INVESTIGATIONS OF THE METABOLISM OF N-ALKYL AGENTS WITH ANTINEOPLASTIC ACTIVITY

by

Charles Joseph Brindley

A thesis submitted for the degree of

Doctor of Philosophy

in the

University of Aston in Birmingham

October 1982

Investigations of the metabolism of N-alkyl agents with antineoplastic activity

by

Charles Joseph Brindley

Submitted for the degree of Doctor of Philosophy, 1982 in the University of Aston in Birmingham

The metabolism and the pharmacokinetics of the antitumour agents, HMM (2,4,6-Tris[dimethylamino]-1,3,5-triazine) and NMF (N-methylformamide) was investigated. An attempt was made to discover a pharmacokinetic or metabolic explanation for the pharmacological activities and toxicities of these compounds. HMM was studied in-vitro and the disposition of NMF was investigated in-vivo.

The kinetic properties of the metabolism of HMM was compared with those of a series of derivatives of HMM and with those of AP (1-phenyl-2,3-dimethylamino-pyrazolon-5). The rate of N-demethylation of HMM and N-desmethyl derivatives, seemed to be related to their lipophilicities and also to their antitumour activity. HEM (2,4,6-Tris[diethylamino]-1,3,5-triazine) was readily dealkylated. However, even though it is more lipophilic than HMM, it is devoid of antitumour activity.

TetraMM (2,4-Bis[methylamino]-6-dimethylamino-1,3,5-triazine) and TriMM (2,4,6-Tris[methylamino]-1,3,5-triazine) are demethylated at a rate slower than that for HMM and PMM (2,4-Bis[dimethylamino]-6-methylamino-1,3,5-triazine), which may partly explain the long plasma elimination half-lives of the HMM metabolites, TetraMM and TriMM, compared to those of HMM and PMM.

The fate of N-alkylformamides in-vivo was studied by determining the plasma disposition and urinary excretion of these compounds in mice and patients. From the resulting plasma concentration-time profiles, pharmacokinetic parameters were evaluated which showed that NMF exhibited good oral bioavailability in both mice and patients. After administration to mice of equimolar doses of two closely related derivatives of NMF, DMF (N,N-dimethylformamide) and NEF (N-ethylformamide), the area under the plasma concentration-time curves for these agents were significantly lower than that for NMF. These results suggest that the marginal antitumour acitvity of DMF and NEF compared to the dramatic activity of NMF, may have a pharmacokinetic basis.

A comparison of the urinary excretion of ^{14}C-methyl-labelled NMF (^{14}C-NMF) with that of unlabelled NMF indicates that only 26.4% of drug was excreted unchanged and 50% as an unidentified metabolite of NMF, probably a conjugate of a hydroxylated derivative of NMF.

The plasma disposition of NMF and DMF in mice, within 24 hours after administration, could not be described by a linear pharmacokinetic model and appeared to be dependent on the dose administered. However, after 60 hours after administration of ^{14}C-NMF, radioactivity was eliminated from the plasma with a half-life of 71.1 hours.

Key words: N-alkylmelamines, N-alkylformamides, metabolic N-dealkylation, enzyme kinetics, pharmacokinetics.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to Dr. Andreas Gescher for his unceasing guidance and friendship throughout my research with the Cancer Chemotherapy Research Group at the University of Aston.

I also wish to thank Dr. Ernest Harpur, Dr. John Slack and Professor Malcolm Stevens for their interest anc co-operation concerning this work, and Miss Karen Ensor for her skilful technical assistance.

I acknowledge the support of the Cancer Research Campaign who funded the research project.

My thanks also to Mrs Hazel Brown for her persistence and care in the typing of this thesis.

CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

HMM	Hexamethylmelamine	2,4,6-Tris(dimethylamino)-1,3,5-triazine
PMM	Pentamethylmelamine	2,4-Bis(dimethylamino)-6-methylamino-1,3,5-triazine
TetraMM	N^2, N^2, N^4, N^6, Tetra-methylmelamine	2,4-Bis(methylamino)-6-dimethylamino-1,3,5-triazine
TMM	N^2, N^2, N^4, N^4, Tetra methylmelamine	2-Amino-4,6-bis(dimethylamino)-1,3,5-triazine
TriMM	N^2, N^4, N^6, Trimethylmelamine	2,4,6-Tris(methylamino)-1,3,5-triazine
HEM	Hexaethylmelamine	2,4,6-Tris(diethylamino)-1,3,5-triazine
HMPMM	Hydroxymethylpenta-methylmelamine	2-Hydroxymethylamino-4,6-bis(dimethylamino)-1,3,5-triazine
HeptaMM	Heptamethylmelamine	2,4-Bis(dimethylamino)-6-trimethyl-1,3,5-triazinium chloride
AP	Aminopyrine	1-Phenyl-2,3-dimethylaminopyrazolo 5
GSH	Glutathione	L-ß-glutamyl-L-cysteinyl-glycine

NMF	N-Methylformamide
NEF	N-Ethylformamide
DMF	N,N-Dimethylformamide
DMSF	N,N-Dimethylthioformamide
^{14}C-NMF	N, ^{14}C-(Methyl)-formamide
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
GC	Gas chromatography
IP	Intraperitoneal
PO	By mouth
IV	Intravenous
IM	Intramuscular
AUC	Area under the plasma concentration-time curve
v	Initial velocity
S	Substrate concentration
Km	Michaelis constant
Vmax	Maximum velocity of an enzymic reaction

PREFACE

This study compares some biochemical and pharmacological properties of the N-methyl-containing antitumour agents, HMM and NMF, with those of their inactive derivatives, e.g. HEM, NEF and DMF. The metabolism of N-methyl agents which are capable of N-demethylation but are not themselves cytotoxic antitumour agents was also investigated.

The thesis is organised into two parts. Part one (the N-alkylmelamines) is concerned with the in-vitro metabolism of HMM and some closely related derivatives, and AP. The enzymes which catalyse the N-demethylation of the N-alkylmelamines and AP were characterised by determining the enzyme kinetic constants, Km and Vmax. The effect of several xenobiotics on the in-vitro metabolism of N-methyl-containing compounds was also investigated. Part two (the N-alkylformamides) deals with the metabolic fate in-vivo of NMF and that of related derivatives, in mice and in patients.

To Cate and my parents

PART ONE

The N-alkylmelamines

SECTION 1

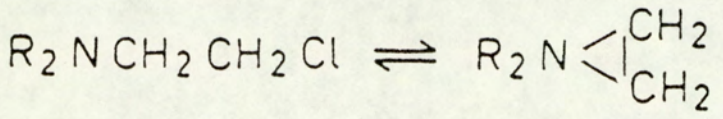
INTRODUCTION

1.1 The Discovery of Hexamethylmelamine (NSC 13875)

Interest in developing chemotherapeutic agents to treat advanced stages of cancer has grown since 1946 when Gilman and Philips (1946) demonstrated that the use of nitrogen mustards represented a significant advance in the management of lymphoid tumour.

In the 1950s investigations were performed into the possible use of a series of melamine derivatives, synthesised from cyanuric chloride (Kaiser et al., 1951) and employed in the production of synthetic resins and fibres. There is a structural similarity between these melamines and the aziridium ion formed on hydrolysis of the nitrogen mustards (fig. 1a) and this suggested possible antitumour activity for the derivatives. When triethylenemelamine (fig. 1b) was found to be active, a search for analogues led to the discovery of hexamethylmelamine (fig. 1c).

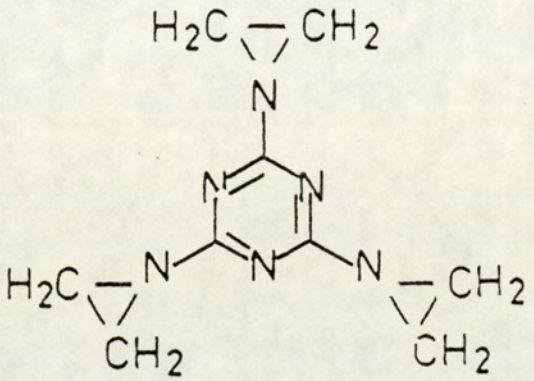
I



R = alkyl or aryl substituent

aziridium ion

II



III

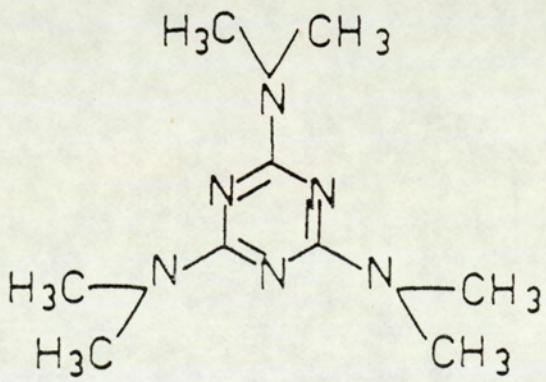


Fig. 1. Chemical structures of N-alkyl-containing antitumour agents:
I; nitrogen mustard, II; triethylenemelamine, and III; HMM.

1.2 Antimtumour Activity of Hexamethylmelamine and Pentamethylmelamine in Animals

Hexamethylmelamine (HMM) has shown significant activity against the Dunning leukaemia of the rat, the mouse adenocarcinoma 755 (Venditti, 1975) and the M5076/A reticulum cell sarcoma (Brindley et al., 1982b). HMM is also active against human lung tumours grown in mice deprived of T-lymphocytes (Mitchley et al., 1975), and the ADJ/PC6A plasmacytoma undergoes complete regression when treated with this agent, even when well established (Mitchley et al.,1975).

Marginal activity of HMM has been demonstrated against the mouse sarcoma 180 (Buckley et al., 1952) and the Walker 256 carcinoma (Hendry et al., 1951).

Venditti (1975) reported the inactivity of HMM as judged by the National Cancer Institute (NCI) screen of P388 leukaemia, Lewis lung carcinoma and B16 melanoma. By 1975, HMM was the only non-hormonal agent missed by this screening method (Venditti, 1975).

Pentamethylmelamine (PMM), the N-demethylated derivative of HMM, is slightly less effective against the mouse sarcoma 180 compared with HMM (Lake et al., 1975), but has similar activity in several human tumours grown in immune-deprived mice (Clinical Brochure, National Cancer Institute, 1978 ; Connors et al., 1977) and in the M5076/A reticulum cell sarcoma (Hahn, 1980). However the therapeutic index of HMM against the ADJ/PC6A plasmacytoma is superior to that of PMM (Rutty and Connors, 1977).

1.3 Clinical Pharmacology of HMM and PMM

Clinical trials of HMM employed as a single agent, or in combination with other chemotherapeutic agents, demonstrated activity in human tumours of the lung (Takita and Didolkar, 1974; Stolinsky and Bateman, 1973; Wampler et al., 1972; Bergevin et al., 1973; Wilson and de la Garza, 1965), ovary (Bergevin et al.,1973; Wilson and de la Garza, 1965; Wharton et al., 1979; Bonomi et al., 1979; Johnson et al., 1978; Bolis et al., 1979; Omura 1981; Vogl et al., 1979b) and Hodkins disease and non-Hodkins lymphoma (Borden et al., 1977).

The poor water solubility of HMM (Cumber and Ross, 1977) renders it unsuitable for parenteral use and it is administered orally in the clinic. The reduced bioavailability of HMM (D'Incalci et al., 1978) when given orally, must be due to first pass metabolism by the liver because HMM is well absorbed from the gut (Ames et al., 1979). The plasma concentration of HMM only represented a small percentage of the plasma concentration of total radioactivity when radio-labelled HMM was administered orally to man (Ames et al., 1979).

The major dose-limiting toxicity symptoms of HMM are gastrointestinal, neurological and haematopoietic (Hahn, 1980). Phase two investigations have shown that the average tolerated dose is 8 mg/Kg/day for 21 days (Bergevin et al., 1973). The mechanism by which HMM causes nausea and vomiting is not known but it is probably a central effect, rather than local irritation, because it

usually occurs several days after treatment (Wilson et al., 1969). Takita and Didolkar (1974) have reported the onset of gastrointestinal toxicity two to three days after drug administration. At therapeutic doses, HMM produced mild to moderate marrow toxicity which was reversible (Legha et al., 1976).

Because of the increased water solubility of PMM (Cumber and Ross, 1977) and its similar activity against some murine tumours compared to HMM, PMM has undergone phase one clinical investigations which however, have so far failed to demonstrate complete or partial responses in man (Smith et al., 1980; Ihde et al., 1980; Casper et al., 1980; Lichtenfield et al., 1980). In these reports, the major dose-limiting toxicity was reported to be nausea and vomiting.

1.4 Mode of Action of HMM

1.4.1 Mechanisms Not Involving Metabolism of HMM

The structural similarity of HMM compared to triethylenemelamine (TEM), led to the postulation that HMM acts as an alkylating agent (Wilson and de la Garza, 1965: Nadkarni et al., 1954). However, the following evidence supports the view that HMM does not act by this mechanism:

(1) Neither HMM nor its demethylated metabolites react positively with 4,nitrobenzylpyridine (NBP) (De Milo and Borkovec, 1968); an in-vitro test reagent demonstrating alkylating activity (La Breque et al., 1968).

(2) The required dose of HMM necessary for the effective treatment of human tumours is two hundred times higher than that for TEM. Also, the LD_{50} of HMM in rats and mice is one hundred fold greater than that of TEM (Philips and Thiersch, 1950). This suggests the possibility that the toxicity and cytotoxicity of HMM is brought about by a different mechanism to that of TEM.

