).

The attempt at reproducing metiamide-induced agranulocytosis in a beagle dog model was ambitious in that an animal model of this type has not been previously described. Although neither of the two good oxidiser model dogs progressed to agranulocytosis, one of them (dog 78) did display a mild leucopenia during the test which appeared to be drug-related. Metiamide appeared to have no effect whatsoever on the white blood cell counts of the two poor oxidiser model dogs.

The range of oxidative abilities observed in the beagle dog population was quite small (3-4 fold), and it is probable that in a larger population of dogs a greater variability in oxidative ability might be observed with possible toxicological consequences for the dogs with abnormal oxidative abilities. The range of oxidative abilities observed in the tested population was also probably diminished by the fact that twelve of the twenty dogs came from only two litters.

Many problems are inherent in attempts to set up animal models for adverse drug reactions in man. However, the discovery of suitable animal models remains a most worthwhile objective. Eventually it may be possible to include these animal models as an integral part of the

preclinical toxicity testing of new drugs, allowing the influence of polymorphic metabolism on the toxicity of drugs to be assessed before the drugs reach man.

The major obstacle to the further development of suitable animal models for man is cost. The animals most similar to man in their response to and metabolism of xenobiotics tend to be the larger mammals (for example,primates) which are also the most expensive animals to buy, breed and maintain. In order to develop an animal model large numbers of these animals would have to be investigated in order to select suitable individuals for the model. A large amount of time and money would need to be invested in a project of this type with no guarantee of success.

A complementary approach would be to encourage the attitude whereby 'idiosyncratic' responses to drugs by both animals and man are recognised as rare opportunities to investigate both the biochemical variation which exists in normal populations, and its toxicological consequences. The more detailed study of these individuals may well provide the basis for reducing the occurrence of idiosyncratic responses to drugs, where a proportion of the idiosyncracy may be a definable genetically-determined metabolic factor which may be isolated and modelled for in experimental animals.

CHAPTER FOUR

STUDIES OF PATIENTS WHO DEVELOPED AGRANULOCYTOSIS ATTRIBUTABLE TO METIAMIDE ADMINISTRATION

4.1 Introduction

Metiamide was taken by about 800 patients during its early clinical trials, most of whom benefitted from its therapeutic activity. Thirteen patients, however, developed severe neutropenia whilst on metiamide therapy. What were the causes of the adverse effect in these patients and why did these patients differ from the rest of the treated patients in their sensitivity to metiamide?

The work described in this Chapter was undertaken in an attempt to answer these two questions by studying some of the possible contributory factors which, in these susceptible patients, may have culminated in the occurrence of metiamide-induced agranulocytosis.

The problem was approached on two fronts; firstly by investigating the possibility of there being a metabolic basis to the occurrence of the blood dyscrasia, and secondly at the cellular level by examining the ability of blood cells from agranulocytosis patients to withstand oxidative stress.

A number of adverse drug reactions are known to be due to the atypical metabolism of drugs by certain individuals in the population. The classical example of this

is the polymorphic acetylation of isoniazid and its relationship to the occurrence of neuropathy.

Individuals who are slow acetylators are more likely to develop peripheral neuropathy than those who are rapid acetylators (Hughes et al., 1954). Similarly prolonged apnoeic reactions may occur after normal doses of the muscle relaxant succinyl choline in certain individuals. These individuals have a genetically-determined cholinesterase varient with a low affinity for the succinylcholine substrate (Lehman & Liddell, 1964).

The discovery of a genetic polymorphism in the commonest metabolic reaction, namely oxidation, opens up a whole new area for investigating the possible contribution of an individual's metabolic constitution towards the predisposition to the toxic effects of certain drugs. Numerous drugs are metabolised in the body by oxidation, and many of these oxidation reactions may be influenced by this genetic polymorphism. The consequences of polymorphic drug oxidation for the toxicity of many drugs remains to be evaluated, but it may now be possible to make a logical and systematic approach to the investigation of these adverse-effects and their relationship to polymorphic drug oxidation.

The influence of polymorphic oxidation on the metabolic handling of drugs may be assessed by the use of phenotyped panels of volunteers (Idle et al., 1979 a). The patients who suffered the adverse reaction may then be phenotyped for their oxidative ability by the use of the debrisoquine phenotyping procedure. If an individual's metabolic oxidative ability contributes to the propensity to develop adverse reactions, then it might be expected that the susceptible patients would all have similar oxidative abilities and that they would be more frequently, either very good oxidisers (EM phenotype), or poor oxidisers (PM phenotype), depending on whether the toxicity is due to the parent drug or an oxidative metabolite thereof.

If, however, the oxidation polymorphism has no influence on the propensity to develop an adverse effect, then the affected patients would be expected to display a range of oxidative abilities similar to that seen in the population at large.

However, this involvement of oxidative ability is only one of many factors which may contribute to the occurrence of adverse drug reactions. In most cases of adverse drug reactions, susceptibility probably results from a multifactorial situation in which the structure

of the drug, the dose and duration of dosage, sex of patient, age of patients and environmental factors all combine with an individual's genetically determined metabolic constitution to produce the adverse effect. If a relationship between a combination of these factors and the occurrence of the adverse reaction can be demonstrated, then some insight into the biochemical mechanisms underlying the occurrence of these adverse effects might be gained. Eventually, certain drug regimens may be able to be adjusted for each individual so as to reduce the risk of adverse-reactions to drugs on a predictive, rather than a "trial and error", basis.

A different type of approach is needed in order to investigate the problems of adverse drug reactions when they are due to specific defects in cellular organisation which are uncovered by drugs. Such a defect is believed to underlie chlorpromazine-induced agranulocytosis (Pisciotta, 1969), whereby a fundamental defect in cell division in granulocyte precursor cells of sensitive patients is uncovered by the drug. Cell cultures from patients who recovered from agranulocytosis, show an increased sensitivity to chlorpromazine compared to controls (Pisciotta, 1971).

A second type of cellular defect underlies the haemolytic

anaemia seen in some individuals after the ingestion of certain drugs. These individuals possess variant forms of enzymes in the pentose phosphate pathway of the red blood cell. The pentose phosphate pathway normally maintains the cellular supplies of NADPH and reduced glutathione and thus protects the cell against oxidative damage. When these individuals ingest certain drugs their erythrocytes experience oxidative stress which cannot be withstood, due to insufficient supplies of reduced glutathione, and the erythrocytes lyse (Beutler, 1972).

By far the most common defect in the pentose-phosphate pathway is in the enzyme glucose-6-phosphate dehydrogenase (G-6-PD). It has been estimated that about 100 million people carry the trait for G-6-PD deficiency (Carson & Frisher, 1966). They belong to a wide range of races and a number of enzyme variants have been described (Beutler, 1972; Grimes & De Gruchy, 1974). Deficiency of the enzyme is not restricted to the erythrocyte but may occur in other tissues, in fact in caucasians most tissues, including leucocytes are affected (Beutler, 1972).

A deficiency of this enzyme in leucocytes would tend to make these cells more sensitive to damage from

drugs such as metiamide which are known to associate with them. A deficiency in this enzyme might therefore contribute to the aetiology of metiamide-induced agranulocytosis.

The metiamide-induced agranulocytosis patients and control patients were therefore traced and investigated with respect to metabolic oxidation and erythrocytic G6PD enzyme activity. The case histories of these patients were also examined in an attempt to uncover any other factors which might have contributed to the occurrence of their blood dyscrasia. Two carbimazole-induced agranulocytosis patients were also investigated to test whether or not the same underlying factors contributed to the occurrence of agranulocytosis associated with metiamide therapy and agranulocytosis brought about by another thiourea based drug, carbimazole (see Chapter 1.p 25).

4.2. Materials and methods

Agranulocytosis patients

The names and addresses of the consultants whose patients developed metiamide-induced agranulocytosis were obtained from Smith, Kline and French Ltd. company

files. These consultants were then approached for permission to investigate the agranulocytosis patients and examine their case histories. The patients gave their informed consent to participating in these studies and ethical approval was obtained from St. Mary's Hospital ethics committee.

Control patients

The consultants were also asked to select a number of patients who had taken metiamide during the clinical trials for similar periods of time to the agranulocytosis patients (at least eight weeks), but did not at any time show a neutropenic reaction to metiamide. These patients were used as 'controls' with which the agranulocytosis patients could be compared.

Carbimazole-induced agranulocytosis patients

Two carbimazole-induced agranulocytosis patients were also investigated. These two patients were sisters and were originally reported in the literature by Hamilton & Saunders (1977).

Debrisoquine assays

All patients were investigated for their metabolic

oxidative status using debrisoquine. The assays for debrisoquine and its metabolites were performed as described previously (Chapter 2).

Glucose-6-phosphate dehydrogenase assay

The method used for estimation of the activity of glucose-6-phosphate dehydrogenase in the agranulocytosis patients erythrocytes was adapted from the method recommended by the World Health Organisation (1967).

Preparation of haemolysate

1. Venous blood (5 ml) was collected in lithium heparin tubes from which the granules had been removed.
2. The blood was immediately centrifuged at 3,000 r.p.m. for 10 minutes.
3. The plasma, buffy coat and a small portion of the red cells were aspirated.
4. The remaining red cells were washed three times with saline (0.9%, w/v), the saline being removed each time by centrifugation and aspiration.

5. The saline was aspirated after the last washing and the packed red cells (200 μl) added to distilled water (3.8 ml) in a test-tube. The mixture was allowed to stand for 10 minutes to complete haemolysis.

6. The resulting haemolysate was centrifuged at 3,000 r.p.m. and the supernatent was withdrawn and stored at 0-4^{o}C until analysis.

Assay

Reagents: Glucose-6-phosphate (Sigma Chemical Company), NADP (Sigma Chemical Company), Tris (BDH), Mg Cl_2 (BDH).

Procedure: The reagents were added to a test-tube in the following order; water (2.2 ml), 1.0 M Tris (pH 8, 0.40 ml), 0.1 M Mg Cl_2 (0.40 ml), haemolysate (0.20 ml) and 10 mM G-6-P (0.40 ml).

The reaction was started by the addition of 2.0 mM NADP (0.40 ml). The blank was made up identically, except the G-6-P and NADP were excluded, the volume being made up with water.

The optical density of the reaction mixture was then

followed in a U.V. spectrophotometer (Pye Unicam SP30) at 340 nm at 25°C for 20 min.

Treatment of results

The rate of change in optical density is calculated from the linear portion of the optical density versus time graph. The enzyme activity in International Units may then be calculated as follows:-

$$\text{Activity (I.U./g Hb.)} = \frac{(\ \Delta\ \text{O.D./min})\ 10^5}{6.22\ (\text{gHb./100}^{+}\text{ml})\ (\text{Enz.vol}^{*})}$$

\+ g haemoglobin per 100 ml blood

* μl packed red cells/ml reaction mixture

Haemoglobin content of patients blood

Samples of the patients blood were routinely analysed for haemoglobin content using a Coulter counter.