(3) Some human tumours refractory to alkylating agents have been reported to be sensitive to HMM when this drug was used as a secondary treatment (Takita and Didolkar, 1974; Vogl et al., 1979b;Stolinsky and Bateman, 1973; Wampler et al., 1972). Recent reports have however cast doubt on this finding (Bolis et al., 1979; Omura, 1981) and HMM employed as a secondary agent may not be as effective as first thought; which indicates cross-resistance between alkylating agents and HMM.

There is some evidence that diamino-dihydro-s-triazenes act as folic acid antagonists (Baker and Ashton, 1972) and HMM may act in a similar manner. However HMM did not inhibit dihydrofolate reductase in-vitro (Worzalla et al., 1974b;Borkovec and De Milo, 1967), which would disprove this hypothesis.

HMM (10^{-4}M) inhibited the in-vitro incorporation of radioactive thymidine and uridine into DNA and RNA (Heere and Donnely, 1971). This has been interpreted as evidence that HMM inhibits DNA and RNA synthesis. HMM also exhibited a greater inhibition of uptake of RNA and DNA precursors into Ehrlich Ascites cells, than protein precursors (Heere and Donnely, 1971). This is interesting since some alkylating agents have also demonstrated this property (Heere and Donnely, 1971).

1.4.2 Metabolism of HMM

1.4.2.1 Evidence for Metabolic Activity

That metabolic activation of HMM is required to elicit its antitumour activity has been shown in the following investigations:

(1) HMM is directly cytotoxic in-vitro only after prolonged exposure to cells (Rutty and Abel, 1980; Bateman et al., 1979; D'Incalci et al., 1980).

(2) HMM, as well as its demethylated derivatives PMM and tetramethylmelamine (TetraMM), requires activation in-vitro to give an increase in survival time in the bioassay described by Rutty and Connors (1977).

These results suggest that intermediates of HMM metabolism are the cytotoxic species responsible for the antitumour activity of HMM. These reactive intermediates may interfere with a biochemical pathway, or pathways, e.g. C1-unit metabolism, in a way as yet unknown. It is possible that they react directly with tumour tissue macromolecules.

HMM (Ames et al., 1981; Garattini et al., 1981) and PMM (Ames et al., 1981) are activated by liver (Ames et al., 1981; Garattini et al., 1981) and tumour (Garattini et al., 1981) microsomal preparations to reactive intermediates which covalently bind to microsomal macromolecules. Covalent binding of metabolites of HMM to tissue macromolecules of liver and tumour was also shown in-vivo (Rutty et al., 1978; Garattini et al., 1981). The binding was not solely due to formaldehyde, released during the demethylation of HMM, because both ring and methyl-labelled HMM were used in some of these investigations (Ames et al., 1981; Rutty et al., 1978).

1.4.2.2 Isolation and Identification of HMM Metabolites

Since metabolism of HMM is thought to be important for its mode of action, many investigations have been performed concerning its metabolism in-vitro and in-vivo.

HMM undergoes extensive demethylation, most certainly in the liver (Rutty et al., 1978), and a number of demethylated metabolites have been isolated in the urine of rats, rabbits, mice and man, after HMM administration (Ames et al., 1979; Worzalla et al., 1974b;Rutty et al., 1978; Worzalla et al., 1973; Bryan et al., 1968).

After administration of HMM to rats, the alkylmelamine appeared to be rapidly metabolised because the unchanged drug could not be detected in the urine (Worzalla et al., 1973). Ames et al. (1979) found demethylated metabolites of PMM in human urine a few minutes after administration of PMM, and the presence of unchanged drug in human urine was also undetectable (Ihde et al., 1981). HMM and PMM appear therefore to be eliminated from the body mainly by metabolism.

Four major metabolites were isolated in the urine of rats after administration of HMM; N^2,N^4,N^6trimethylmelamine (TriMM), N^2,N^4dimethylmelamine, monomethylmelamine and melamine (Worzalla et al., 1974b). The two major metabolites detected in human plasma were PMM and TetraMM (Broggini et al., 1981). TriMM and DMM were also identified in small amounts (Benvenuto et al., 1981).

After administration of HMM or PMM to mice, the area under the plasma concentration-time profiles of TetraMM and TriMM have been seven to ten fold greater than those of the parent compound (Broggini et al., 1981). This persistence

of demethylated metabolites in the plasma was also shown for TriMM after PMM administration to the rabbit (Ames et al., 1979), and may be partly explained by the slow demethylation rate of TetraMM and TriMM as compared to HMM and PMM. Differences in the rates of N-demethylation have also been shown in-vitro (Rutty and Connors, 1977; Broggini et al., 1981), but these investigations used only one substrate concentration.

It was therefore considered that a detailed study of the demethylation of HMM and some of its demethylated metabolites, was relevant in order to support or refute the hypothesis that the long plasma elimination half-life of TetraMM and TriMM, as compared to HMM and PMM, reflect differences in the affinities of the differently methylated melamines by the metabolising enzymes.

After administration of ^{14}C-ring-labelled HMM, 89% and 79% of the total radioactivity could be recovered in the urine after 72 hours in man and rats respectively (Worzalla et al., 1974b). The radioactivity was still attached to the unchanged ring moiety which shows how stable the s-triazene ring is. However, when TEM was administered to rats 72 to 88% was recovered as cyanuric acid in the urine (Nadkarni et al., 1954). Only a small percentage of radioactivity was found as $^{14}CO_2$ in the breath of rats, after administration of TEM labelled in the ethylenimino moiety (Smith et al., 1958). However, when HMM was labelled in the methyl moiety and administered to rats (Worzalla et al., 1973) and mice (Gescher and Raymont, 1981) 24% to 33% of total radioactivity

could be recovered as exhaled $^{14}CO_2$. This evidence clearly indicates a different metabolic pathway for HMM compared to TEM.

HMM appears to undergo oxidative N-demethylation by a similar pathway in humans, rats and house flies (Worzalla et al., 1974b; Chang et al., 1964; Chang et al., 1968) as indicated by the appearance of identical demethylated metabolites after HMM administration. The proposed metabolic scheme (fig.2) suggests preferential demethylation of N-dimethyl moieties.

The demethylation of HMM is thought to proceed via a C-hydroxylation to N-hydroxymethylpentamethylmelamine (HMPMM) which subsequently decomposes to PMM with the concomitant loss of formaldehyde (De Milo and Borkovec, 1968). Succeeding demethylations yield melamines with four, three two or one methyl group(s).

Metabolically generated N-hydroxymethyl compounds have been thought to be transient species which readily rearrange to eliminate formaldehyde (Testa and Jenner, 1976). However Gescher et al. (1979) have demonstrated appreciable stability of N-hydroxymethyl metabolites of some N-methyl-containing xenobiotics, including HMM and PMM, and have identified HMPMM as an in-vitro metabolite by high pressure liquid chromatography and gas chromatography-mass spectrometry.

The stability of some carbinolamines suggests that they may escape breakdown in the liver and go to extrahepatic

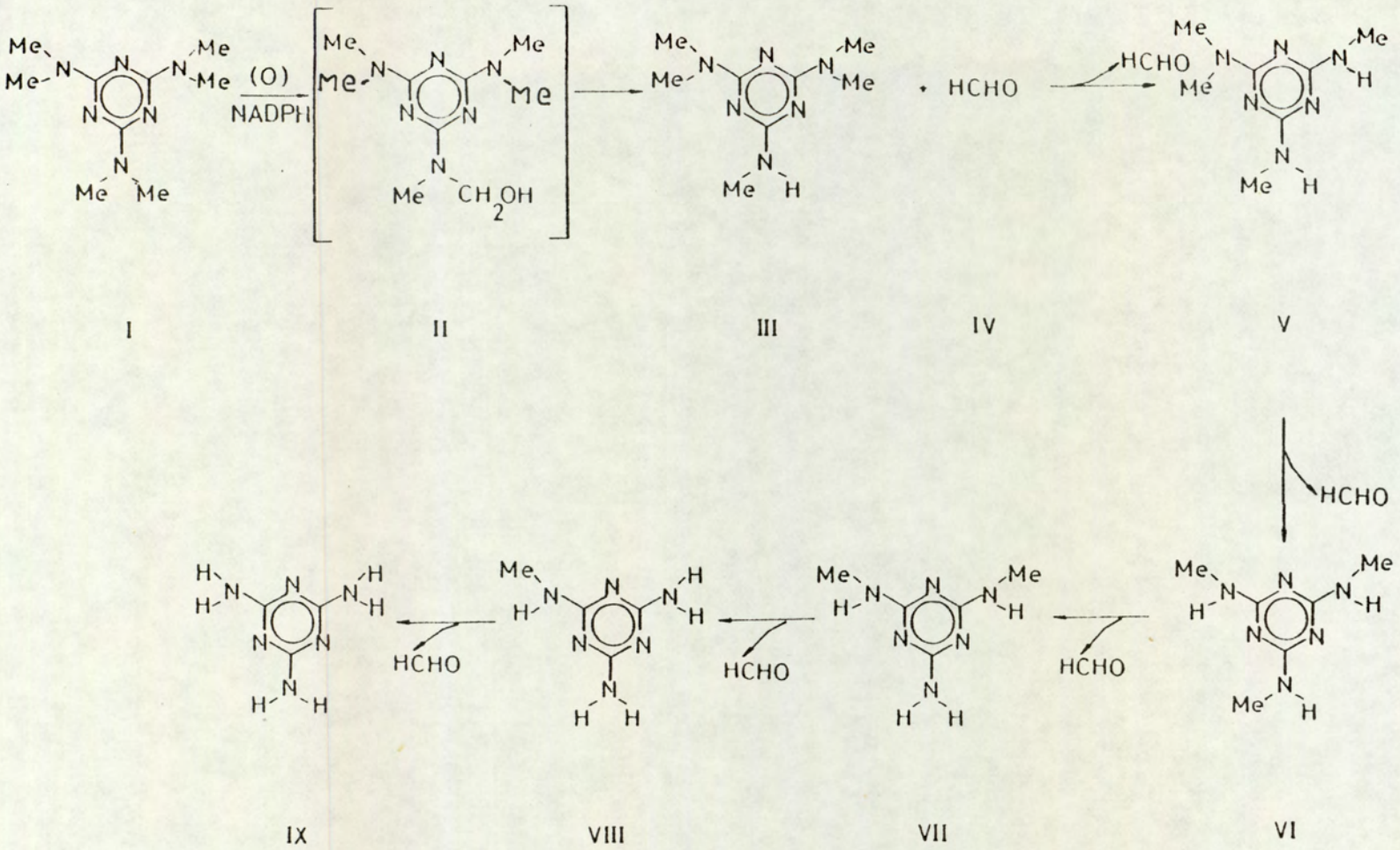


Fig. 2. Suggested pathway for the oxidative N-demethylation of HMM in humans, mice, rats and house flies. HMM (I) is metabolised to HMPMM (II) which chemically decomposes to PMM (II) and formaldehyde (IV). Successive demethylations yield TetraMM (V), TriMM (VI), dimethylmelamine (VII), monomethylmelamine (VIII), and melamine (IX). Each oxidative step is thought to proceed via a N-hydroxylated intermediate with the subsequent production of formaldehyde. The metabolic scheme suggests preferential demethylation of dimethyl moieties.

tissues,which may have toxicological consequences for the host (Gescher et al., 1979). However, direct evidence for the present of HMPMM in body fluids is lacking. Rutty and Connors (1977) have shown that TetraMM, TriMM, dimethylmelamine, monomethylmelamine and melamine are considerably less active against the ADJ/PC6A plasmacytoma when compared to the activity of HMM and PMM. They concluded that melamines require four or more N-methyl groups in the molecule for activity against this tumour system.

Lake et al. (1975) also found that toxicity and antitumour activity were reduced as the number of N- methyl groups in the melamine molecule decreased but at equitoxic doses the N-methyl melamines demonstrated comparable anti-tumour activity.

Whereas the metabolism of HMM and PMM in-vivo involves an activation step, probably due to the formation of methylols, metabolic generation of melamines with less than five methyl groups, from HMM or PMM, appears to represent a deactivation pathway.

1.4.2.3 Mechanism of Action of HMPMM

HMPMM is a major in-vitro metabolite of HMM (Gescher et al., 1980) and is highly cytotoxic when incubated with murine (Rutty and Abel, 1980) and human (Bateman et al., 1979; D'Incalci et al., 1980) tumour cell lines. The toxicity of HMPMM to cultured L1210 leukaemia and Walker 256 carcinoma appears to be solely due to formaldehyde, whereas in the ADJ/PC6A plasmacytoma it seems to act differently (Rutty and Abel, 1980). The cytotoxicity of HMPMM to L1210 leukaemia and Walker 256 carcinoma can be reversed with semicarbazide which traps formaldehyde; however this is not the case for the cytotoxicity of HMPMM to ADJ/PC6A plasmacytoma (Rutty and Abel, 1980).

It is possible that the methylols act by a mechanism analogous to alkylating agents; i.e. these intermediates of HMM may react covalently with nucleophilic centres of proteins and nucleic acids. Chemically, N-hydroxymethyl compounds are characterised by their ability to aminomethylate nucleophiles (Weitzel et al., 1964) and HMPMM has shown the capacity to aminomethylate PMM (Bořkovec and De Milo, 1967).

Some antitumour agents which are thought to be alkylating agents have the ability to covalently bind to DNA and form interstrand cross-links (Ewig and Kohn, 1977) when assayed by the alkaline elution assay pioneered by Kohn, et al., 1979). However, when HMM was metabolised by a liver metabolising system, and assayed by the alkaline elution

technique, neither HMM metabolites (Ross et al., 1981) nor direct addition of HMPMM (Muindi, 1981) could demonstrate DNA-DNA cross-linking activity. Although both formaldehyde and HMPMM produced extensive DNA-protein cross-linking in L1210 leukaemia cells (Muindi et 1981), it appears that the mode of action of HMPMM is not attributable to DNA cross-linking.

1.4.2.4 The Enzyme System Catalysing the Metabolism of the N-alkylmelamines

The N-demethylation of HMM is catalysed by a microsomal enzyme system and requires the presence of NADPH and molecular oxygen (Rutty and Connors, 1977; Ames et al., 1981; Garattini et al., 1981). Inhibitors, e.g. SKF525A and carbon monoxide, and inducers, e.g. phenobarbitone, of cytochrome P450-dependent drug metabolism, also influence the demethylation of HMM: phenobarbitone markedly stimulated the in-vivo (Worzalla et al., 1972; Gescher and Raymont, 1981) and in-vitro (Worzalla et al., 1972; Ames et al., 1981) metabolism of HMM, whilst SKF525A and carbon monoxide inhibited both HMM and PMM metabolism in-vitro (Ames et al., 1981).

The enzyme system catalysing the metabolism of HMM is therefore believed to involve the cytochrome P450-dependent monooxygenases of the liver.

In view of the importance of metabolism for the activity of HMM, it was considered relevant to attempt to further characterise the hepatic enzymes which catalyse the metabolism of HMM and its desmethyl metabolites.

1.5 Aims and Scope of the Present Investigation

HMM is active against a number of murine and human tumours. Its mode of action is not well understood, but it appears that metabolic activation is required to elicit its antitumour activity. The metabolic pathway which HMM undergoes is oxidative N-demethylation. Many drugs with N-methyl moieties, e.g. aminopyrine (Gram et al., 1968) and imipramine (Bickel and Baggiotini, 1966) are metabolised in this way. However, these compounds unlike HMM, are not antineoplastic agents. If one assumes that this biotransformation is important for the antitumour activity of HMM, one can postulate either of two explanations:

(1) HMM and aminopyrine (AP) undergo identical N-demethylations to different products; relatively stable methylols in the case of HMM, which may be the ultimate cytotoxic species in-vivo, and formaldehyde in the case of AP, which is rapidly metabolised to formate and carbon dioxide.

(2) Different enzymes located in different tissues and different cell compartments, catalyse the biotransformation of antineoplastic as well as non-antineoplastic N-methyl compounds; or these compounds react with the enzymes in different ways. Even though one of the products of these enzyme reactions is the same, i.e. formaldehyde which is a cytotoxic species, its differential formation may make its precursors, the N-methyl drugs, cytotoxic agents in some cases (HMM) and innocuous chemicals in others (AP).

In order to distinquish between these two possibilities an attempt was made to characterise the hepatic enzymes which metabolise HMM, its metabolites, and for comparison, AP. Such investigations were thought to reveal differences in the affinities of the metabolising enzymes for the substrates. The metabolism of HMM in the liver may be completely unimportant as a bioactivation step and its metabolism in the tumour cell may be the important event, leading to the actual antineoplastic species. However, very little is known about the enzymes which metabolise HMM in tumour tissues and the enzyme levels appear to be low (Garattini et al., 1981). It was therefore decided to investigate the hepatic HMM metabolising system and conclusions might be applicable to the enzyme system which may metabolise HMM in the tumour.

HMM is active against the ADJ/PC6A plasmacytoma whereas hexaethylmelamine (HEM) is devoid of antitumour activity in this tumour model (Rutty and Connors, 1977). The N-de-ethylation of HEM was investigated by characterising the enzymes responsible for its metabolism, in an attempt to explain the fact that N-methyl moieties are required in the melamine molecule for antitumour activity.

As previously mentioned in section (1.4.2.2), another rationale for studying the demethylation of HMM and its desmethyl metabolites was to explain the long plasma elimination half-life of TetraMM and TriMM as compared to HMM and PMM.

There have been examples in the literature which show that different N-methyl-containing xenobiotics undergo N-demethylation by pathways which are characterised by particular features. For example, the N-demethylation of the carcinogen dimethylaminoazobenzene was dependent on the endogenous compound glutathione (GSH), whereas the N-demethylation of AP was not influenced by GSH (Levine and Finkelstein, 1979). One of the many roles of hepatic GSH is to detoxify potentially harmful electrophilic compounds (Chasseaud, 1976). It also acts as an intra-cellular reductant and has the capacity to:

(1) Provide the reducing capacity for some enzyme reactions, e.g. it is a coenzyme of enzymes such as formaldehyde dehydrogenase and GSH peroxidase, and

(2) Protect proteins and cell membranes by reacting with peroxides and radicals which may be generated during xenobiotic metabolism.

In order to characterise HMM metabolising enzymes with respect to their dependence on GSH, the experimental conditions described by Levine and Finkelstein (1970) were used and the metabolism of HMM was compared with that of AP.

The antitumour agent adriamycin also reduces hepatic GSH levels (Doroshaw et al., 1979). Since HMM has been used in conjunction with adriamycin in the treatment of ovarian cancer (Vogl et al., 1979) it was considered relevant to investigate whether adriamycin influences the metabolism of HMM.

The N-demethylation of the carcinogen dimethylnitrosamine, and AP has been shown to be influenced by the presence of organic solvents (Argus et al., 1980; Kawalek and Andrews, 1980). Therefore the influence of organic solvents on the metabolism of HMM and AP was investigated to further characterise differences in the metabolism of N-methyl containing compounds; differences, which in some way may provide explanations for the fact that HMM is bioactivated by oxidative N-demethylation whereas AP appears to be detoxified by this process.

SECTION 2

MATERIALS

2.1 Substrates and their Derivatives Employed in the In-Vitro Investigations

The compounds listed below were synthesised in our laboratories.

The following melamine derivatives were prepared according to the methods of Thurston et al. (1951) and Paget and Hammer (1958) by Dr. R. J. Simmonds, Prof. M. F. G. Stevens and Mr. S. P. Langdon.

2,4,6-Tris (dimethylamino)-1,3,5-triazine

2,4-Bis(dimethylamino)-6-methylamino-1,3,5-triazine

2-Amino-4,6-bis(dimethylamino)-1,3,5-triazine

2,4-Bis(methylamino)-6-dimethylamino-1,3,5-triazine

2,4,6-Tris(methylamino)-1,3,5-triazine

2,4,6-Tris(diethylamino)-1,3,5-triazine

3-(4-Carboxymethylphenyl)-1,1-dimethyltriazene was prepared by Prof. M. F. G. Stevens according to the method of Connors et al. (1976) by treatment of the appropriate aryldiazonium salt with aqueous dimethylamine.

2,4-Bis(dimethylamino)-6-trimethyl-1,3,5-triazinium chloride was synthesided by Prof. M. F. G. Stevens according to the method of Dovlation et al. (1977).

3-O-[2,4-Bis(dimethylamino)-1,3,5-triazin-6-yl]-D-glucopyranose was synthesised according to a novel synthesis (Simmonds et al., 1982) by reacting the protected sugar with 2-Chloro-4,6-bis(dimethylamino)-1,3,5-triazine.

2.2 Miscellaneous Chemicals

Aminopyrine

dimethylsulphoxide

dioxane

folin's reagent

paraformaldehyde and

trichloracetic acid were obtained from BDH Chemicals Limited, Atherstone, United Kingdom.

Bovine serum albumin (BSA)

diethylmaleate

glucose-6-phosphate (G7879)

glucose-6-phosphate dehydrogenase (G68878)

methyliodide

nicotinamide adenine dinucleotide phosphate (N0505) and

reduced glutathione were purchased from Sigma Company Limited, Poole, United Kingdom.

Packing materials for the GC columns were obtained from Phase Separations Limited, Queensferry, United Kingdom.

Adriamycin was a gift from Dr. S. Chahwallah, University of Aston in Birmingham, United Kingdom.

2.3 Animals

Animals were obtained from Bantin and Kingman Limited, Hull, U.K. In order to avoid stress they were held for at least one week in an animal house to acclimatize.

They were fed on Heygates Modified 418 breeding diet ad libitum.

2.4 Buffer solutions and reagents

Magnesium free Earle's medium (Earle, 1943) consisted of:

$NaHCO_3$, 2.2g

NaCl, 6.8g

D-Glucose, 1.0g

KCl, 0.4g

NaH_2PO_4, 0.14g

and was made up to 1L with distilled water and the pH adjusted to 7.4.

Nash reagent (Nash, 1953) contained:

Ammonium acetate, 112.5g

acetylacetone, 1.5ml

acetic acid, 2.25ml

and distilled water made up to 250ml

Lowry reagent (Lowry, 1951) consisted of the following solutions:

2% Na_2CO_3. 10 H_2O in 0.1M NaOH

1% $CuSO_4$. 5 H_2O and

2% K or Na tartrate.

SECTION 3

METHODS

3.1 Estimation of Initial Velocity by Colourimetry

3.1.1 Preparation of Microsomes

Male Balb C mice (20-25g) were used throughout the initial velocity measurements. The animals were killed by cervical dislocation and the livers were removed between 9 and 11 a.m. and rinsed in cold 0.25M sucrose solution. The livers were weighed after blotting with filter paper. One part of liver was then homogenised with four parts of 0.25M sucrose solution in a glass homogeniser with a teflon pestel. The resultant homogenate was then centifuged in a M.S.E. Superspeed 50 ultracentrifuge at a rotor speed equivalent to 9000g and at a temperature of 0-4^{o}C for 20 minutes. The resultant supernatent was decanted and this fraction was centrifuged for a further 90 minutes at a rotor speed equivalent to 105,000g. The supernatent obtained after this centrifugation was discarded and the microsomal pellet remaining was resuspended in Earle's medium to yield a microsomal concentration equivalent to 200mg of liver in 0.5 ml.

3.1.2 Enzyme Concentration

The microsomal pellet contains the proteins which catalyse xenobiotic hydroxylation reactions. 0.1 ml of the microsomal suspension was diluted with 5 ml distilled water. 1 ml of this solution was assayed for protein content according to the method of Lowry et al. (1951). Absorbances were read using a Beckman Acta V spectrophotometer or Cecil C.E.5095 spectrophotometer, against control samples without protein, at 750 nm. Concentrations were calculated from a standard curve using BSA as the protein source (fig. 3).

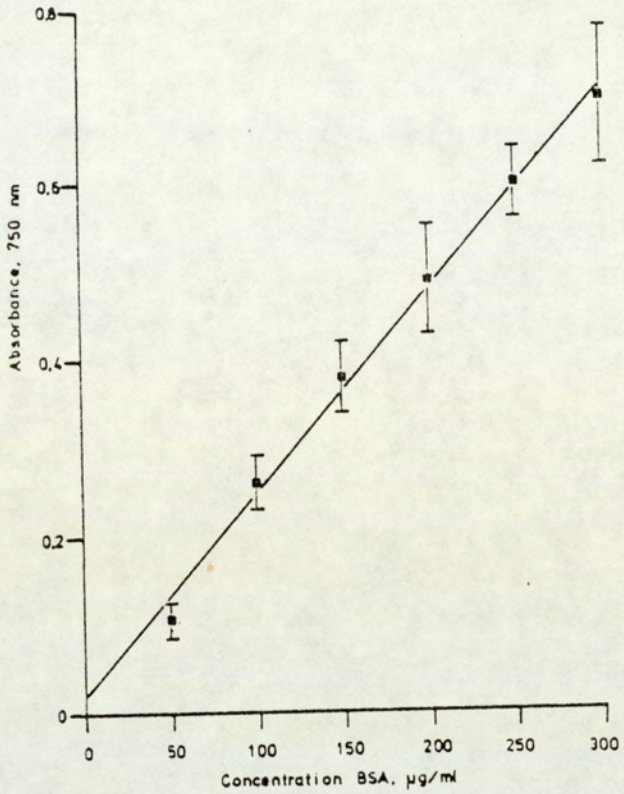


Fig. 3. Standard curve for the estimation of protein concentration.

The absorbance of various concentrations of bovine serum albumin (BSA) measured at 750 nm. Each value is the mean (± s.d.) of four determinations.

The sensitivity of the assay described in section (2.1) is determined by the rate of demethylation of substrate and the extinction coefficient of the formaldehyde chromagen formed in the Hantzsch reaction. This limitation in sensitivity usually necessitates the use of a microsomal concentration equivalent to about 250 mg liver in the incubation (Poland and Nebert, 1973). In the present investigation a microsomal concentration of about 200 mg liver was used in the incubation throughout the in-vitro studies.

Investigators have used between one and four mg protein per ml although less than 2 mg has been recommended (Fouts, 1970). A protein concentration of 1-2 mg per ml was used throughout this investigation.

The rate of N-demethylation of HMM was estimated at different enzyme concentrations to ensure linearity between the oxygenation rate of substrate (v) and the concentration of microsomal protein. The specific activity of the enzyme source (nmol product formed per mg protein per minute) has to be independent of microsomal concentration in order to ensure that the following conditions are met:

(1) The method being employed to follow the reaction rate truly reflects the velocity of the conversion of substrate to product, at all enzyme concentrations used.

(2) The concentration of the substrate and any required co-factor is greatly in excess of the enzyme concentration. (Zeffren and Hall, 1973).

3.1.3 Assay Conditions

3.1.3.1 Cofactors

The reaction mixtures consisted of 0.5 ml microsomal suspension and 0.1 ml of the following cofactors dissolved in Earle's medium:

NADP, (400µm); $MgCl_2$, (5mM); glucose - 6 - phosphate, (4mM); which generate 0.4mM NADPH. The glucose -6- phosphate solution also contained 15 units per ml glucose -6- phosphate dehydrogenase. The figures in parenthesis indicate the final concentration in the incubation mixture.