Statistical treatment of data

The debrisoquine metabolism data from agranulocytosis patients and control patients was analysed by means of a non-parametric significance test. Such tests are

used when no simple assumptions can be made regarding the distribution of the sample data. For the debrisoquine data, the distribution of metabolic ratios within the control population is by definition not normally distributed, there being two distinct modes corresponding to the EM phenotype and the PM phenotype. The Wilcoxon rank sum test was therefore used to analyse the data (Colquhoun, 1971).

4.3 Results

4.3.1 Patient case-histories

Of the original thirteen metiamide-induced agranulocytosis patients, five were eventually traced and investigated. Of the remaining eight patients, one had died, one had chronic renal failure, one refused to participate in the study, one was living in America, three had moved address and could not be contacted and one is currently under investigation.

Table 4.1 lists the details of the five patients who were investigated.

Detailed case histories for each of these patients are given below.

Table 4.1 Details of metiamide-induced agranulocytosis patients

Patient No.	Sex	Age (y)	Weight (kg)	Trial centre
A1	F	49	62	Edinburgh
A2	M	64	68	Bristol
A3	M	45	54	Portsmouth
A4	M	55	64	Exeter
A5	F	67	60	Dundee

Patient A1

Patient A1, a 49 year-old woman, was diagnosed to have Zollinger-Ellison syndrome with resulting recurrent duodenal ulceration. Dicyclomine (Merbentyl) and isopropamide-iodide-trifluoperazine (Stelabid) had been taken for some weeks before the trial but these drugs were stopped a few days before the metiamide treatment was started.

Metiamide was prescribed at 300 mg q.i.d. after an oral absorbtion study with 200 mg [^{3}H]metiamide revealed a maximum blood level of less than 3 μM. The white blood

cell counts during metiamide treatment may be seen in Fig.4.1.

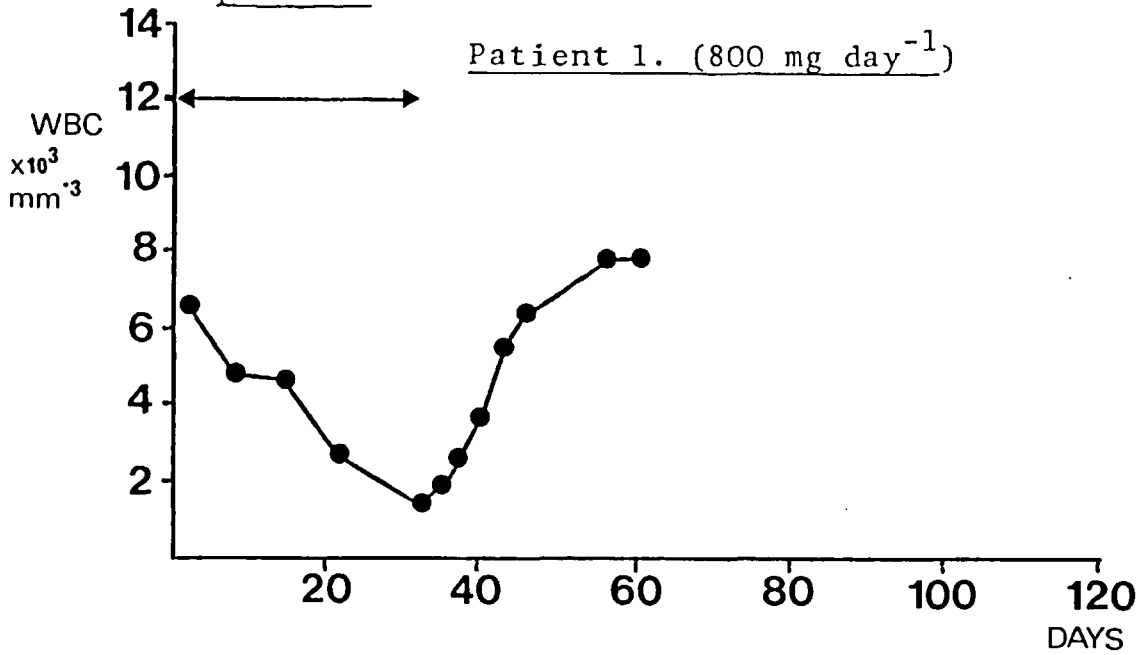
On day 16 of treatment a mild sore throat developed, on day 21 it was worse, and a blood cell count revealed a definite leucopenia. Hower, due to a breakdown in the warning system, the metiamide was continued. On day 27 the patient complained of a very sore throat and fever. An antibiotic was prescribed by her G.P. and was taken from day 27-31.

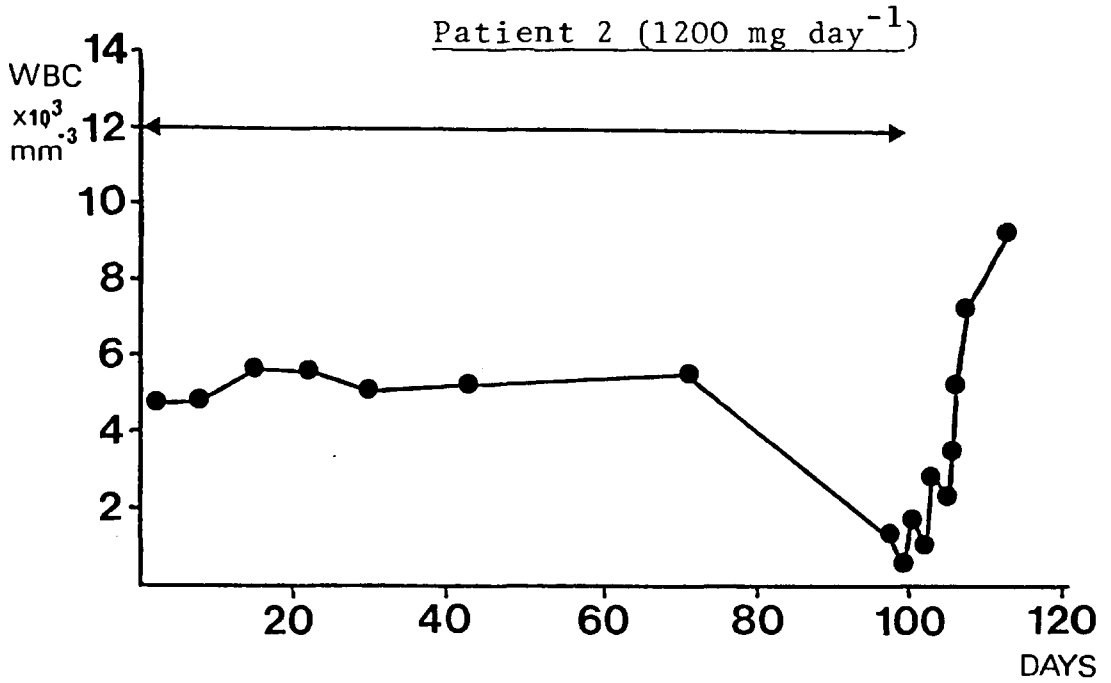
The patient was admitted to hospital on day 31 and the metiamide treatment was discontinued. A bone marrow biopsy was performed on day 35 and this revealed a small number of myeloid precursor cells present, but no adult neutrophils. The megakaryocyte and erythocyte series were normal. A slight increase in lymphocytes and plasma cells was noted but it was felt that this was secondary to the agranulocytosis. The haematologist commented that the bone-marrow picture was consistent with a maturation arrest of the neutrophil series.

Patient A2

Patient A2, a 64 year-old man, was admitted to the metiamide trial due to recurrent duodenal ulceration

Fig.4.1 W.B.C. counts of metiamide-induced agranulocytosis patients.





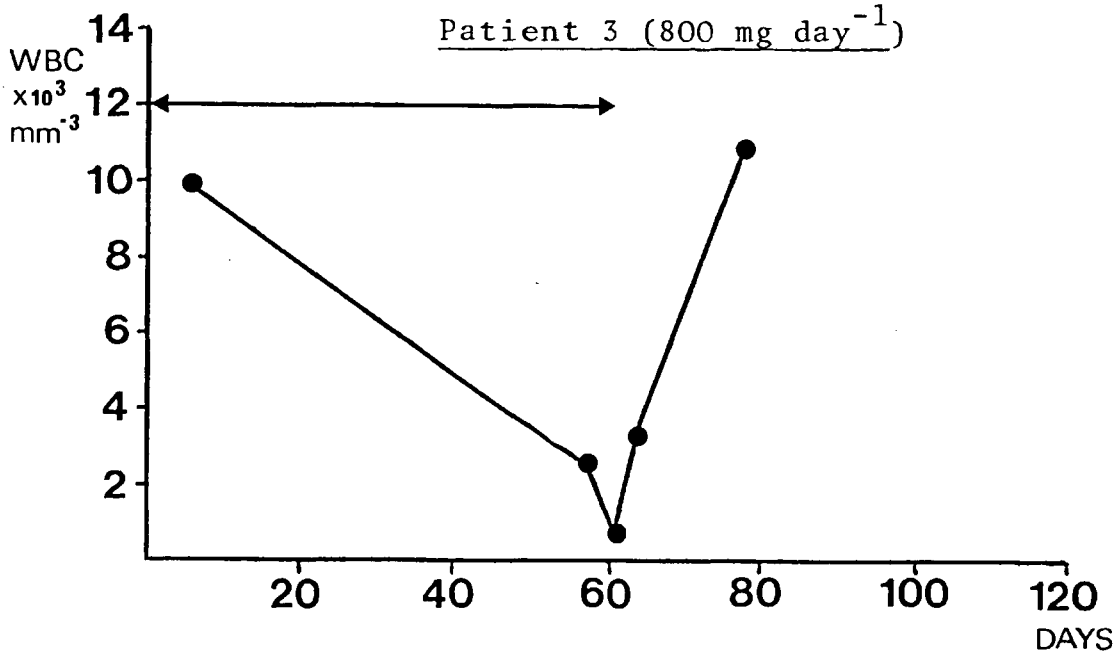
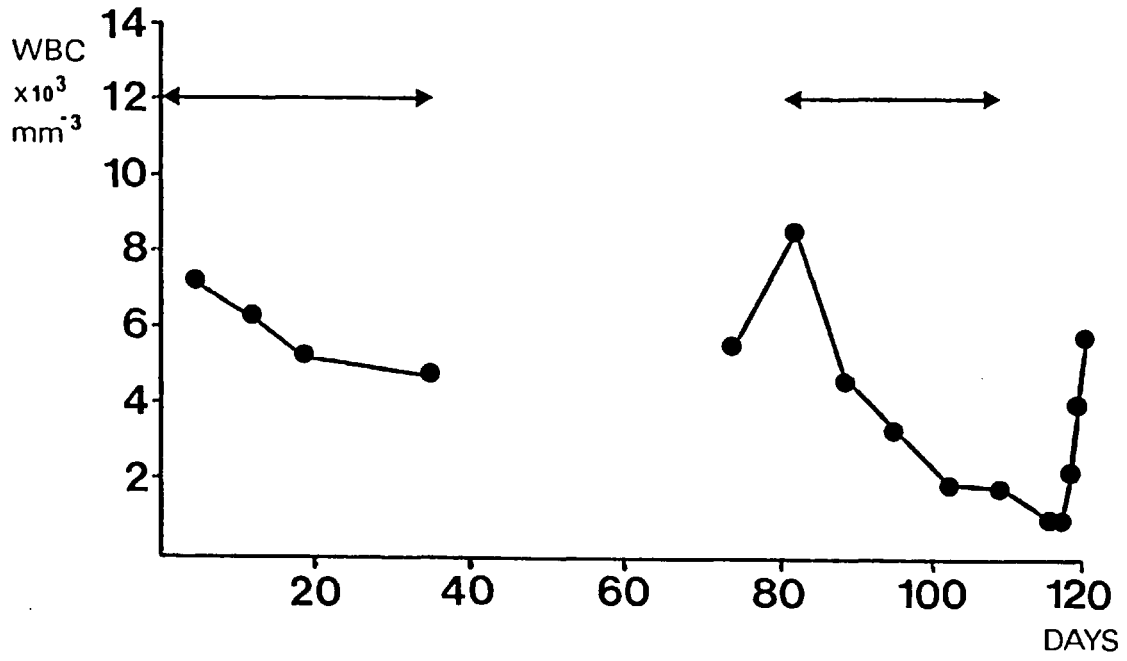
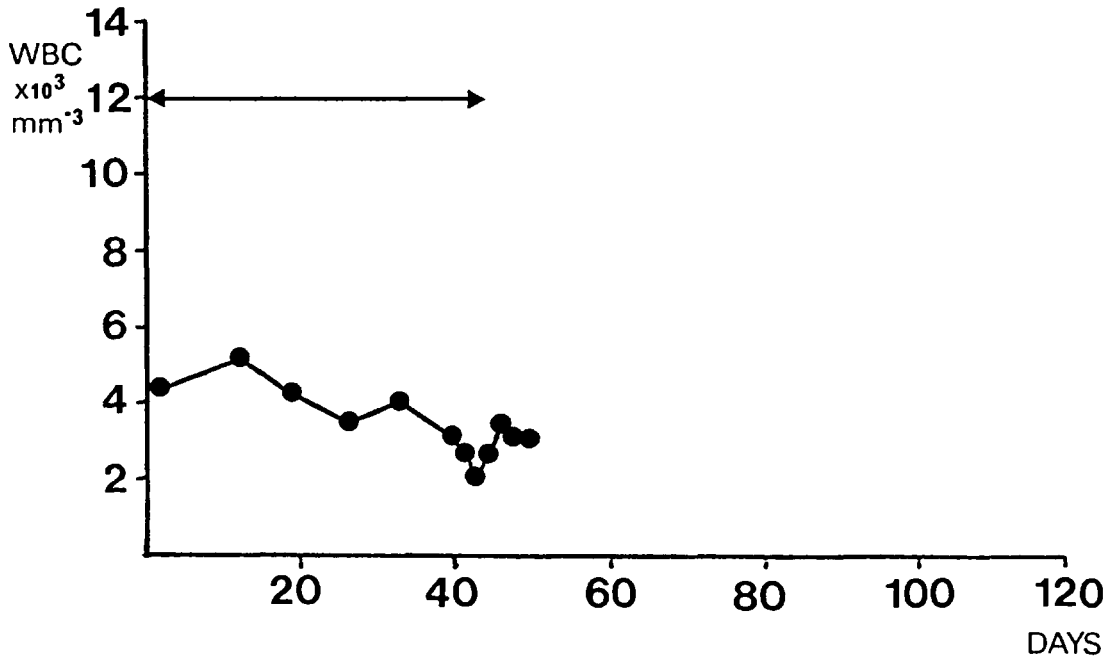