3.1.3.2 Substrate concentration

The experimental conditions were worked out under which the rate of demethylation of HMM and AP (nmol formaldehyde formed per mg protein) was linear with time. Initial velocity was then estimated on variation of substrate concentration. Substrate concentrations suggested for studies of this kind vary from $\frac{1}{3}$ - 3 times Km (Wilkinson, 1961) and 1/5 - 5 times Km (Cleland, 1967). The Km was approximated in the present study by plotting initial velocity against substrate concentration and estimating substrate concentration at one half the observed maximum velocity.

If only a short range of concentrations are used it may be difficult to detect the true shape of the kinetic curve (Lenk, 1976). The lower limits of substrate concentration were set by the sensitivity of the analytical assay. The upper limit of substrate concentration was dependent on the solubility of substrate in the incubation mixture. Because of the lipophilic nature of the melamines, their solubility in the

aqueous system was limited, and at concentrations above 5mM, problems in achieving an even distribution in the assay medium arose.

Substrate concentrations in the present study varied between 0.02 mM and 5 mM. N^2,N^2,N^4,N^4-Tetramethylmelamine (TMM) was dissolved in dimethylsulphoxide, and heptamethylmelamine and the glucose derivative of TMM were dissolved in distilled water. All other substrates were dissolved in acetone.

0.1 ml of the substrate solution was added to the reaction mixture which was then made up to a volume of 2.5 ml with Earle's medium. The reaction mixture was incubated at $37^{o}C$ with shaking at a constant speed, in open 25 ml glass beakers. The shaking rate of the incubator effects the even distribution of the substrate and a high shaking rate was used to improve the oxygen supply for the monooxygenase reaction.

3.1.4 Estimation of Formaldehyde by the Nash Colourimetric Assay

The reaction was initiated by addition of substrate and terminated after 20 minutes by addition of 0.5 ml 20% trichloracetic acid solution. The mixture containing trichloracetic acid was transferred to glass centrifuge tubes (15 ml) and spun at approximately 1500g for 10 minutes in a M.S.E. Minor or Haereus Christ Labofuge 6000 bench centrifuge to remove precipitated proteins.

Formaldehyde produced during the demethylation reaction was measured according to the method of Werringloer (1978) which was itself an adaptation of the method introduced by Nash (1953) and is based on the Hantzsch reaction. 2ml of the resultant supernatent was added to 1ml of Nash reagent and heated at 60^{o}C for 10 minutes. The chromaphore obtained was analysed for optical density in a Beckman Acta V or Cecil CE 5095 spectrophotometer at a wavelength of 412nm, and formaldehyde concentration was estimated by comparing the optical density against a formaldehyde standard curve.

Standard solutions were prepared by dissolving paraformaldehyde in 1M NaOH solution (1ml) and adjusting to a final volume of 100ml with distilled water. Formaldehyde solutions were prepared by dilution of standard solutions with Earle's medium, and calibration curves were constructed from absorbance values corrected using control values without added formaldehyde, using linear regression analysis, e.g. fig. (4).

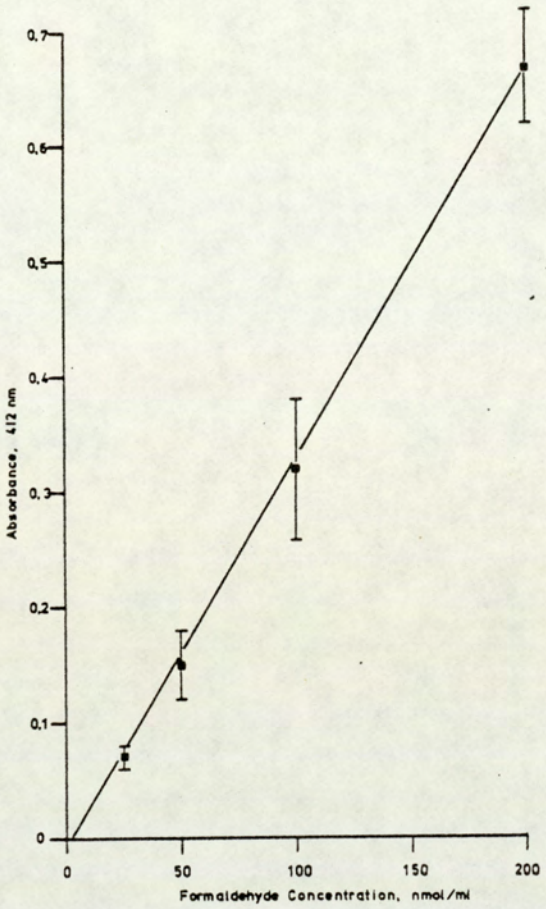


Fig. 4 Calibration curve for the estimation of formaldehyde. The absorbance of various formaldehyde solutions at 412 nm.

3.2 Estimation of Initial Velocity by Gas Chromatography.

The microsomes were prepared and the incubation was performed as described in sections (3.1 to 3.1.3.2).

3.2.1 Extraction of N-alkylmelamines from the Incubation Mixtures.

3.2.1.1 N-methylmelamines

The reaction mixtures were initiated by addition of 0.1ml of substrate and terminated with 0.5ml, 1M sodium hydroxide solution. Incubations treated in this way were added to Astell roll-bottles (15ml) which contained 12ml diethylether and were sealed with silicone stoppers and agitated by spinning on an Astell roll-bottle apparatus for 30 minutes at a temperature of approximately $4^{o}C$. 10ml of the ether fraction was decanted into glass Quickfit tubes (15ml) containing anhydrous magnesium sulphate and left for 60 minutes at $4^{o}C$ to remove any contaminating water in the solvent. 6ml aliquots of the dried ether was then evaporated using a Buchi rotor evaporator. 0.1ml distilled, dried acetone, containing the internal standard, was added to the remaining residue. The internal standards employed were HEM in the case of HMM and PMM, and HMM in the case of HEM.

3.2.1.2. HEM

The extraction was performed as described above (sectio 3.2.1.1) except that diethylether was replaced by ethylacetate.

3.2.2 Recovery of the N-alkylmelamines

25 to 100 µm of the substrate under examination was added to the incubation mixtures containing 0.5ml 1M NaOH and extracted as described in section (3.2.1). These samples were compared to standard solutions and the recovery of the

substrate from the incubation mixture was calculated.

3.2.3 Gas Chromatographic Conditions

N-alkylmelamines and their dealkylated metabolites were quantified from samples of incubation mixtures by a gas chromatographic assay similar to that described by D'Incalci et al. (1979).

Analysis was performed on a Pye-Unicam 204 gas chromatograph fitted with a flame ionization detector. The column used was a glass tube 2m long and of 3mm internal diameter packed with Chromosorb W-AWDMCS, 100-120 mesh and coated with 3% OV17. Columns were conditioned for at least 24 hours at 250^{o}C with a carrier gas flow rate of 40 ml/ minute. The operating conditions were as follows:

column oven temperature: 210^{o}C

injector port temperature: 250^{o}C

detector temperature: 250^{o}C

During analysis, nitrogen was used as the carrier gas at a flow rate of 30ml/minute. Air and hydrogen flow rates were 300 and 30ml per minute respectively.

3.3 Estimation of the Kinetic Constants

3.3.1 Plotting Methods

The kinetic parameters Km and Vmax can be defined as follows:

1) Vmax (maximum velocity constant), is the maximum velocity of the reaction which is approachable as the enzyme approaches saturation by the substrate, and it reflects the breakdown of the enzyme-substrate complex after insertion of oxygen.

2) Km (Michaelis constant), is the reciprocal affinity of the enzyme for the substrate in respect to a specific metabolic pathway of the substrate employed. It also describes the ease with which the enzyme-substrate complex is formed and the substrate concentration for which any observed oxygenation velocity is one half the maximum velocity.

Numerical values for the kinetic constants Km and Vmax were obtained from pairs of values of initial velocity and substrate concentration, by graphical methods. Three linear plotting methods derived from the Michaelis-Menten equation (1913) were used to estimate the kinetic constants:

1) Lineweaver-Burke; 1/v against 1/S (Lineweaver and Burke, 1934)
2) Hanes; S/v against S (Hanes,1932)
3) Hofstee; v against v/S (Hofstee, 1959)

Intercepts and slopes were calculated from linear regression analysis of each experiment using the experimental points on the linear portions of the curve obtained. The relationships between intercepts, slopes and kinetic constants are shown in fig.(5).

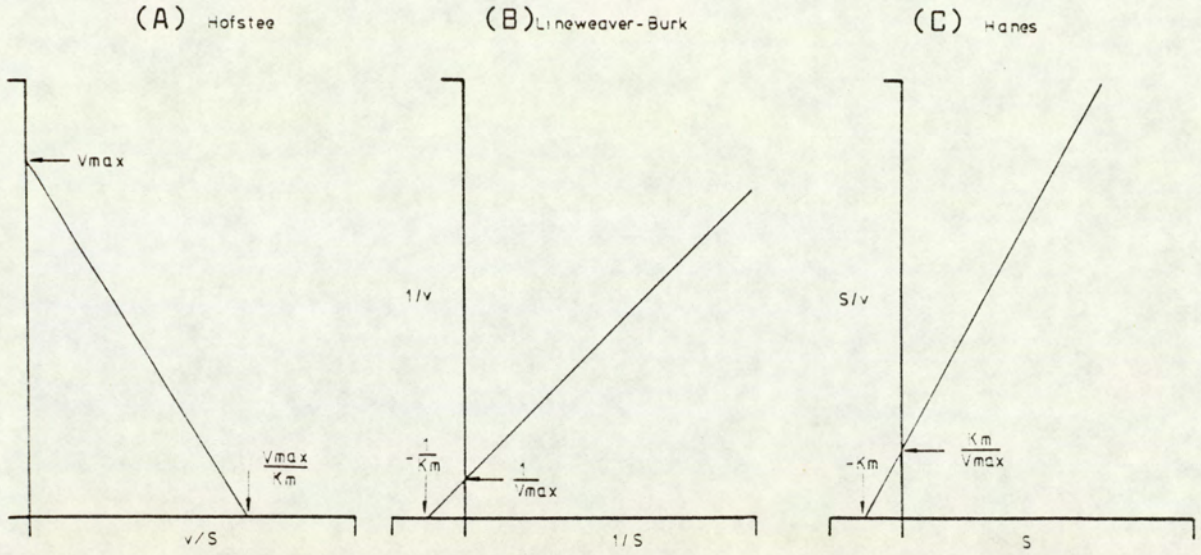


Fig. 5. Graphical methods for the determination of the kinetic constants, Km and Vmax. Each plot represents a linear transform of the Michaelis-Menten equation devised by (A) Hofstee (1959), (B) Lineweaver and Burke (1934) and (C) Hanes (1932). Initial velocity is represented by (v) and substrate concentration by (S).

The Lineweaver-Burke plot is the most commonly used, but it does possess inherent disadvantages which are discussed by Dowd and Riggs (1965). The main disadvantage is that experimental points may be concentrated towards the left hand side of the graph and values of initial velocity at low concentrations, which often cannot be determined with the required accuracy, have such an important influence on the shape of the curve. An attempt was made in the present investigation to reduce these errors by varying the concentration of substrate accordingly and putting more experimental weight on values for initial velocity at lower concentrations.

3.3.2 Validity of Kinetic Analysis

These in-vitro investigations were not aimed at measuring absolute values for the kinetic constants but were thought to reflect differences in the affinity of the metabolising enzymes for closely related substrates.

Measurement of formaldehyde formed from the demethylation reaction does not distinguish between different methyl moieties in reactions of xenobiotics with more then one methyl group. AP for example, can theoretically generate three moles of formaldehyde and therefore at least three compounds may serve as substrate for demethylation, each possessing distinguishable kinetic constants (Archakov et al., 1974).

Measuring the rate of disappearance of substrate also lacks sensitivity. The most sensitive method of measuring drug oxidations is by determining the rate of formation of oxygenated substrate (Lenk, 1976).

If this metabolite is unstable, e.g. HMPMM, the decomposition product, e.g. PMM, can be measured.

In the present investigation kinetic constants were obtained from the following methods and the values were compared:

1) measurement of total formaldehyde produced during the reaction by a spectrophotometric determination,
2) measurement of the disappearance of substrate and appearance of demethylated product by gas chromatography.

3.4 Product Inhibition

The following experiments were performed,in an attempt to assess the effect of the N-demethylated product on the metabolism of the parent substrate:

1) the effect of addition of PMM on the metabolism of HMM,
2) the effect of addition of TetraMM on the metabolism of PMM,
3) the effect of addition of TriMM on the metabolism of TetraMM.

Initial velocity measurements were performed by the GC assay according to the method described in section(2.1). The substrate concentrations in the incubation were 50 μM for all the methylmelamines employed.

3.5 Effect ofGSH-depleting Agents and Polar Solvents on the Metabolism of N-methyl-containing Xenobiotics.

3.5.1 Preparation of the 9000g supernatent

Livers were excised from male Cba/ca mice (20-25) and the supernatent obtained, after centrifuging at 9000g according to section (3.1.1) was used as the enzyme source.

3.5.2. Enzyme Concentration

A quantity of 9000g supernatent equivalent to 200mg liver was used for each in-vitro incubation.

3.5.3 Pretreatment of mice with GSH-depleting agents.

One hour before excision of the liver, mice were injected intraperitoneally (IP) with methyliodide (75mg/Kg) or diethylmaleate (0.7ml/Kg) suspended in arachis oil; or adriamycin (30mg/Kg) dissolved in 0.9% saline.

3.5.4 In-vitro Addition of Methyliodide.

100 μM methyliodide was added to the incubation mixture immediately before the addition of substrate.

3.5.5 Pretreatment of mice with Polar Solvents

16 hours prior to the excision of the liver, mice were injected IP with 0.1 ml acetone or dioxane (30mmole/Kg) in 0.9% saline.

3.5.6 In-vitro Addition of Polar Solvents

100μM acetone or dioxane was added to the incubation mixture immediately before the addition of substrate.

3.6 Comparison of Mean Data

Data was analysed using the two-tailed t-test for independent means, and comparisons were not considered significant when the two-tailed probability (p) was greater than 0.05.

SECTION 4

RESULTS

4.1 Properties of the Analytical Methods Used to Determine the N-demethylation of HMM.

In order to determine the enzymatic kinetic constants for HMM and its derivatives it was necessary to establish that the enzyme-catalysed reaction was linear with time. Fig. (6) shows that the rate of production of formaldehyde during the metabolism of 2mM HMM and 2mM AP was linear with time after 20 minutes.

It is also required that the rate of formaldehyde production is linear with microsomal protein concentration. Fig.(7) shows that the rate of formaldehyde produced during the N-demethylation of 2mM HMM was directly proportional to microsomal concentration up to 2.1 mg microsomal protein per ml.

Fig.(8) shows that the addition of microsomes to standard formaldehyde solutions did not significantly change the extent to which it reacted with Nash reagent.

The limits of sensitivity of the Nash assay for formaldehyde, as described by Werringloer (1978), see section (2.1.4), were found to be between 20-25 μM.

In addition to measuring the rate at which N-methyl compounds were metabolised by estimating the formation of the metabolite formaldehyde and its precursors, the disappearance of substrate and appearance of desmethyl metabolites was measured by GC. Calibration curves for HMM, PMM, HEM and TetraMM were constructed from standard solutions containing these agents. The calibration curves proved to be straight lines ($r > 0.99$), passing through the origin. Examples of

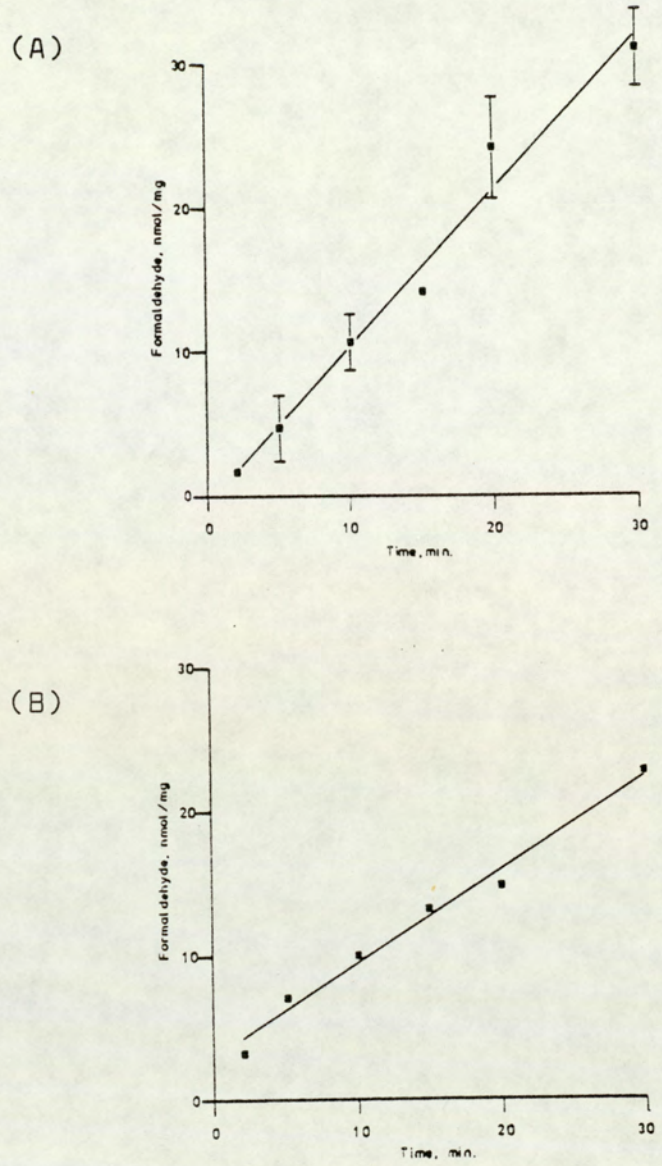


Fig. 6. Progress curves for the N-demethylation of 2mM HMM (A) and 2mM AP (B) incubated with mouse liver microsomes (1.5-2 mg/ml). The reaction was followed by estimating formaldehyde production using the colorimetric assay. Experimental points are means ($\pm$ s.d.) of at least five determinations, or in the case of values without error bars, the means of two determinations.

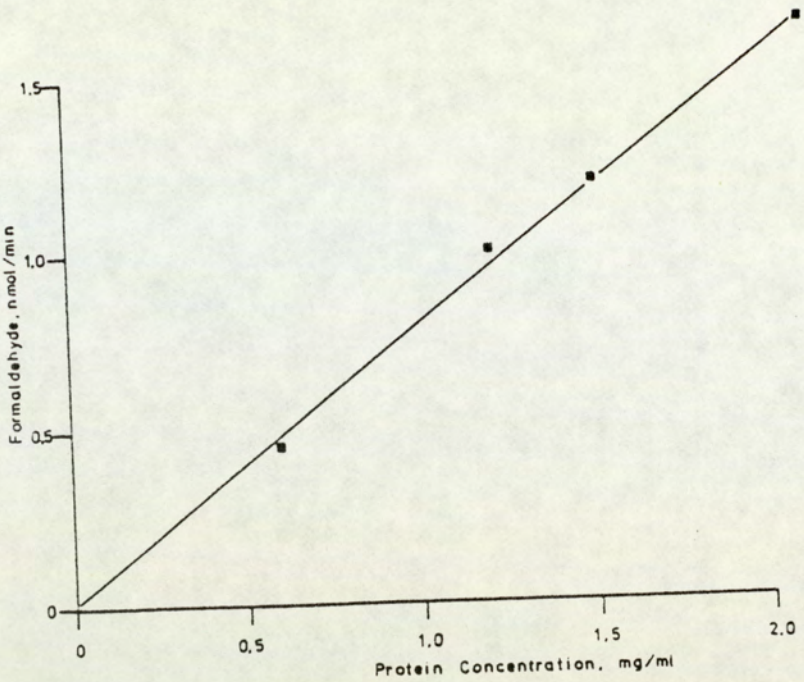


Fig. 7. Linearity of formaldehyde formation, from the N-demethylation of HMM, with the concentration of microsomal protein (0.6-2.1mg/ml). Incubations contained 2mM HMM and were terminated after 20 minutes. Each value is the mean of duplicate determinations from one experiment.

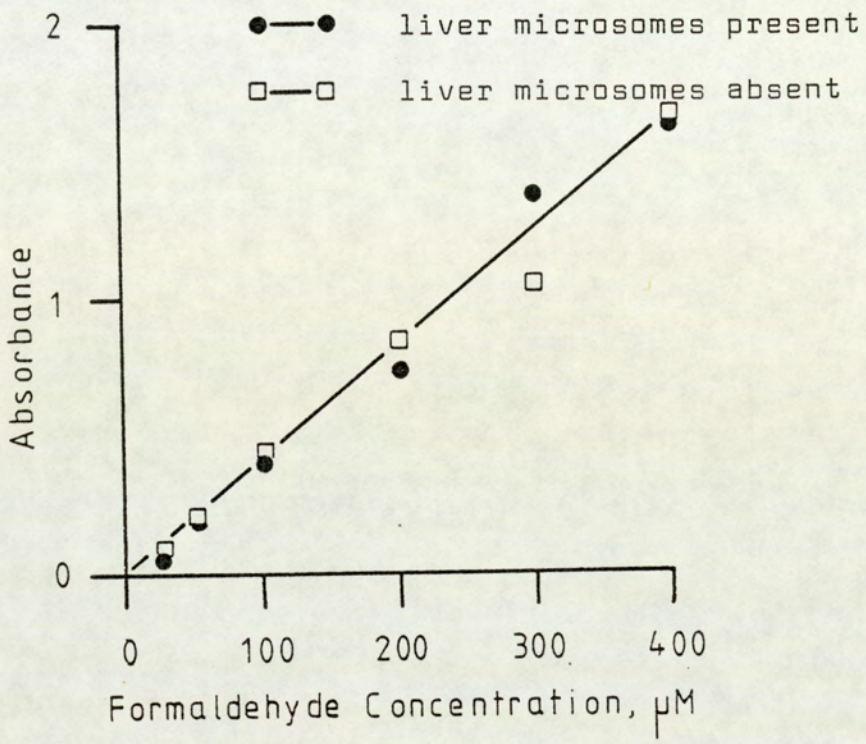


Fig. 8 The effect of addition of mouse liver microsomes (2mg protein/ml) on the absorbance of various formaldehyde concentrations. Absorbance was measured at 412nm in the presence (●—●) and absence (□—□) of liver microsomes. Each value is the mean of duplicate determinations from one experiment.

calibration curves of HMM and HEM from one experiment are shown in fig. (9).

Fig. (10) shows the chromatogram obtained after injection of a standard solution containing HMM, PMM, TetraMM TriMM and Hexaethylmelamine (HEM). Retention times were 2.1, 2.4, 2.7, 3.0 and 3.7 minutes respectively. Extracts from liver microsomal preparations, with and without the addition of HMM, PMM and HEM are shown in figs. (11A-B). The retention times of HMM and derivatives were identical to those of known samples shown in fig. (10).

After extraction of the N-alkylmelamines from incubation mixtures, the recoveries of the respective agents were HMM 90.8 $\pm$ 4.7% (n=9), PMM 89.4 $\pm$ 7.5% (n=19), TetraMM 89.5 $\pm$ 6.0% (n=10), HEM 76.6 $\pm$ 12.5% (n=5).

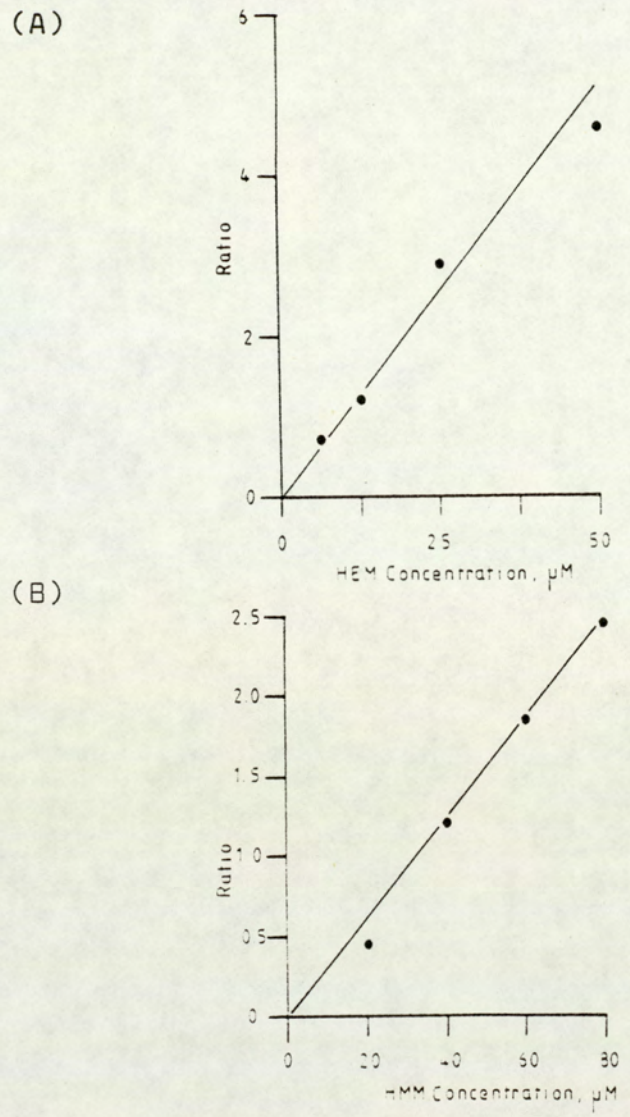


Fig. 9. The linearity of the GC detector response (peak height ratio) with various concentrations of HEM (A) and HMM (B). Each value is the mean of duplicate determinations from one experiment.