Fig.4.1 (continued)

Patient 4 (800 mg day^{-1})



Patient 5 (1200 mg day^{-1})



⟷ Period of metiamide administration.

since 1943. He received an aluminium compound (Actal) for some weeks before the metiamide trial, but it was discontinued a few days before the metiamide was started.

Metiamide was prescribed at 200 mg q.i.d. On day 90 of the trial the patient complained of a sore throat, general malaise and fever. Metiamide was stopped on day 99 and a bone marrow biopsy taken on day 103 revealed megakaryocytes in normal numbers and the erythroid series was active and normal in appearance. The myeloid series were plentiful but there was a marked reduction in metamyelocytes and segmented neutrophils. The bone marrow picture was considered compatible with the recovery phase following agranulocytosis.

The patient recovered clinically by day 106.

The patient's family history revealed a high incidence of cardiovascular disease but was otherwise unremarkable.

Patient A3

Patient A3, a 45 year-old man, had a 7-year history of duodenal ulceration for which he had a vagotomy and

and pyloroplasty in July 1975. One week later he had a massive haemorrhage from a post-bulbar duodenal ulcer which was undersown at operation. He had copious aspirates post-operatively and a further bleed from an anastomotic ulcer for which he had a partial gastrectomy on August 20th, 1975.

Post-operatively the patient continued to have copious aspirates of between 2 and 41 day^{-1} and further bleeding. Metiamide (100 mg) was infused intravenously on September 5th and within 24h the aspirates had ceased. He was treated with metiamide by mouth and his general condition improved until he was discharged home, taking metiamide 200mg twice daily and 400mg at night.

On day 56 of metiamide treatment he developed a staphylococcal infection of his abdominal wound and one eye. Three days later he was admitted to hospital and metiamide withdrawn. His total WBC count was then 700 cells mm^{-1} with 15% neutrophils. A bone marrow aspirate from the right iliac crest (day 68) showed evidence of recovering agranulocytosis.

Cimetidine was infused intravenously (day 61) at a dose of 1g day^{-1} to control his acid secretion. On day 63 his WBC count was 34,000 cells mm^{-3} with 47% neutrophils.

Treatment was continued with cimetidine by mouth (200 mg q.i.d., the patient maintained his recovery and was eventually discharged home.

On investigation both the patient's previous history and family history proved unremarkable.

Patient A4

Patient A4, a 55 year-old man, underwent a polygastrectomy for chronic duodenal ulceration in 1972 and was thereafter reasonably well until he developed a large stomal ulcer. He was treated by excision of the fistulous elements and reconstruction of the stomach. He underwent a successful 1-month trial on metiamide (800 mg day^{-1}) in July 1975 which controlled his acid secretions. A second course of metiamide (800 mg day^{-1}) was started in October 1975.

He developed a sore throat and noticed some scratches were not healing. Three days later (day 109) he was admitted to hospital. On admission he had general malaise, sore throat, multiple infected skin lesions and a perianal abscess with local spreading cellulitis.

Examination of the bone marrow showed erythropoiesis was normal but neutrophil granulopoiesis was severely

depressed with a maturation arrest at the promyelocyte stage. The eosinophil series showed a mild increase in numbers. Megakaryocytes were somewhat reduced in number but were producing platelets actively. The overall bone-marrow picture was thought to be compatible with a diagnosis of drug-induced agranulocytosis.

The patient was treated by barrier nursing and with antibiotics, and was eventually discharged home. Investigation of the family history revealed nothing remarkable.

Patient A5

Patient A5, a 67 year-old woman, was entered into the metiamide trial due to recurrent duodenal ulceration. She was prescribed metiamide (1200 mg day^{-1}) for one month and her ulcer healed. She was then placed on placebo in the maintenance trial and her ulcer returned. She was then restarted on metiamide (200 mg q.i.d. and 400 mg nocte).

After 40 days treatment a marked neutropenia was observed, the white cell count falling to 2,200 cells mm^{-3} on day 43. Metiamide was withdrawn on day 44 and the white cell count rose.

Two bone-marrow biopsies were performed and they both revealed evidence of the recovery phase following drug-induced agranulocytosis.

Control patients

Eleven patients were investigated to act as controls with which to compare the agranulocytosis patients. All the control patients had received metiamide at a minimum dose level of 800 mg day^{-1} for a period exceeding two months with no apparent effect on the white blood cell count. The patients were selected by Mr.Celestin in Bristol and Dr.Saunders in Dundee on the basis of the criteria outlined above. Details of these patients are shown in Table 4.2.

Table 4.2 Details of metiamide control patients

Patient No.	Sex	Age (years)	Weight (kg)	Trial centre
C1	M	53	78	Bristol
C2	M	35	62	Bristol
C3	M	40	72	Bristol
C4	M	44	97	Bristol
C5	M	42	79	Bristol

Patient No.	Sex	Age (years)	Weight (kg)	Trial centre
C6	M	31	93	Bristol
C7	M	47	74	Bristol
C8	M	52	65	Bristol
C9	M	38	54	Bristol
C10	M	55	78	Dundee
C11	M	31	64	Dundee

Carbimazole-induced agranulocytosis patients

Patient A6

Patient A6, a 29 year-old female, presented in 1970 with symptoms consistent with a clinical diagnosis of thyrotoxicosis. Treatment with carbimazole (20 mg t.d.s.) was instituted. After one week of treatment she developed myalgia, malaise and fever. Seventeen days after starting treatment she developed a sore mouth, and sore throat. She was admitted to hospital where a total white blood cell count of 1100 mm^{-3} was found with a total absence of neutrophils. Bone-marrow aspiration showed a maturation arrest with no myeloid neutrophils beyond the myelocyte stage. Haemoglobin

levels and platelet counts were within normal limits. A diagnosis of carbimazole-induced agranulocytosis was made and the drug withdrawn.

A rapid improvement in her clinical and haematological condition occurred following treatment with antibiotics and steroids. Her thyrotoxicosis was subsequently controlled with potassium perchlorate followed by partial thyroidectomy.

Patient A7

Patient A7, a 42 year-old female (sister of patient A6) presented in 1975 with symptoms of thyrotoxicosis. Treatment with carbimazole (15 mg t.d.s.) was instituted but owing to patient error she took only 5 mg t.d.s. She took this dosage for 24 days and then suffered an ulcerated mouth and a sore throat. She was treated with penicillin and the carbimazole was discontinued.

The white cell count at this time was 3,500 cells mm^{-3} with 12% neutrophils. Haemoglobin levels and platelet counts were normal. Two weeks later her white cell count had risen to 4800 cells mm^{-3} with 60% neutrophils. Her thyrotoxicosis was subsequently controlled by

radioactive iodine.

4.3.2 The metabolism of debrisoquine in agranulocytosis and control patients.

The metabolism of debrisoquine was studied in the agranulocytosis and the control patient groups using the experimental protocol described in Chapter 2 (vide supra).

The pair of alleles which influence the 4-hydroxylation of debrisoquine in man are also known to influence, to some extent, the aromatic oxidation of debrisoquine to produce 5-, 6-, 7- and 8-hydroxydebrisoquine. However, other factors, and possibly other gene loci may be involved in the aromatic oxidation since not all good 4-hydroxylators of debrisoquine produce large amounts of these phenolic metabolites. The agranulocytosis and control patient groups were therefore investigated for all aspects of their metabolism of debrisoquine, including aromatic oxidation, to determine whether or not differences between the two groups could be detected in any of the possible metabolic oxidations (Tables 4.3 and 4.4).

Table 4.3. The metabolism of debrisoquine in agranulocytosis patients.