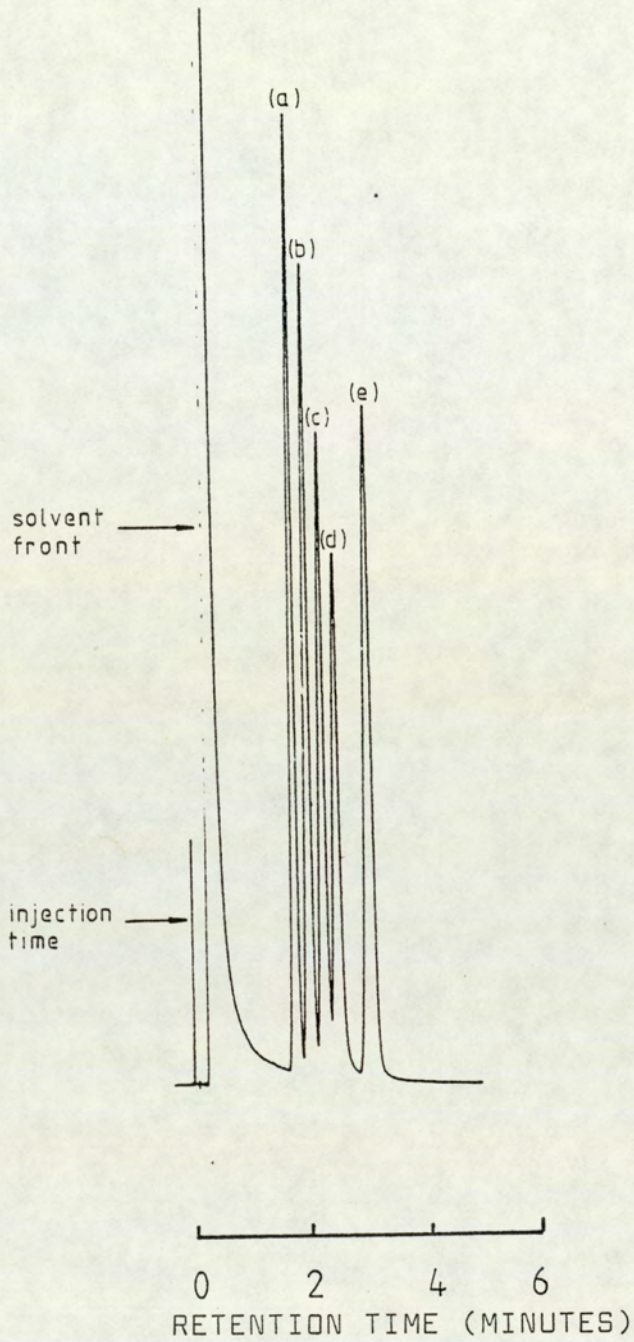


Fig. 10 Gas chromatogram of a standard solution of the N-alkylmelamines by flame ionization detection. HMM (a), PMM (b), TetraMM (c), TriMM (d) and HEM (e) were dissolved in acetone to give a concentration of 50µM.

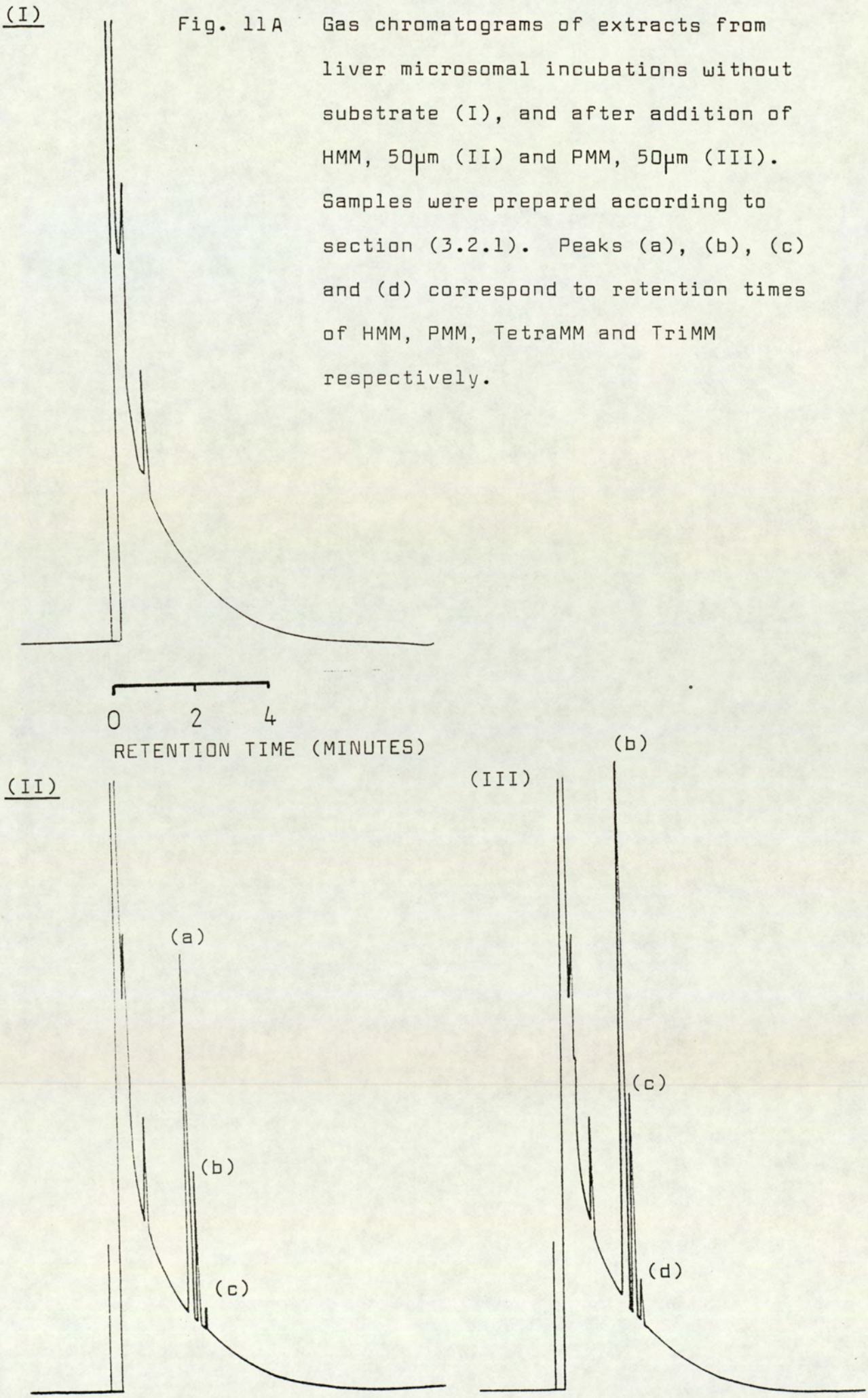


Fig. 11A Gas chromatograms of extracts from liver microsomal incubations without substrate (I), and after addition of HMM, 50µm (II) and PMM, 50µm (III). Samples were prepared according to section (3.2.1). Peaks (a), (b), (c) and (d) correspond to retention times of HMM, PMM, TetraMM and TriMM respectively.

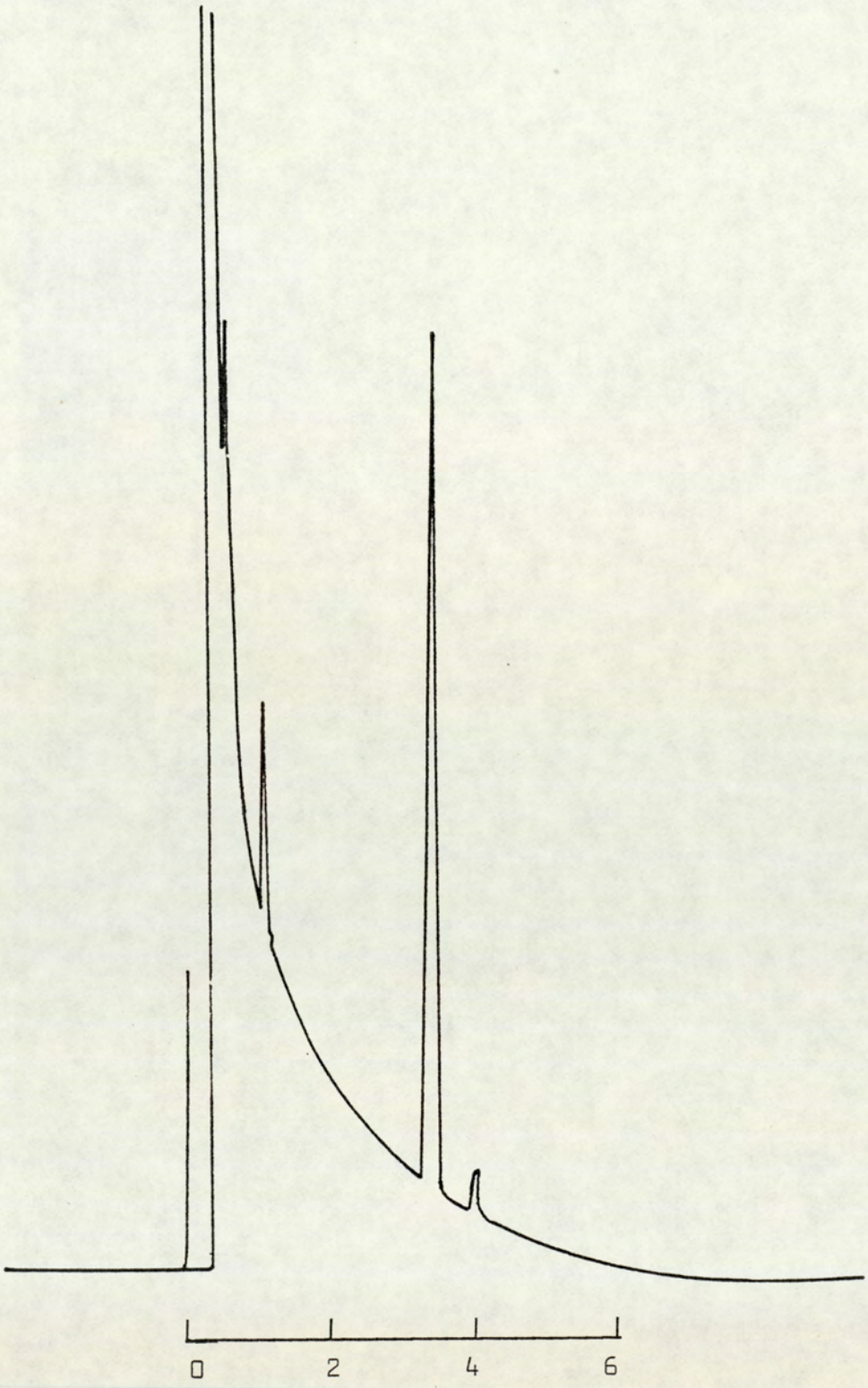


Fig. 11B Gas chromatogram of liver microsomal extract containing 50um HEM. The sample was prepared according to section (3.2.1). Peaks (a) and (b) correspond to retention times of HMM and PMM respectively.

4.2 Properties of the Enzymatic N-demethylation of HMM

4.2.1 Kinetic Parameters Characterising the N-demethylation of HMM and its Derivatives.

Figs.(12A-H)show the double-reciprocal plots obtained from the kinetic analysis of the enzymatic N-demethylation of HMM and its N-desmethyl metabolites; and for comparison, the plot for AP is also shown.

The Lineweaver-Burke plots obtained from the determination of formaldehyde produced during the demethylation reaction were curvilinear, concave-downwards; whereas those plots obtained from determining substrate disappearance or the appearance of desmethyl metabolite by GC were linear (figs. 12, A,B, G and H).

The linear portions of the double-reciprocal plots consisting of values for substrate concentrations above 50 μM, in the case of the formaldehyde determinations, were applied to compute apparent Michaelis constants. Two other plotting methods according to Hofstee (1959) and Hanes (1932) (described in section 2.3.1) were also employed to calculate the kinetic constants from both assays. A comparison of these different plotting methods is shown in fig.(13) using PMM as an example.

The kinetic parameters derived from the plots in fig.(13)are shown in table (1). The values obtained by the three different methods were not substantially different from each other.

The kinetic parameters determined from the colourimetric and GC assays are shown in tables (2) and (3) respectively where each individual constant is the mean of values computed by the three methods.

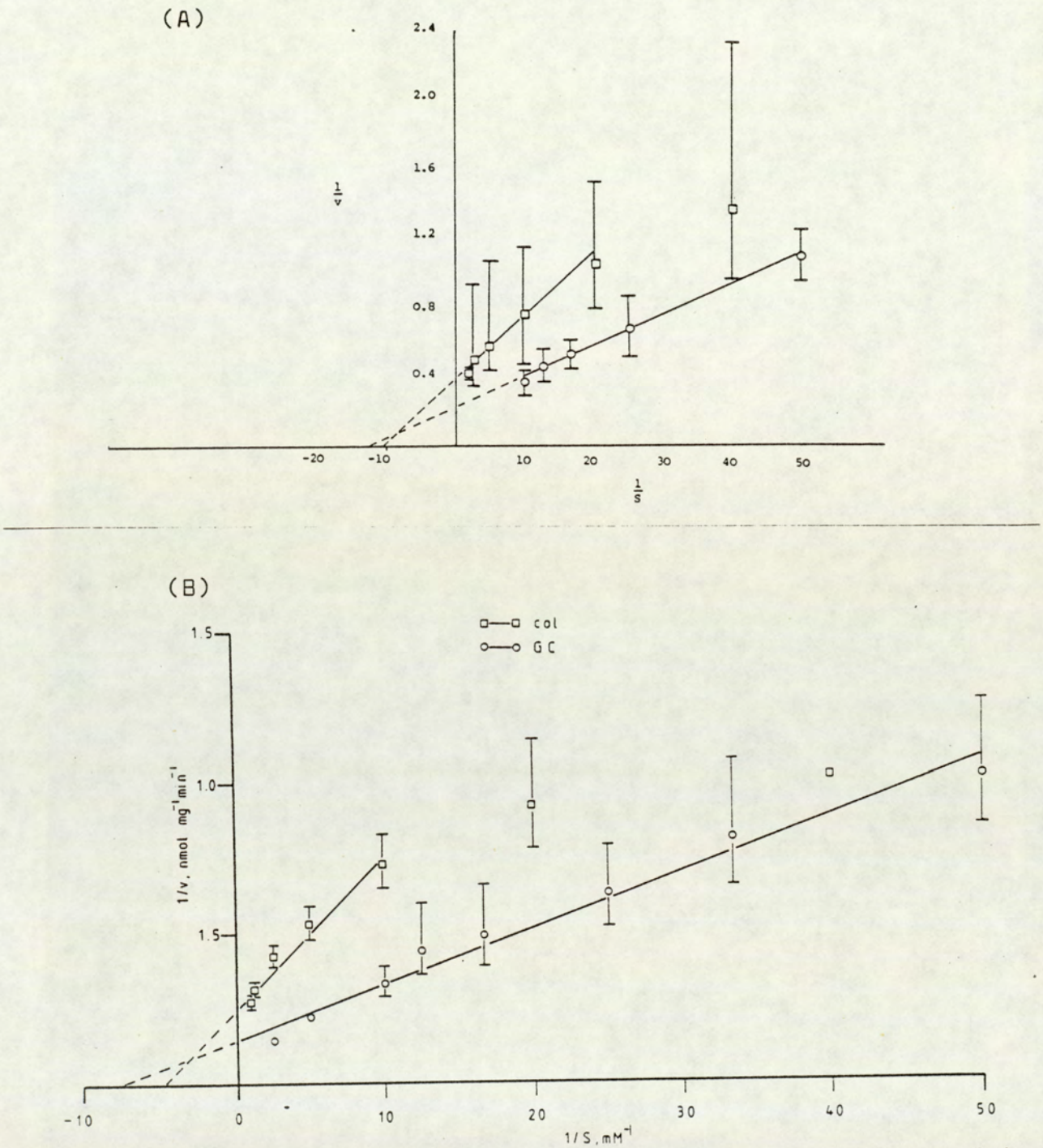


Fig. 12A-B Double-reciprocal plots for the metabolism of HMM (A) and PMM (B). Initial velocity (v) is expressed as nmoles of formaldehyde formed per minute per mg microsomal protein as determined by the colourimetric assay (□) and also as nmoles HMM or PMM metabolised per minute per mg microsomal protein (GC assay) (○). Experimental points are means (± s.d.) of at least four experiments or in the case of points without error bars, the means of two determinations.