Patient No.	% Dose in 0-8h urine as:-						
	Deb	4OH	5OH	6OH	7OH	8OH	Total
A1	24.7	20.6	3.8	3.7	4.5	1.2	58.5
A2	10.0	15.0	0.4	2.6	0.8	nd	28.8
A3	6.4	26.8	4.3	2.7	4.8	1.7	46.7
A4	10.1	14.4	nd	nd	2.4	0.3	27.2
A5	24.4	28.8	1.7	1.7	3.5	nd	57.1
A6	10.3	20.8	2.0	2.5	5.7	nd	41.3
A7	3.2	11.8	2.2	8.2	9.0	0.4	34.8

Table 4.4 The metabolism of debrisoquine in control patients.

Patient No.	% Dose in 0-8h urine as:-						
	Deb	4OH Deb	5OH Deb	6OH Deb	7OH Deb	8OH Deb	Total
C1	9.8	28.5	3.5	1.6	4.1	nd	47.5
C2	11.5	19.3	4.9	2.7	8.9	nd	47.3
C3	29.3	6.1	nd	nd	nd	nd	35.4
C4	30.3	36.9	nd	nd	nd	nd	67.2
C5	9.7	20.8	0.4	2.3	5.6	nd	38.8

cont./

Table 4.4(cont.)

Patient No.	Deb	4OH Deb	5OH Deb	6OH Deb	7OH Deb	8OH Deb	Total
C6	14.2	31.2	1.0	0.7	2.1	nd	49.2
C7	31.3	25.5	1.6	0.8	4.0	nd	63.2
C8	7.0	15.8	0.3	0.8	2.1	nd	26.0
C9	14.6	29.0	0.9	0.6	1.4	nd	46.5
C10	16.6	4.7	nd	nd	nd	nd	21.3
C11	20.7	34.3	nd	0.5	1.1	nd	56.6

These results may be expressed as metabolite ratios, which are obtained by dividing the amount of unchanged debrisoquine recovered in the 0-8h urine by the amount of each metabolite recovered in the urine in the same period (Tables 4.5 and 4.6)

$$\text{Metabolic ratio} = \frac{\%\text{ dose excreted in 0-8h urine as debrisoquine}}{\%\text{ dose excreted in 0-8h urine as metabolite}}$$

Table 4.5 Debrisoquine metabolite ratios for agranulocytosis patients

No.	4OH Deb	5OH Deb	6OH Deb	7OH Deb	8OH Deb	Total Phenolic Ox.	Total Ox.
A1	1.2	6.5	6.7	5.5	20.6	1.9	0.7
A2	0.7	25.0	3.8	12.5	-	2.6	0.5
A3	0.2	1.5	2.4	1.3	3.8	0.5	0.2

cont./

Table 4.5(cont.)

No.	4OH Deb	5OH Deb	6OH Deb	7OH Deb	8OH Deb	Total Phenolic Ox	Total Ox
A4	0.7	-	-	4.2	33.7	3.7	0.6
A5	0.8	14.4	14.4	7.0	-	3.5	0.7
A6	0.5	5.2	4.1	1.8	-	1.0	0.3
A7	0.3	1.5	0.4	0.4	8.0	0.2	0.1

Table 4.6 Debrisoquine metabolite ratios for control patients

No.	4Oh Deb	5OH Deb	6OH Deb	7OH Deb	8OH Deb	Total Phenolic Ox	Total Ox
CI	0.3	2.8	6.1	2.4	-	1.1	0.3
C2	0.6	2.3	4.3	1.3	-	0.7	0.3
C3	4.8	-	-	-	-	-	4.8
C4	0.8	-	-	-	-	-	0.8
C5	0.5	24.3	4.2	1.7	-	1.2	0.3
C6	0.5	14.2	20.3	6.8	-	3.7	0.4
C7	1.2	19.6	39.1	7.8	-	4.9	1.0
C8	0.4	23.3	8.8	3.3	-	2.2	0.3
C9	0.5	16.2	24.3	10.4	-	5.0	0.5
C10	3.5	-	-	-	-	-	3.5
C11	0.6	-	41.4	18.8	-	33.1	0.6

The metabolite ratios for each group may now be compared by means of the Wilcoxon rank sum test (Table 4.7).

Table 4.7 Comparison of debrisoquine metabolite ratios for agranulocytosis and control group patients by means of the Wilcoxon rank sum test.

Metabolite ratio	R value	2P<
4OH Deb	0.41	0.68
5OH Deb	1.40	0.16
6OH Deb	1.99	0.05 *
7OH Deb	1.45	0.15
8OH Deb	1.99	0.05 *
Total phenolic oxidation	1.99	0.05 *
Total oxidation	0.95	0.34
Alicyclic oxidation / aromatic oxidation	2.13	0.03 *

* significant at the 5% level.

It may be seen that the extent of 4-hydroxylation of debrisoquine was not significantly different in the two groups of patients. However the agranulocytosis

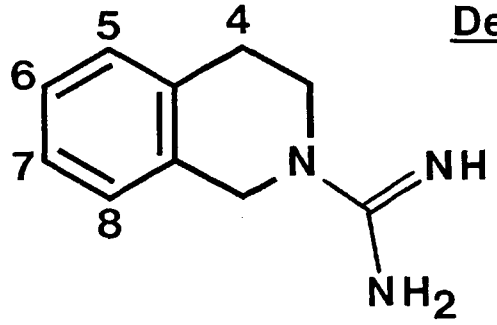
patients had significantly lower metabolite ratios for 6- and 8-hydroxydebrisoquine compared to the controls. A similar trend was apparent for the 5- and 7- hydroxydebrisoquine metabolites but did not reach levels of statistical significance.

The agranulocytosis patients also had significantly lower metabolite ratios when total aromatic oxidation was considered, but did not have significantly lower ratios when total debrisoquine oxidation, including 4-hydroxylation, was considered.

A number of conclusions may be drawn from these results. Firstly, all seven of the agranulocytosis patients were of the EM phenotype determined by the 4-hydroxylation of debrisoquine, and six out of the seven patients had metabolic ratios lower than 1.0 (the median value for a white caucasian population; Evans et al., 1980). Thus all the agranulocytosis patients were good oxidisers of debrisoquine and consequently a number of other drugs including metiamide. However, since the frequency of the extensive metabolising allele is high in the British population, a large number of patients would have to be investigated to confirm this observation.

A second interesting observation was that not only were all the agranulocytosis patients good 4-hydroxylators of debrisoquine but were significantly better at aromatic oxidation, to produce the phenolic metabolites of debrisoquine, than controls.

Thus, for the agranulocytosis patients the favoured position for the insertion of oxygen into the debrisoquine molecule tended to be in the aromatic ring rather than in the 4-position. This may be due to the manner in which the debrisoquine molecule and the oxidised cytochrome P450 complex come together. A slightly different spatial arrangement of the debrisoquine-oxidised cytochrome P450 complex might produce a type of oxidised debrisoquine intermediate which on rearrangement would favour the 5-, 6-, 7- and 8-positions rather than the 4-position in the molecule. It is possible that this is controlled by a third allele which may occur at the same locus as the EM and PM alleles and determine extensive oxidation but of a slightly different nature to the EM allele.



Debrisoquine

A hypothesis such as this might also explain why although a significant correlation occurs between the extent of alicyclic and aromatic oxidation of debrisoquine on a population basis, certain individuals have been observed who do not fit this pattern. Some subjects have been observed to excrete large amounts of the phenolic metabolites but relatively small amounts of 4-hydroxydebrisoquine whilst others are very good 4-hydroxylators of debrisoquine but produce very small amounts of the phenolic metabolites.

It may well be that it is this exceptional oxidative ability, reflected in the aromatic oxidation of debrisoquine that is the principle metabolic determinant which, when in combination with other factors, may result in the occurrence of metiamide-induced agranulocytosis.

3.3.3 Other factors involved in the occurrence of of metiamide-induced agranulocytosis

The case-histories and family-histories of the agranulocytosis and control metiamide patients were investigated in order to determine whether or not other factors could be shown to contribute to the propensity of some individuals to develop the blood dyscrasia.

Dose of metiamide

The daily dose of a drug is an obvious factor to consider as contributing to the aetiology of a toxic effect. The incidence of neutropenic reactions to the antithyroid drugs has been observed to be dose-dependent (McGavack & Chevalley, 1954; Wiberg & Nuttall, 1972). The dose of metiamide received by each patient in terms of mg of metiamide per kg body weight per day was calculated.

The agranulocytosis patients were found to have received a significantly higher dose than the control patients (agranulocytosis patients, 15.7 $\pm$ 3.8 mg kg^{-1}; control patients, 11.6 $\pm$ 2.3 mg kg^{-1}; $2P < 0.05$; Fig 4.2).

Age of patient

It has been noted that agranulocytosis due to certain phenothiazines more commonly occurs in middle-aged to elderly patients (Pisciotta, 1969). The amount of active haemopoietic tissues in the body decreases with increasing age (Custer & Ahlfeldt, 1932); Custer,1949), it being gradually replaced by fatty cells. It might therefore be expected that an increased susceptibility

Fig.4.2 Daily dose of metiamide in control and agranulocytosis patients

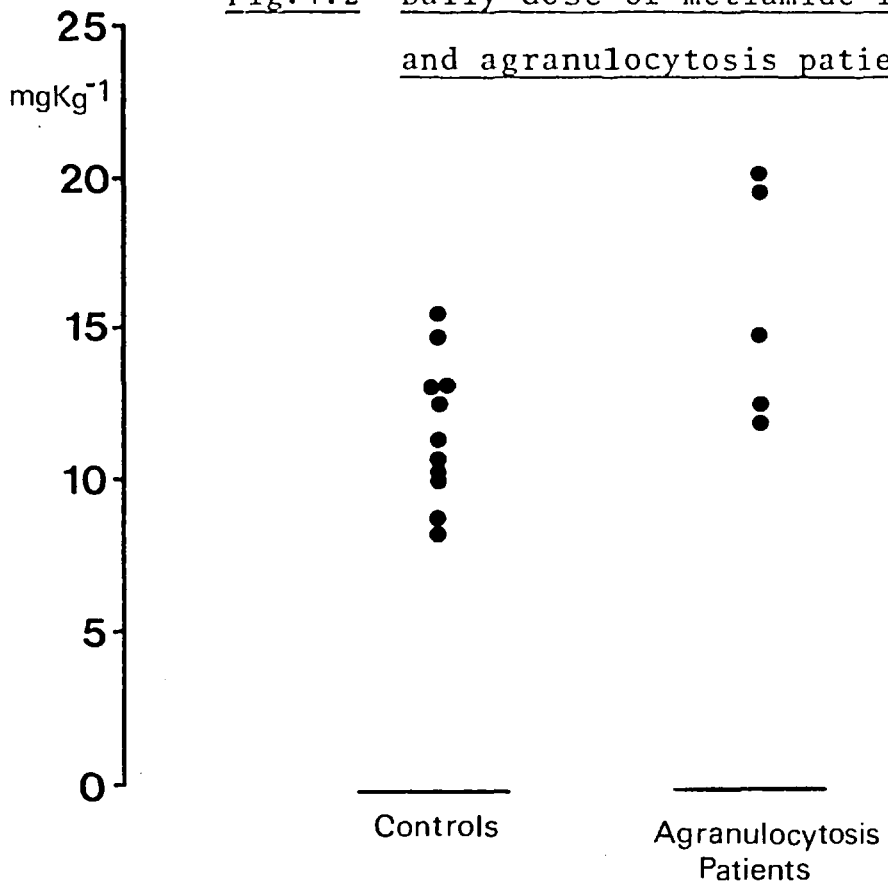
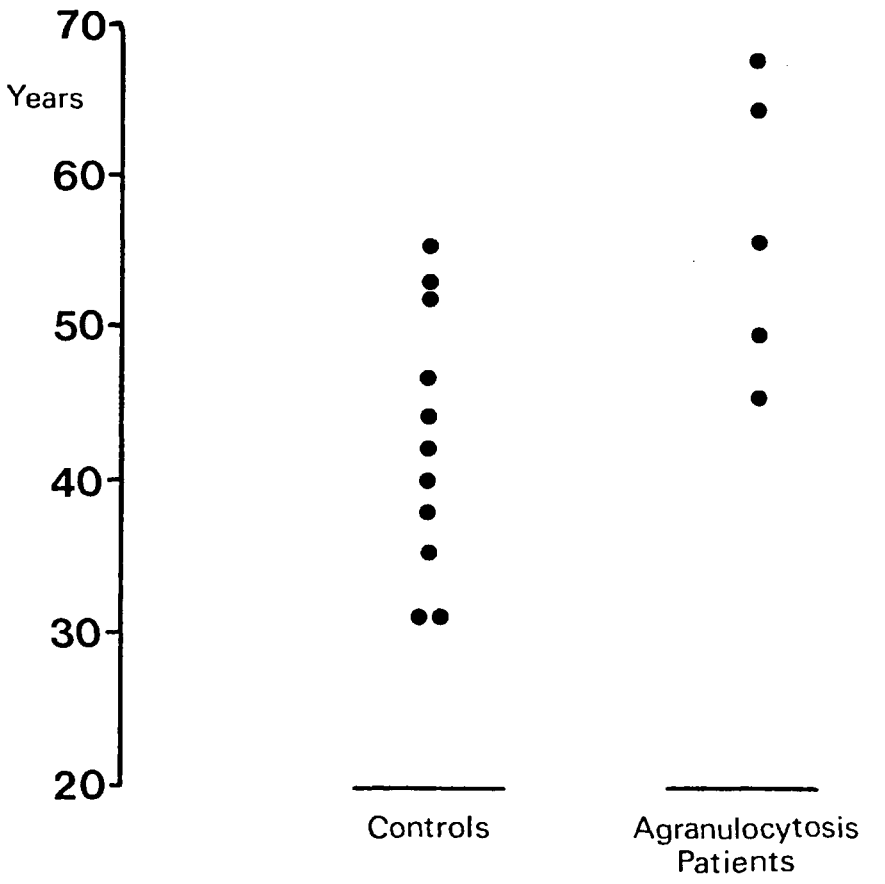


Fig.4.3 Ages of control and agranulocytosis patients



to drugs which act on the haemopoietic tissue might be found in older patients, as these patients would be receiving a higher effective dose of the drug per unit mass of active haemopoietic tissue.

The metiamide-induced agranulocytosis patients were in fact significantly older at the time of the metiamide clinical trials than the control patients. (agranulocytosis patients 56.0 $\pm$ 9.4 years control patients 42.5 $\pm$ 8.5 years; $2P < 0.05$; Fig 4.3).

Susceptibility Index

Three factors have been identified, each of which appear to contribute to the occurrence of metiamide-induced agranulocytosis, namely dose of metiamide, age of the patient and metabolic oxidative ability. Individuals who display high levels of all three of these factors would thus be expected to be highly susceptible to the haemotoxic effects of metiamide.

An index was therefore calculated for the agranulocytosis patients and the control patients which included all three of these factors:-

Susceptibility index = (Age) (Dose) (Oxidative ability)

The higher the suceptibility index the greater the probability of developing metiamide-induced agranulocytosis.

The oxidative abilities of the patients were assessed as the percentage of the total urinary excretion of debrisoquine as its oxidised metabolites in the 0-8h urine. The susceptibility indexes for the agranulocytosis patients and the control patients are shown in Fig.4.4. The metiamide-induced agranulocytosis patients were found to have a significantly higher susceptibility index than the control patients (assessed by the Wilcoxon rank sum test, $2P < 0.003$).

Table 4.8 lists some of the factors which have been studied in agranulocytosis and control patients, and whether or not they are statistically different in the two groups. It may be seen that certain factors such as body weight and daily dose of metiamide were not significantly different between the two groups. However, when the factors are combined to give the daily dose of metiamide per unit body weight it emerges that the agranulocytosis patients received a statistically significantly higher dose than controls ($2P < 0.04$).

Fig.4.4 Susceptibility indexes of control and agranulocytosis patients

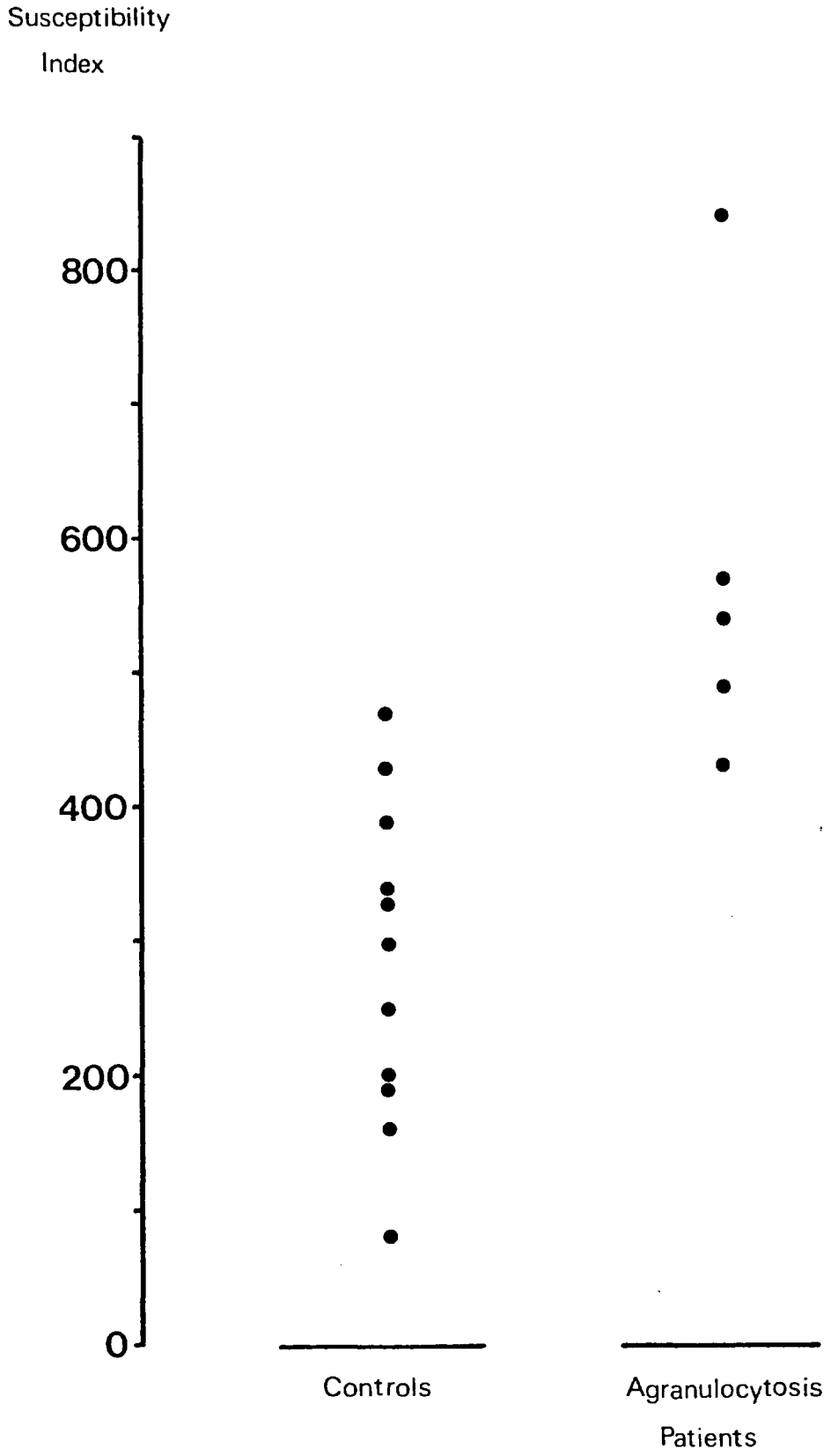


Table 4.8 Analysis of the relative importance of a number of factors possibly contributing to the propensity to develop metiamide-induced agranulocytosis

FACTOR	STATISTICAL TEST	2P<
1.Constitutional		
Age (y)	t	0.04
Weight (kg)	t	ns
2.Dose of metiamide		
Daily dose (gd^{-1})	t	ns
Daily dose per unit body weight ($g\ kg^{-1}\ d^{-1}$)	t	0.04
3.Metabolic		
% Total recovery as 4OH Deb	R	ns
% total recovery as Deb phenols	R	0.04
% total recovery as oxidative metabolites	R	ns

FACTOR	STATISTICAL TEST	2P<
4. Composite		
Age x Dose kg^{-1}	R	0.005
Age x total oxidative ability	R	0.03
Dose kg^{-1} x total oxidative ability	R	ns
Susceptibility index (age x dose kg^{-1} x oxidative ability)	R	0.003

t - two sample t-test

R - Wilcoxon rank sum test

3.3.4 Glucose-6-phosphate dehydrogenase enzyme activity

Two metiamide-induced agranulocytosis patients and two carbimazole-induced agranulocytosis patients were investigated with respect to their erythrocyte G-6-PD enzyme activity (Table 4.9). Normal values for G-6-PD activity are 4.6-13.5 I.U./g Hb. (World Health Organisation, 1967).

Table 4.9 G6PD enzyme activity in agranulocytosis patients' erythrocytes.

Patient No.	Enzyme activity ($I.U.\ gHb^{-1}$)
A1	6.2
A2	5.7
A6	9.5
A7	6.8

4.4 Discussion

The studies described in this chapter were undertaken in an attempt to identify some of the factors which underlie the occurrence of metiamide-induced agranulocytosis. The investigations carried out were designed to test the hypothesis that the haematotoxicity of metiamide is related to its metabolic oxidation in the body to form highly reactive and hence toxic metabolites. Individual susceptibility to metiamide toxicity might therefore be related to metabolic oxidative ability plus a number of other unidentified factors which would tend to increase the susceptibility of the haemopoietic tissue.

Investigation of the agranulocytosis patients metabolic oxidative abilities, using debrisoquine, revealed that all seven patients were probably homozygous extensive metabolisers. Of the eleven control patients investigated, three were probably heterozygotes and eight probably homozygous extensive metabolisers. The agranulocytosis patients were significantly better at aromatic oxidation, producing the phenolic metabolites of debrisoquine, than the control patients.

These findings give some supportive evidence to the hypothesis that a high metabolic oxidative ability is a contributory background factor in the occurrence of metiamide-induced agranulocytosis.