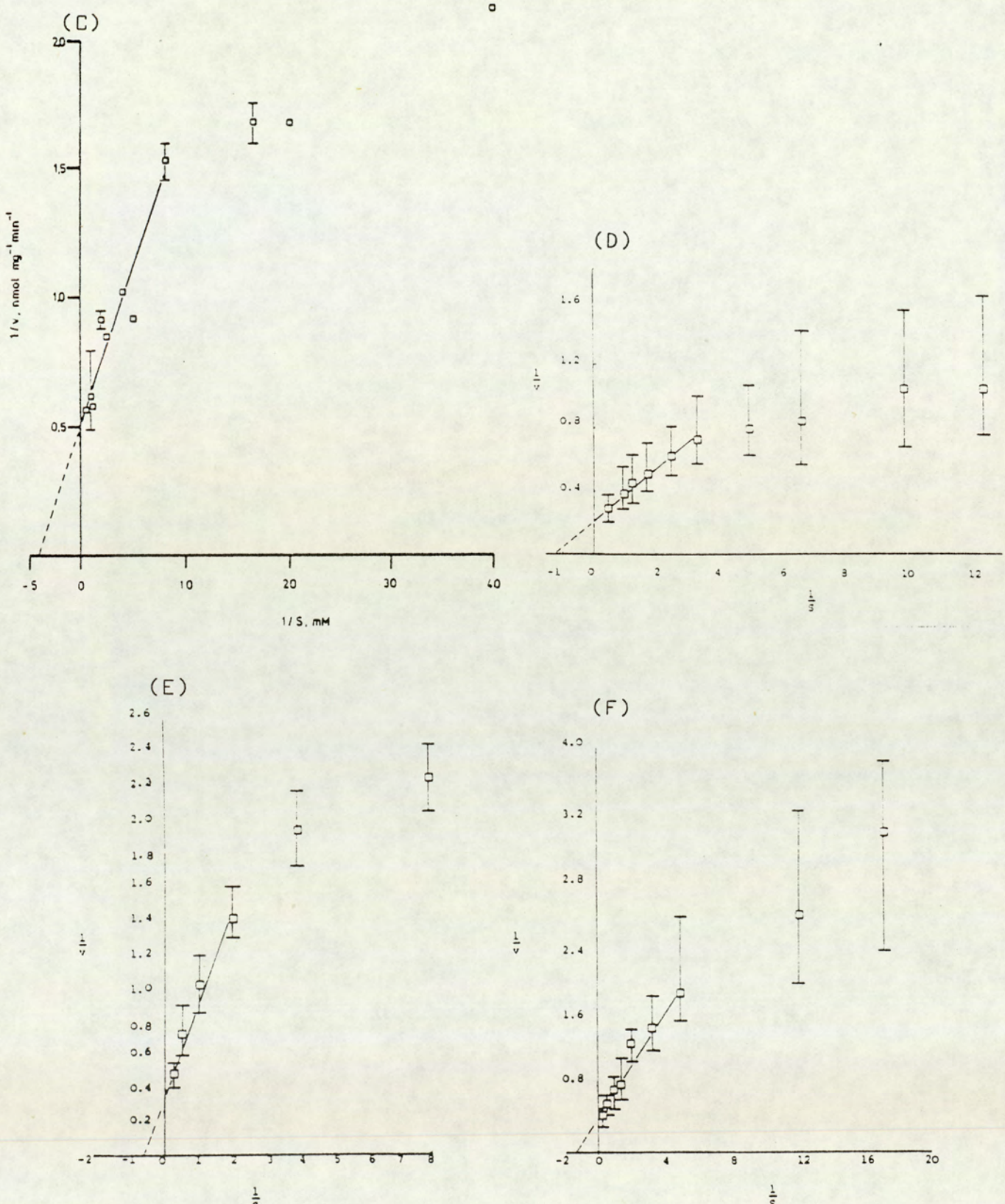
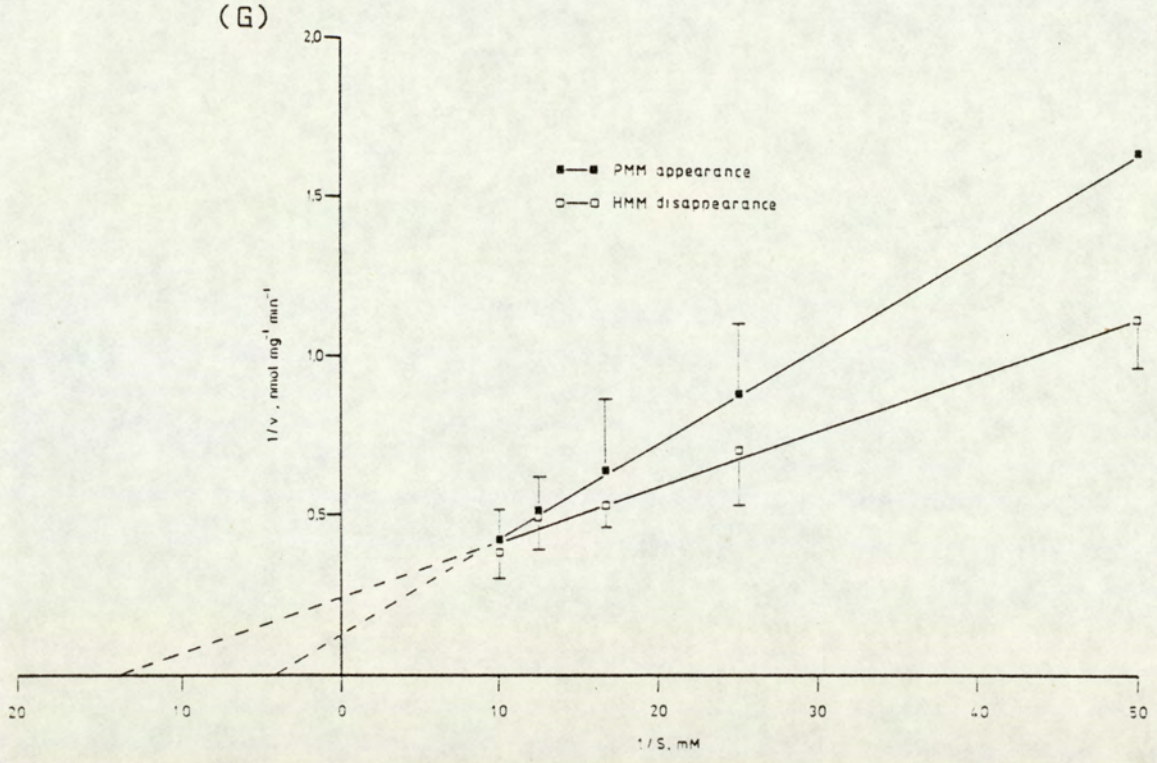


Fig. 12C-F Double-reciprocal plots for the metabolism of TMM (C), TetraMM (D), TriMM (E) and AP (F). Initial velocity (v) is expressed as nmoles per minute per mg microsomal protein as determined in the colourimetric assay. Experimental points are the means (± s.d.) of three to eight experiments or in the case of points without error bars, the means of two determinations.



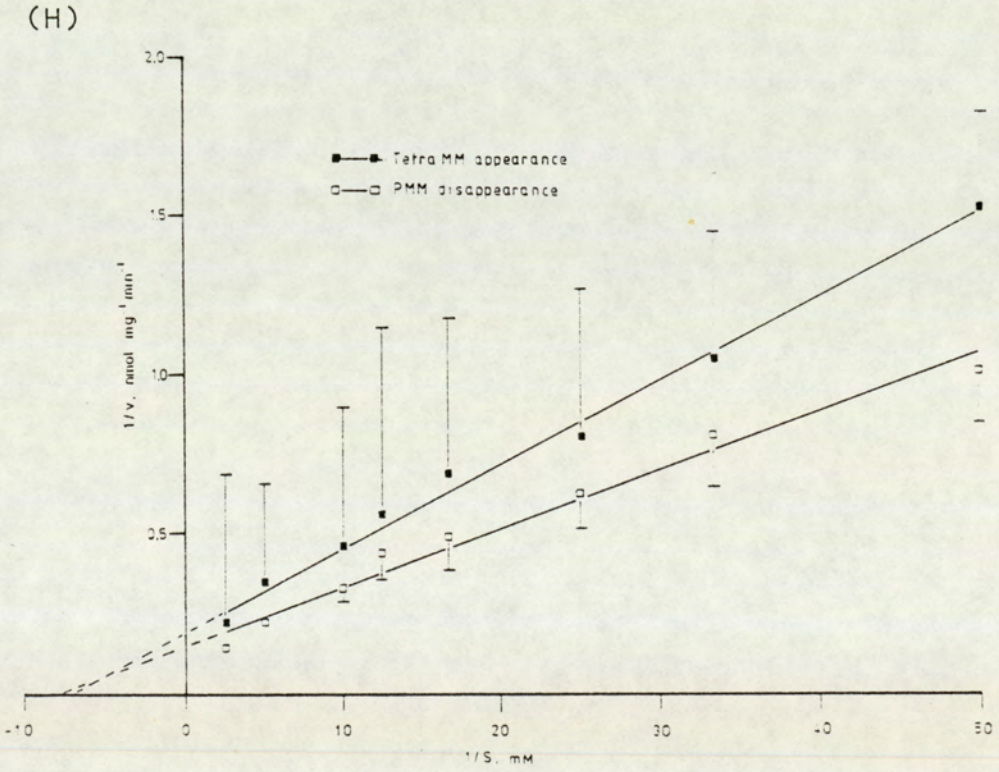


Fig.12(G-H)Double-reciprocal plots for the metabolism of HMM(G) and PMM (H) estimated by GC.(v) is expressed as nmoles demethylated product formed (■) or substrate metabolised (□) per minute per mg microsomal protein. Experimental points are means (± s.d.) of at least three experiments, or in the case of points without error bars, the means of two determinations.