However, since about 50% of a British population are also homozygous extensive oxidisers, it is apparent that although a high oxidative ability might be a prerequisite for the propensity to develop metiamide-induced agranulocytosis, it does not necessarily follow that all good oxidisers are susceptible to the haemotoxic effects of metiamide. A number of other factors must be considered in the etiology of the dyscrasia.

One of these factors would appear to be the duration of drug dosage. The earliest a neutropenic response was

observed in the agranulocytosis patients was after 3 weeks treatment with metiamide whilst in most cases a period of at least 8 weeks continuous administration was needed before changes occurred in the white blood cell counts. Many of the patients in the metiamide trial only received the drug for a period of one month and were thus probably not exposed to the drug for a long enough time period, for the haemotoxic effect to be observed. Pisciotta (1969) in a study of chlorpromazine-induced agranulocytosis also noticed a definite relationship between period of drug administration and onset of neutropenia. A period of at least 10 days but usually 20-30 days of continuous drug administration was needed before haematotoxicity was observed.

The size of the daily dose given to the patients appear to be another contributory factor. The metiamide-induced agranulocytosis patients were found to have been given significantly higher doses (in g metiamide per kg body weight) than the control patients. This type of dose-dependent effect has also been observed in the occurrence of haematotoxicity due to the antithyroid drugs (McGavack & Chevalley, 1954; Wiberg & Nuttall, 1972). The higher the doses of these compounds which are given to patients, the higher the incidences of both total granulocytopenic reactions and fatal granulocytopenic reactions (Table 4.10).

A third contributory factor would appear to be the age of the patient. The metiamide-induced agranulocytosis patients were found to be significantly older than the metiamide control patients at the time that they received metiamide. This observation probably reflects the decrease in the amount of active haemopoietic tissue which occurs with age (Custer, 1949). Thus, in older patients a smaller mass of active haemopoietic tissue would receive a higher effective dose of the drug than younger patients.

Table 4.10 The dose-dependent haematotoxicity of several antithyroid compounds (after McGavack & Chevalley, 1954).

Drug	Daily Dose (g)	Granulocytopenic reactions (% of patients)	
		Total	Fatal
Thiourea	1.0 - 2.0	0.5	0.1
Thiourea	2.0 - 5.0	4.0	1.5
Methylthiouracil	0.3 - 0.6	0.3	0
Methylthiouracil	0.6 - 1.4	3.0	2.0
Propylthiouracil	0.1 - 0.15	0.1	0
Propylthiouracil	0.25- 0.5	0.3	0.1
Methimazole	0.015-0.025	0	0
Methimazole	0.03 -0.06	1.0	0.1

In a study of chlorpromazine-induced agranulocytosis patients, Pisciotta (1969) observed that middle-aged and elderly females appeared more vulnerable than other groups to the haematotoxic effects of the drug. This observation may reflect an interaction between the age and body-weight factors in these patients on the propensity to develop haematotoxic reactions to this type of bone-marrow suppressive agent.

It is apparent from these studies that no single factor determines the individual susceptibility to metiamide-induced agranulocytosis, but rather a number of factors which in certain individuals combine to produce a situation whereby a patient has an increased risk of displaying the blood dyscrasia if exposed to the drug.

A susceptibility index, derived from the dose of drug, age of patient and metabolic oxidative ability of the patient, thus shows a highly significant difference between the agranulocytosis patients and the control patients (as assessed by means of the Wilcoxon rank sum test; $2P < 0.003$). An index of this type might be used to reduce the possibility of the occurrence of haematotoxic side effects of certain drugs, since two of the contributory factors, namely age of patient and oxidative ability of the patient, could be determined

prior to drug administration. A third contributory factor, the dosage of the drug, might then be adjusted so as to minimise the possibility of haematotoxic side-effects occurring in certain 'high risk' patients. Alternatively a different form of treatment might be initiated.

The G-6-PD enzyme activities of the agranulocytosis patients were all within normal limits indicating that, at least in the erythrocytes, these patients had no inability to protect the cell against oxidative stress. Patients recovered from chlorpromazine-induced agranulocytosis have been shown to have normal levels of reduced glutathione in their leucocytes in the presence and absence of chlorpromazine (Pisciotta & Daly, 1960). The involvement of an inherent inability of granulocytes to protect themselves against oxidative stress therefore appears unlikely in this type of drug-induced blood dyscrasia.

There remains the possibility, however, of a specific defect in the susceptible patients granulocytes, which is uncovered by metiamide, similar to the defect described by Pisciotta (1971) in the granulocytes of chlorpromazine-induced agranulocytosis patients. This is unlikely to be the case since Epstein-Barr stimulated

lymphocyte cultures of lymphocytes from metiamide-induced agranulocytosis patients and carbimazole-induced agranulocytosis patients showed no increased sensitivity to metiamide, its metabolites or carbimazole compared to normal controls. (N.Carver, J.R.Idle & R.L.Smith, personal communication).

The biochemical mechanisms underlying the occurrence of metiamide-induced agranulocytosis therefore appear not to be caused by a cellular defect in sensitive patients which is uncovered by the drug, but rather due to a direct, dose-dependent, toxic action of metiamide on the bone-marrow of susceptible patients, probably due to the formation of a toxic oxidative metabolite. Susceptibility appears to be influenced by a number of contributory factors which includes dose of metiamide, age of patient and metbolic oxidative ability of the patient.

CHAPTER FIVE

DISCUSSION

The occurrence of adverse reactions to drugs has been recognised as a major problem in the treatment of disease for many years. In fact the scale of the problem is probably much greater than is generally realised. On the whole, only the most severe (i.e. life-threatening) adverse reactions are reported in the literature or to government bodies, which results in most of the published data being underestimates of the true incidences of adverse reactions. One detailed study revealed that 5 per cent of patients admitted to a hospital had an adverse drug reaction, while approximately 14 per cent of patients developed a reaction to at least one drug during their hospitalisation (Cluff et al., 1965).

The frequent occurrence of adverse drug reactions expends large amounts of valuable hospital resources in terms of beds and treatment quite apart from having serious clinical consequences for the individual patient. When the adverse effect is serious enough to warrant the withdrawal of the drug from clinical use, vast amounts of time and money invested by the drug company into the development of the drug, may be lost.

The history of metiamide is an excellent example of this type of problem, whereby a completely new type of drug, the first histamine H_2-receptor antagonist, received clinical trials in 700-800 patients. The trials were a great success, the drug producing the required pharmacological effect. However a few patients developed a severe adverse reaction and the drug had to be withdrawn. Thus a valuable compound with good therapeutic potential was lost to medicine. Fortunately, cimetidine was rapidly developed from metiamide and has proved to be one of the most successful drugs of the last decade.

It is apparent, therefore, that there is an urgent need to minimise the occurrence of adverse drug reactions. The major obstacle to achieving this end is, however, that in a large number of drugs the underlying mechanisms of the drugs toxicity are not known, nor is it known why some individuals appear to be more susceptible to the toxic effects of these drugs, than others.

The detailed study of individual cases of drug-induced adverse reactions is therefore of profound importance in attempts to gain some insight into the underlying biochemical mechanisms of drug toxicity. By the study of these individuals and of the factors which distinguish

them from the rest of the population it may be possible to reduce the risk of adverse reactions in susceptible patients. The aim of the work described in this thesis was to investigate some of the factors which might underlie the occurrence of metiamide-induced agranulocytosis.

Many sulphur-containing compounds are known to be potentially haematotoxic, thus, it was considered highly probable that the haematotoxicity associated with metiamide was related to the presence of the thiourea moiety in its structure.

A second possibility was that the haematotoxicity associated with metiamide was related to its histamine H_2-receptor antagonist activity. However, replacement of the thiourea group in metiamide with a cyanoguanidine group to produce cimetidine (also a histamine H_2-receptor antagonist), completely removed the haematotoxic properties of the molecule without major changes in pharmacological potency. Ranitidine, another histamine H_2-receptor antagonist which is chemically quite different from metiamide and cimetidine, has also shown no evidence of haematotoxicity in animals or in man.

The mechanisms by which thiourea and its derivatives exert their toxic effects have not been fully elucidated.

Most of the work carried out on the toxic properties of the thiourea moiety have concentrated on the toxicity of these compounds in the lung, particularly mechanisms of binding of thiourea-containing substrates to lung macromolecules. There appears to be some good evidence that the toxicity of these compounds is related to their metabolic oxidation to produce highly reactive and hence toxic metabolites, which then bind to macromolecules in the lung and liver. The cytochrome P-450 mono-oxygenase system has been shown to be, at least in part, responsible for this metabolic oxidation (Lee et al., 1980). Although thiourea-containing compounds have also been reported to be sustrates for the flavoprotein amine oxidase described by Ziegler (Poulsen et al., 1974).

Some indirect evidence for the formation of these reactive metabolites of metiamide comes from the urinary excretion of the urea analogue of metiamide. This metabolite may be formed by the further oxidation of the sulphenic or sulphinic acids of metiamide, releasing some form of 'active sulphur' in the tissues. A certain proportion of these reactive metabolites would be rapidly reduced back to the parent thione by glutathione in physiological conditions. Other thiourea-containing compounds have been reported to deplete glutathione concentrations in

liver, lung and bone-marrow of animals, and that the change in tissue glutathione concentrations are both time-and dose-dependent (Ziegler, 1978). Glutathione thus appears to play some sort of protective role against the potential toxicity of thiourea derivatives and it is possible that an impaired ability to produce an adequate supply of reduced glutathione when challenged with high doses of thiourea derivatives, would lead to toxicity.

The evidence therefore suggests that the toxicity associated with metiamide involves metabolic oxidation in the thiourea moiety to produce highly reactive metabolites which then bind to granulocyte precursor cells in the bone-marrow. How then do the apparent inter-individual differences in the haematotoxicity of metiamide arise?

The hypothesis which has been proposed and tested in these studies is that the genetic polymorphism which influences the metabolic oxidation of a number of drugs in man also influences the metabolism of metiamide. Thus certain individuals in the population would have an increased risk of displaying haematotoxic reactions to metiamide than others. This increased susceptibility could result from two possible situations, either the

polymorphism might directly influence the ability to carry out the metabolic oxidation of the thiourea group, or the polymorphism might influence the disposition of metiamide in the body, resulting in marked variations in the tissue levels of metiamide and its metabolites between patients.

Study of the influence of the oxidation polymorphism on the metabolism of metiamide in man has shown that the subjects of the EM phenotype excrete greater amounts of metiamide and its oxidative metabolites whilst the PM phenotype subjects tend to excrete a greater amount of the conjugated metabolites of metiamide. Thus individuals of the EM phenotype would have higher plasma and presumably bone-marrow concentrations of metiamide and its oxidative metabolites than the PM phenotype subjects, possibly leading to an increased risk of bone-marrow toxicity.

However, the metabolic differences which have been observed between phenotypes are not large, and although oxidative ability appears to be a contributory factor in the aetiology of the blood dyscrasia, other factors are undoubtedly also involved. Some of these other factors have been investigated in this study.

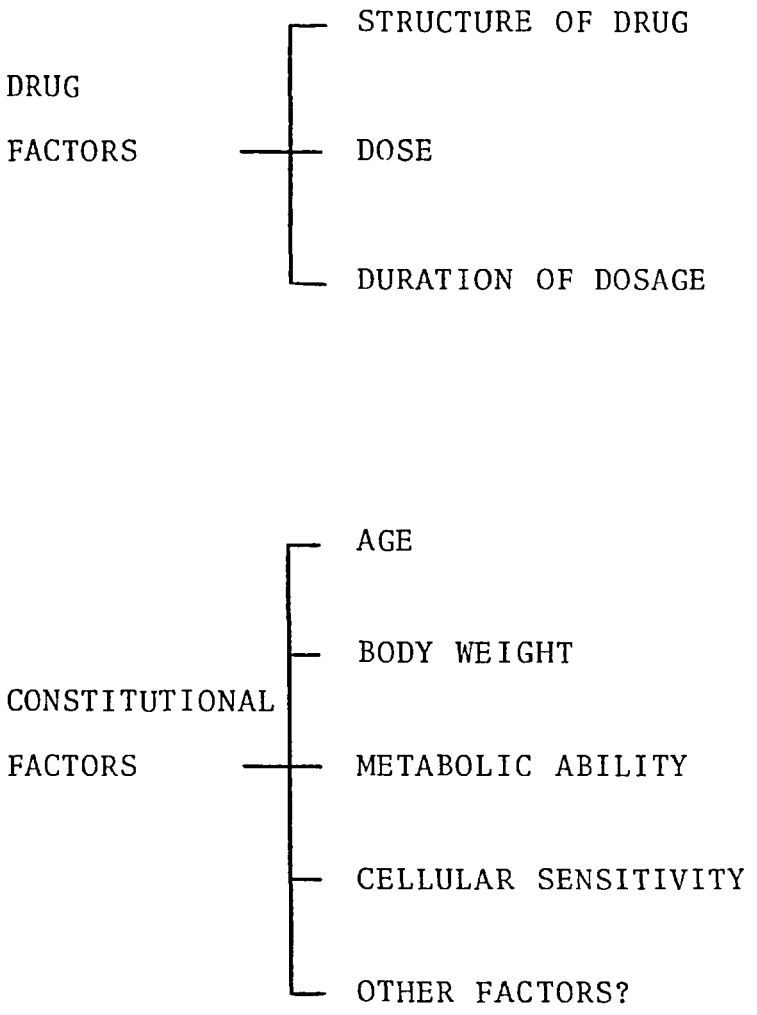
The contributory factors involved in the occurrence of

metiamide-induced agranulocytosis may be considered in two groups, those concerned with the drug and those concerned with the individual patients constitution (Fig.5.1).

The involvement of the structure of metiamide in its haematotoxicity has been previously discussed in relation to the presence of the thiourea group, which when replaced, results in loss of haematotoxicity. The dose-size and the duration of dosage of metiamide also appear to have been contributory factors in the occurrence of the agranulocytosis. The daily doses of metiamide ranged from 400 mgd^{-1} in some patients to 1200 mg d^{-1} in others. However no cases of metiamide-induced agranulocytosis were reported for patients receiving less than 800 mgd^{-1} metiamide. It would appear, therefore, that the occurrence of metiamide-induced haematotoxicity was to some extent dose-dependent, occurring only above a threshold level of dosing.

The duration of metiamide dosage appears to have been important. None of the patients were reported to have had any neutropenic reactions to metiamide until after at least three weeks continuous administration of the drug, while in some cases, toxic reactions were only observed after three months continuous administration

Fig.5.1 Factors probably contributing to metiamide induced agranulocytosis



metiamide.

The dose and duration of dosage may be inter-dependent factors, as the higher the initial dose level, the earlier the neutropenic response occurred. Thus the two patients receiving the highest dose levels in this study (1200 mg $^{d-1}$) progressed to agranulocytosis after the shortest periods of metiamide treatment (25 and 40 days).

Three constitutional factors have been identified which appear to contribute to the propensity of certain individuals to develop the blood dyscrasia, namely age of patient, body weight of patient and oxidative ability of patient. The involvement of age as a factor probably reflects the gradual decline in activity of the haemopoietic tissues with increasing age (Custer & Ahlfeldt, 1932). Other factors may well also be involved in the aetiology of the problem which may explain the large inter-species differences in the haematotoxicity of metiamide. Whether or not these inter-species differences are in the sensitivity of the bone-marrow cells to metiamide, or in cellular repair mechanisms or in turnover of cells in the bone-marrow, remains to be elucidated.

A susceptibility index which combines a number of possible

contributory factors in the occurrence of metiamide-induced agranulocytosis, shows a highly significant difference between patients who developed agranulocytosis and the control patients, who did not. A future area of research might be to investigate the potential for such an index for a number of drugs which are still in clinical use. A number of drugs which might be suitable for this type of investigation are given in Table 5.1. Particularly suitable candidates would be the antithyroid drugs carbimazole and methimazole whose haematotoxicity have many features in common with metiamide-induced haematotoxicity.

By the identification of individuals in the population who because of their particular constitution would be more 'at risk' of developing adverse reactions to certain drugs than the rest of the population, it may be possible to reduce the occurrence of these adverse drug reactions.

Metiamide-induced agranulocytosis has been discussed in the context of a number of possible aetiological factors. It would appear that although oxidation phenotype may have been a contributory factor in the

Table 5.1

Drugs in clinical use for which the calculation of a susceptibility index for individual patients might be relevant.

Acetazolamide

Carbimazole

Chlorpromazine

Penicillamine

Thioridazine

cases of this dyscrasia, it is somewhat overshadowed by other determinants such as dose size, duration of therapy and the patients' age. In clinical trials such as those carried out with metiamide, where a wide range of patients' ages were seen, and dose sizes and durations of therapy were employed, it is difficult to inspect

post hoc a single variable such as inherited metabolic capacity. It is in many ways remarkable that a particular combination of related and independent variables such as those investigated in this study, can be defined to give a crude threshold beyond which an adverse reaction is more commonly encountered. Obviously, it would have been easier to reach a firm conclusion regarding metiamide toxicity in man if doses, for example, had been standardised in the clinical trials. However, normalization of such variables is often impracticable in multi-centre clinical trials of a new drug where, above all, the well-being of the individual patient must be taken into account by the physician. This study highlights the difficulties of attempting to analyse single variables against a multifactorial background of effects.

Oxidation phenotype appears to be associated with a number of other untoward responses to drugs. Individuals who are poor metabolisers (PM phenotype) are:

a) more prone to develop postural hypotension in response to a small oral dose of the adrenergic neurone blocker debrisoquine (Idle et al., 1978),

b) more likely to develop diplopia, blurred vision, dizziness and headache when given sparteine (Eichelbaum et al., 1979),

c) more likely to develop methaemoglobinaemia when given a single dose of phenacetin (I.Kong, T.P.Sloan, J.R.Idle & R.L.Smith, personal communication) and

d) more likely to develop increased lactic acid levels when given a single oral dose of phenformin (Idle et al., 198).

These untoward reactions have all been studied in a prospective manner and thus other variables such as age and dose have been minimised. Additionally, in all four cases the expected adverse response is a minor one and therefore studies of this nature can be justified on ethical grounds. In the case of metiamide, a prospective investigation is obviously impossible.

This raises the question as how best to study the relationship between a drug and its serious adverse responses. Obviously, only animal studies will provide this information. One of the propositions in the case of metiamide was that oxidative metabolism of the drug played a role in the development of the dyscrasia. In accord with thishypothesis, it was necessary to define the oxidative metabolism of metiamide in both man and animals, both in terms of the metabolic pathways available and the extent of the metabolism and variability in each pathway.

As has been shown, metiamide is converted to a number of metabolites in man and animals namely, a sulphoxide, a urea analogue, a hydroxymethyl metabolite and an N-glucuronide. The diallelomorphic gene locus, which regulates the oxidative metabolism of debrisoquine and a number of other drugs in man, has been shown to influence to a minor, yet significant degree, the metabolism of metiamide. The metabolism of metiamide, in relation to debrisoquine oxidation, has been investigated in a number of animal species and strains thereof in an attempt to find a suitable animal model for metiamide metabolism in man. Since the dog is the only species apart from man in which metiamide-induced haematotoxicity has been observed, it was used to investigate the relationship between metiamide haematotoxicity and debrisoquine oxidation, both of which appeared to show wide variations between individuals in the dog population. The findings of these investigations were inconclusive.

It is felt, however, that in situations where the link between drug, its toxic actions and its oxidative metabolism is somewhat stronger, animal models for the human EM and PM phenotypes, such as those recently described for inbred rat strains (Al-Dabbagh et al., 1981), may provide a prospective means of screening drugs for toxicity, where such toxicity may be dose-

related and thus in some cases, a probably function of genetically-determined oxidative metabolic capacity. It is to be hoped that by the use of such models and by an increased awareness of the diversity which occurs in all normal populations and its consequences when uncovered by drugs, a safer and more effective use of drug therapy may be achieved in the treatment of disease.

References

Journals are abbreviated in the form recommended by the Biochemical Society.

Al-Dabbagh, S.G., Idle, J.R. & Smith, R.L. (1981)
J.Pharm. Pharmacol. In the press.

Amann, F.W., Buhler, F.R., Conen, D., Brunner, F., Ritz, R. & Speck, B. (1980).
Lancet, 1, 150.

Ash, A.S.F. & Schild, H.O. (1966)
Br.J.Pharmacol. 27, 427-439.

Baimonova, K.M. & Serebrovskaya, I.A. (1973)
Patol Fiziol Eksp. Ter. 2 74-76; cited in Chem. Abstr. 79, 38994.

Bartels, E.C. (1948)
Am. J. Med. 5, 48-52.

Benjamin, J.E. & Biederman, J.B. (1934)
J.A.M.A. 103, 161-163.

Beutler, E. (1972)
in The Metabolic Basis of Inherited Disease (Stanbury, J.B., Wyngaarden, J.B. & Fredrickson, D.S., eds.), p. 1358, McGraw-Hill, New York.

Black, J.W., Duncan, W.A.M., Durant, G.J., Ganellin, C.R. & Parsons, M.E. (1972).
Nature (London) 236, 385-390.

Black, J.W., Duncan, W.A.M., Emmett, J.C., Ganellin, C.R., Hesselbo, T. & Parsons, M.E. (1973).
Agents & Actions 3, 133-137.

Bock, H.E. (1946).
Agranulozytose. F.Enke Verlag, Studtgart.

Boyd, M.R. & Neal, R.A. (1976).
Drug Metab. Dispos. 4, 314-322.

Bridges, J.W., Kibby, M.R. & Williams, R.T. (1965)
Biochem. J. 96, 829-836.

Brimblecombe, R.W., Duncan, W.A.M., Owen, D.A.A. & Parsons, M.E. (1975).
Fed. Proc. 35, 1931-1934.

Brimblecombe, R.W., Duncan, W.A.M. & Parsons, M.E. (1974)
S.Afr. Med. J. 48, 2253-2255.

Brown (1902)
Amer. Med. 3, 649.

Burland, W.L., Sharpe, P.C., Colin-Jones, D.G., Turnbull, P.R.G. & Bowskill, P. (1975).
Lancet 2, 1085.

Carson, P.E. & Frisher, H. (1966)
Amer. J.Med. 41, 744-761.

Cluff, L.E., Thornton, G. & Seidl, L. (1965).
Trans. Ass. Amer. Physicians 78, 255-268.

Colquhoun, D. (1971).
Lectures on Biostatistics, p. 143, Clarendon Press, Oxford.

Committee on Problems of Drug Safety (1969)
Clin.Pharmacol.Ther. 10, 607-634.

Corcos, J.M., Solar-Bechara, J., Mayer, K., Freyberg, R.H., Goldstein, R. & Jaffa, J.J. (1964)
J.A.M.A. 189, 847-848.

Cross, S.A.M. (1977)
Proc.Europ.Soc.Toxicol. 18, 288-290.

Cummings, A.J., Martin, B.K. & Park, G.S. (1967)
Br. J.Pharmacol. 29, 130-149.

Custer, R.P. (1949)
Atlas of Blood & Bone-marrow, pp. 30-31, W.B.Saunders Co., Philadelphia.

Custer, R.P. & Ahlfeldt, F.E. (1932)
J.Lab.Clin.Med. 17, 960-970.

Dameshek, W.& Colmes, A. (1936)
J.Clin.Invest. 15, 85-97.

De Gruchy, D.C. (1975)
Drug-induced Blood Disorders, pp. 76-117, Blackwell Scientific Publications, London.

Dieke, S.H., Allen, G.S. & Richter, C.P. (1947)
J.Pharmacol.Exp.Ther. 90, 260-270.

Dieke, S.H. & Richter, C.P. (1946)
Proc.Soc.Exp.Biol.Med. 62, 22-25.

Dunlop, D. (1969)
in Hazards of Therapy, Royal College of Physicians of Edinburgh, Publication No.36, p.7, Constable, Edinburgh.

Eichelbaum, M., Spannbrucker, N., Steincke, B. & Dengler, H.J. (1979)
Eur.J.Clin.Pharmacol. 16, 183-187.

Evans, D.A.P., Mahgoub, A., Sloan, T.P., Idle, J.R. & Smith, R.L. (1980)
J.Med.Genet. 17, 102-105.

Feldman, E.J. & Isenberg, J.I. (1976)
N.Engl. J.Med. 295, 1178-1179.

Fleischer, D.& Samloff, I.M. (1977)
N.Engl.J.Med. 296, 342.

Forrest, J.A.H., Shearman, D.J.C., Spence, R.& Celestin, L.R. (1975)
Lancet 1, 392-393.

Frymoyer, J.W. & Jacox, R.F. (1963)
J.Lab.Clin.Med. 62, 891-904.

Girdwood, R.H. (1971)
in The Adverse Effects of Drugs, (Hanson, G.C., ed.), 1st ed., p.70, Beecham Research Laboratories, Brentford, Middlesex.

Girdwood, R.H. (1973)
Blood Disorders due to Drugs and other Agents, pp. 147-186, Exerpta Medica, Amsterdam, London and Princeton.

Griffiths,R., Lee ,R.M. & Taylor ,D.C. (1977)
In Proceedings of the Second International Symposium on histamine H_2-receptor antagonists (Burland,W.L. & Simkins,M.A.,eds.) pp.38-51, Excerpta Medica, Amsterdam.

Grimes, A.J. & De Gruchy, G.C. (1974)
in Blood and its disorders, (Hardisty, R.M. & Weatherall, D.J., eds.), p. 473, Blackwell, Oxford.

Hamilton, D.V. & Saunders, J. (1977)
Curr. Med.Res. Opin. 4, 607-608.

Hughes, H.B., Biehl, J.P., Jones, A.P. & Schmidt, L.H. (1954).
Am.Rev.Tuberc.Pulm.Dis. 70, 266-273.

Huguley, C.M. (1964)
J.A.M.A. 189, 938-941.

Hunter, A.L. & Neal, R.A. (1975)
Biochem. Pharmacol. 24, 2199-2205.

Idle, J.R., Mahgoub, A., Angelo, M.M., Dring, L.G., Lancaster, R. & Smith, R.L. (1979)
Br.J.Clin.Pharmacol. 7, 257-266.

Idle, J.R., Mahgoub, A., Lancaster, R. & Smith, R.L.(1978)
Life Sci. 22 , 979-984.

Idle, J.R., Oates, N.S., Shah, R.R. & Smith, R.L. (1981)
Br.J.Clin.Pharmacol. in the press.

Idle, J.R., Sloan, T.P., Smith, R.L. & Wakile, L.A.(1979a)
Br.J.Pharmacol. 66, 430-431 P.

Idle, J.R. & Smith, R.L. (1979)
Drug Metab.Rev. 9, 301-317.

Imman, W.H.W. (1970)
Proc.R.Soc.Med. 63, 1302-1304.

Jaffe, E.R. (1972)
N.Engl.J.Med. 286, 156-157.

Jirousek, L. & Cunningham, L.W. (1968)
Biochem.Biophys.Acta 170, 160-171.

Lam, D.C.C. & Lindsay, R.H. (1979)
Biochem.Pharmacol. 28, 2289-2296.

Lambo, T.A. (1957)
Br.Med.J. 2, 1048.

Landsteiner, K. (1947)
The Speciticity of Serological Reactions. Harvard
University Press, Cambridge.

Landsteiner, K. & Jacobs, J. (1936)
J.Exp.Med. 64, 625-639.

Landsteiner, K. & Van Der Scheer, J. (1936)
J.Exp.Med. 63, 325-339.

Latta, H. (1947)
Bull.Johns Hopkins Hosp. 80, 181-197.

Lee, P.W., Arnau, T. & Neal, R.A. (1980)
Toxicol.Appl.Pharmacol. 53, 164-173.

Lehmann, H. & Liddell, J. (1964)
Prog.Med.Genet. 3, 75-105.

Lindberg, L.G. & Norden, A. (1961)
Acta Med.Scand. 170, 195-199.

Lindsay, R.H., Cash, A.G., Vaughn, A.W. & Hill, J.B.
(1977)
Biochem.Pharmacol. 26, 617-623.

Lindsay, R.H., Kelly, K. & Hill, J.B. (1978)
Ala.J.Med.Sci. 15, 29-41.

Lindsay, R.H., Kelly, K. & Hill, J.B. (1979)
Endocrinology 104, 1686-1697.

Madison, F.W. & Squier, T.L. (1934)
J.A.M.A. 102, 755-759.

Mahgoub, A., Idle, J.R., Dring, L.G., Lancaster, R.& Smith, R.L. (1977)
Lancet 2, 584-586.

Magis, C.C., Barge, A. & Dausset, J. (1968)
Clin.Exp.Immun. 3, 989-1003.

Marchant, B., Alexander, W.D., Robertson, J.W.K. & Lazarus, J.H. (1971)
Metabolism 20, 989-999.

Mazze, R.I., Cousins, M.J. & Kosek, J.C. (1973)
J.Pharmacol.Exp.Ther. 184, 481-488.

McGavack, T.H. & Chevalley, J. (1954)
Am.J.Med. 17, 36-40.

Meyer, B.J. & Karel, L. (1948)
J.Pharmacol.Exp.Ther. 92, 15-31.

Mitchell, J.R., Thorgeirsson, W.P., Black, M.J., Timbrell, J.A., Snodgrass, W.R., Potter, W.Z., Jollow, D.J. & Keisec, H.R. (1975)
Clin.Pharmacol.Ther. 18, 70-79.

Moeschlin, S. (1955)
Acta Haemat. 20, 176-80.

Moeschlin, S. & Wagner, K. (1952)
Acta.Haemat. 8, 29-41.

Morton, J.H. (1947)
Am.J.Med. 2, 53-64.

Mountain, J.T. (1968)
Arch.Environ.Health 6, 357-365.

Parker, F.P. & Kracke, R.R. (1936)
Am.J.Clin.Path. 6, 41-56.

Papapetrou, P.D., Marchant, B., Gavras, H. & Alexander, W.D. (1972)
Biochem.Pharmacol. 21, 363-377.

Pearson, J.R., Binder, C.I. & Neber, J. (1955)
J.A.M.A. 157, 339-341.

Pisciotta, A.V. (1969)
J.A.M.A. 208, 1862-1868.

Pisciotta, A.V. (1971)
J.Lab.Clin.Med. 78, 435-448.

Pisciotta, A.V. & Daly, M. (1960)
Blood 16, 1572-1578.

Poulsen, L.L., Hyslop, R.M. & Ziegler, D.M. (1974)
Biochem.Pharmacol. 23, 3431-3440.

Rachmilewitz, M. & Rosin, A. (1949)
Acta Haemat. 2, 153-167.

Riegelman,S.,Loo,J.C. & Rowland,M.(1968)
J.Pharm.Sci. 57 , 117-123.

Saunders, L. & Natunen, T. (1973)
J.Pharm.Pharmacol. 25, 44P-51P

Schultz, W. (1922)
Dtsch.Med.Wschr. 48, 1495.

Schwarz, E. (1904)
Ges.Inn.Med.Kinderheilk.Wein. 3, 190.

Shah, R.R., Oates, N.S., Idle, J.R. & Smith, R.L. (1980)
Lancet 1, 1147.

Sikkema, S.H., Thewlis, E.W. & Meyer, O.O. (1946)
Blood 1, 411-425.

Sloan, T.P., Idle, J.R., Lancaster, R. & Smith, R.L.
(1978)
Proc.7th Int.Cong.Pharmacol., Paris p.2866.

Smith, R.L. & Williams, R.T. (1961)
J.Med.Pharm.Chem. 4, 137-146.

Specht, N.W. & Boehme, E.J. (1952)
J.A.M.A. 149, 1010-1011.

Spencer, K.E.V. & Sutton,T.J. (1977)
Proc.Europ.Soc.Toxicol. 18, 286-287.

Tait, G.B. (1957)
Lancet 1, 303.

Tasker, J.R. (1955)
Br.Med.J. 1, 950-951.

Taylor, D.C., Cresswell, P.R. & Pepper, E.S. (1979)
Drug Metab.Dispos. 7, 393-398.

Turk, W. (1907)
Wien.Klin.Wschr. 20, 157.

Urban, J. (1972)
Inaugural Dissertation, Eberhard-Karls University, Tubringen.

Wiberg, J.J. & Nuttall, F.Q. (1972)
Ann.Intern.Med. 77, 414-416.

Woolhouse, N.M., Andoh, B., Mahgoub, A., Sloan, T.P., Idle, J.R. & Smith, R.L. (1979)
Clin.Pharmacol.Ther. 26, 584-591.

World Health Organisation (19)
World Health Organisation Technical Report Series, No.366 p.30-32.

Zhislin, L.E. & Ovetshaya, N.M. (1972)
Grg.Tr.Prof.Zabol. 16, 51-52; cited in Chem.Abstr. 77, 97443.

Ziegler, D.M. & Mitchell, C.H. (1972)
Arch.Biochem.Biophys. 150, 116-125.

Ziegler, D.M. (1978)
Biochem.Soc.Trans. 6, 94-96.

Zingale, S.B., Minzer, L., Rosenberg, B. & Lee, S.L. (1963)
Arch.Intern.Med. 112, 63-66.