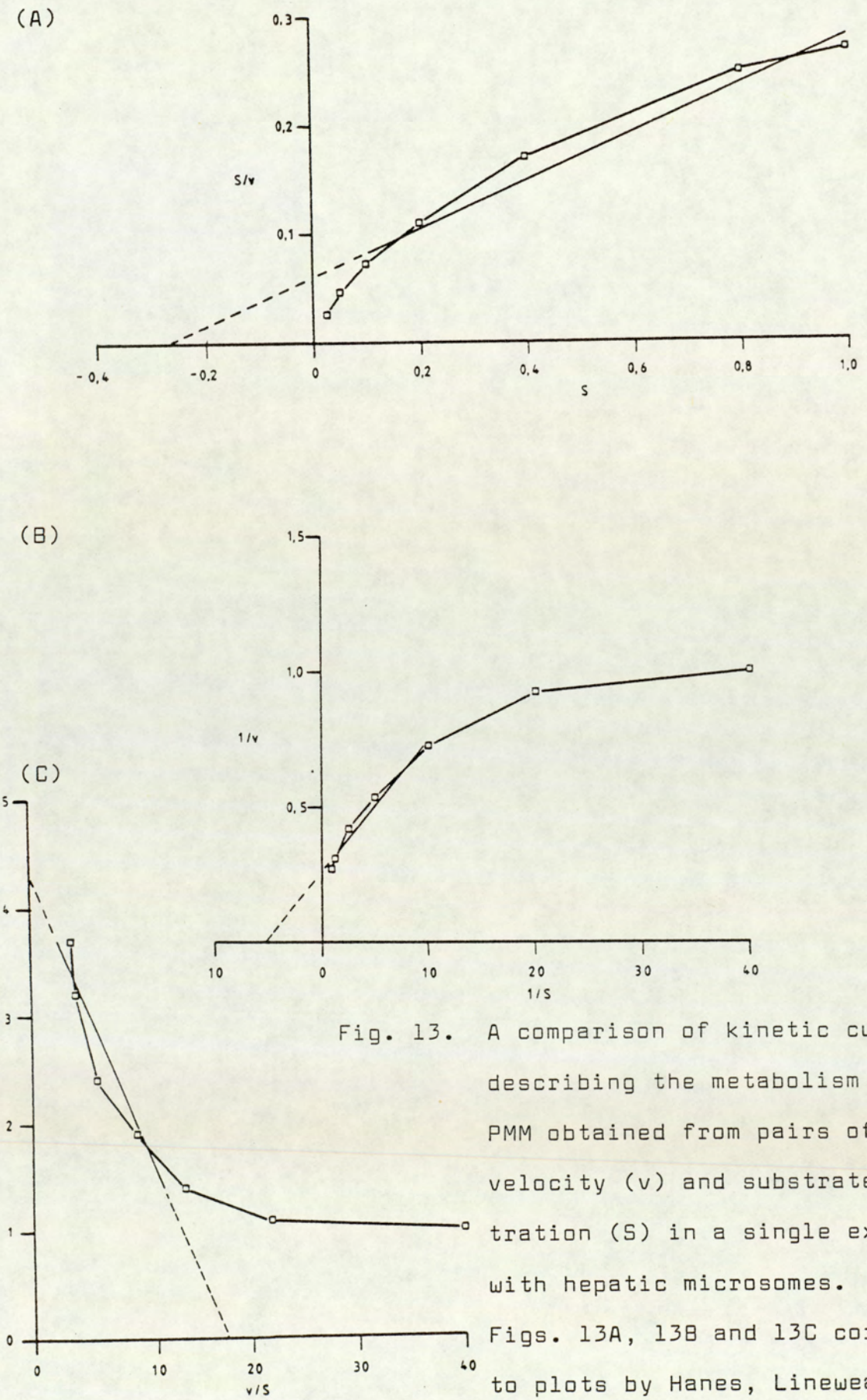


Fig. 13. A comparison of kinetic curves describing the metabolism of PMM obtained from pairs of initial velocity (v) and substrate concentration (S) in a single experiment with hepatic microsomes. Figs. 13A, 13B and 13C correspond to plots by Hanes, Lineweaver-Burk and Hofstee respectively. Initial velocity is expressed as nmoles HCHO formed per minute per mg microsomal protein.

TABLE 1

A Comparison of the Plotting Methods Used to Determine the Kinetic Parameters for PMM

Plotting Method	Kinetic Parameter	
	Km	Vmax
Hofstee (1959)	0.24	4.27
Lineweaver-Burk (1934)	0.19	3.87
Hanes (1932)	0.30	4.56

Values were obtained from one experiment

TABLE 2

Apparent K_m and V_{max} Values for the N-alkylmelamines and AP Determined by the Colourimetric Assay

Substrate	K_m (mM)	Vmax (nmol formaldehyde formed per mg microsomal protein per minute)	n
HMM	0.09 ± 0.01	2.6 ± 0.80	5
PMM	0.23 ± 0.08	4.2 ± 0.06	5
TetraMM	0.91 ± 0.07	5.1 ± 1.50	3
TMM	0.21 ± 0.03	1.8 ± 0.30	4
TRIMM	1.7 ± 0.6	2.9 ± 0.9	7
AP	0.80 ± 0.40	2.5 ± 0.07	8
HeptaMM	3.0	1.1	2
Glucose-TMM	NOT MEASURABLE		

n = number of experiments

TABLE 3

Apparent K_m and V_{max} Values for N-alkylmelamines Determined by the GC Assay

Substrate	K_m (mM)	V_{max} (nmol parent substrate metabolised or product appearance per mg microsomal protein per minute)	n
HMM			
HMM disappearance	0.08 ± 0.02	4.3 ± 0.09	4
PMM appearance	0.22 ± 0.07	6.5 ± 1.9	4
PMM			
PMM disappearance	0.13 ± 0.01	6.1 ± 1.5	4
TetraMM appearance	0.18 ± 0.15	6.4 ± 6.2	4
HEM			
HEM disappearance	0.15 ± 0.05	5.9 ± 1.5	4

n = number of experiments

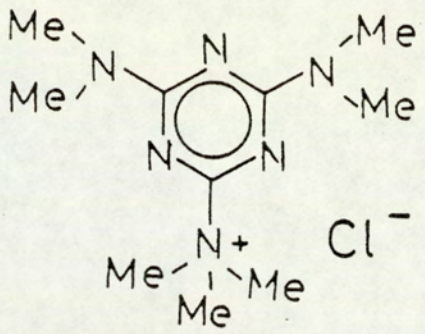
The apparent Vmax values obtained from the GC analysis (table 3) were consistently higher than those values calculated from the colourimetric assay (table 2) whereas the Km values obtained from the two methods were similar.

The apparent Km values for HMM and its N-desmethyl metabolites in table (2) show that a reduction in the number of N-methyl groups in the melamine molecule results in a decreased affinity of the enzymes for the substrates. The Km values for HMM and its N-demethylated metabolites were significantly different from each other ($p < 0.02$).

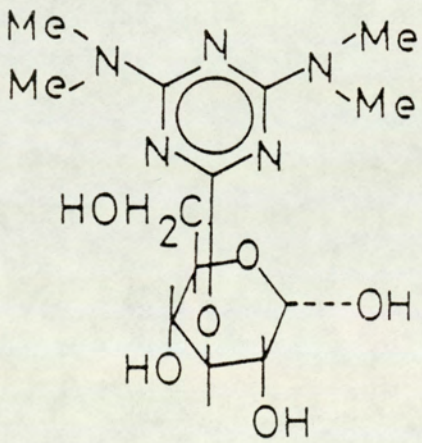
It is however worth noting that in the order HMM, PMM and TetraMM, the decrease in affinity of the mouse liver enzymes for the substrates is accompanied by an increase in maximal velocity (table 2).

HMM is only sparingly aqueous soluble (Cumber and Ross, 1977) making it unsuitable for parental administration. A search for analogues of HMM with enhanced water solubility led to the synthesis of HeptaMM (2,4-Bis(dimethylamino)-6-trimethyl-1,3,5-trazinium chloride) and a glucose derivative of TMM (3-O-[2,4-Bis(dimethylamino)-1,3,5-trizin-6-yl]-D-glucopyranose), see (fig.14).

An attempt was made to follow their in-vitro N-demethylation by estimating formaldehyde production and calculating their respective kinetic constants (table 2). The affinity of the demethylating enzyme for HeptaMM was considerably lower than that for the other melamine derivatives studied as shown by a comparison of Km values (table 2); and the Vmax value was the smallest in the series.



I



II

Fig. 14 Chemical structures of the water soluble melamine derivatives, HeptaMM (I) and 3-O-(2,4-Bis(dimethylamino)-1,3,5-triazin-6-yl)-D-glucopyranose (II).

Kinetic constants could not be calculated for the glucose derivative because even at high substrate concentrations (2mM), levels of formaldehyde produced during the incubation were near the detection limits of the assay.

It therefore appears that an increase in water solubility of a substrate results in a decreased affinity of the demethylating enzyme for that substrate in-vitro.

Replacing the N-methyl groups in the HMM molecule with N-ethyl moieties results in a loss of activity against the tumour models which are sensitive to HMM (see section 1.5). In order to see whether this difference may be explained by markedly different rates of metabolism, the N-dealkylation of HEM and HMM was determined by measuring substrate disappearance by GC and the kinetic parameters obtained are shown in table (3). Km and Vmax values for HEM were not significantly different from the constants for HMM ($p < 0.07$).

4.2.2 Inhibition of N-alkylmelamine Dealkylation by Substrate and Metabolites.

A plot of initial velocity of metabolic formaldehyde generation against substrate concentration (fig. 15) revealed that HMM appeared to inhibit its own metabolism in-vitro at substrate concentrations above 0.5 mM. This inhibitory effect at high HMM substrate concentrations was also shown when substrate disappearance was determined by GC analysis (fig.16). However AP and the N-demethylated metabolites of HMM did not appear to possess this ability to inhibit their own metabolism (fig.15).

This inhibitory effect may also be due to N-demethylated metabolites interfering with the enzymatic demethylation of the parent substrate. In order to verify this hypothesis, the effect of PMM, TetraMM and TriMM on the in-vitro demethylation of HMM, PMM and TetraMM respectively was determined by measuring the disappearance of substrate by GC with and without addition of demethylated metabolite. Fig.(17) shows that PMM inhibited the demethylation of HMM by 54% ($p < 0.005$) and TriMM inhibited the metabolism of TetraMM by 44% ($p < 0.04$). TetraMM seemed to inhibit PMM metabolism but this was not significant. (All the above concentrations were 50µM).

The inhibition of the metabolism of HMM may thus be due partly to the influence of N-demethylated metabolites formed during the in-vitro metabolism of HMM.

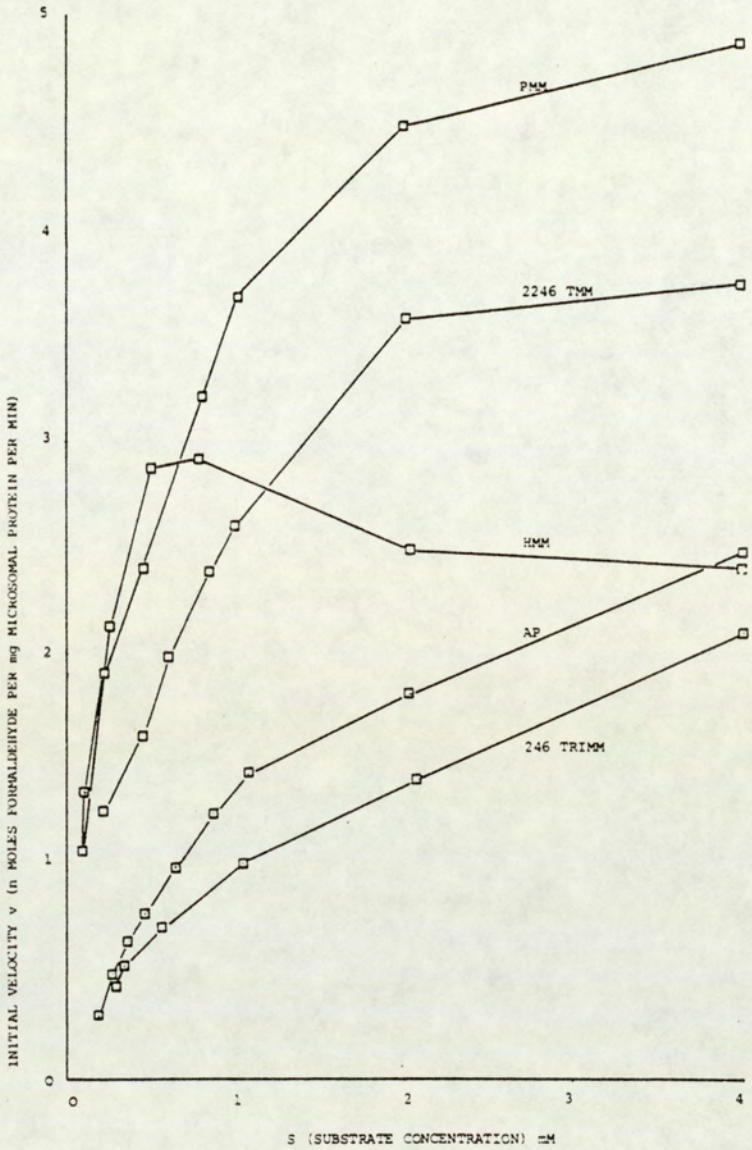


Fig. 15 In-vitro metabolism of N-methylmelamines and AP. Initial velocities (v) were determined by the colourimetric assay and are plotted against substrate concentration (S). Experimental points are the means of two to eight determinations.

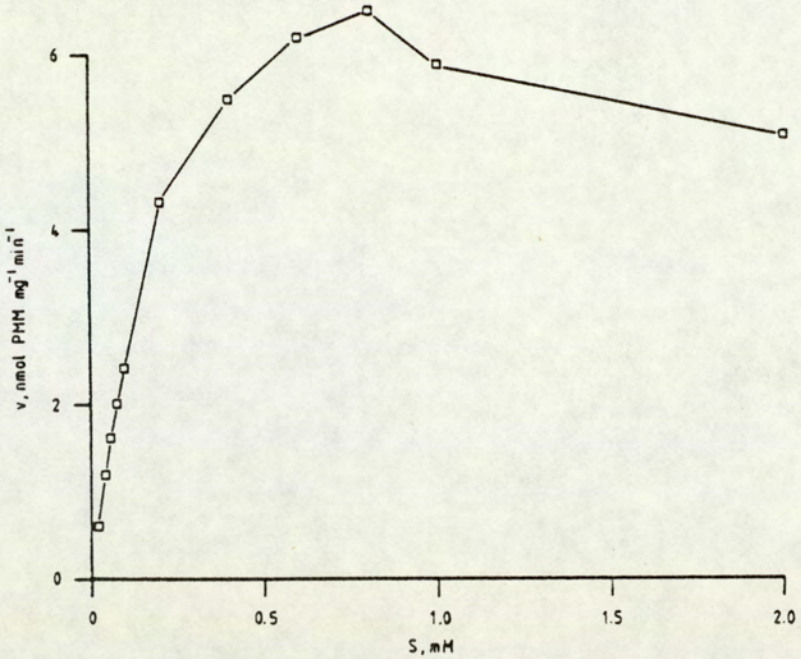


Fig. 16 Inhibition of the <u>in-vitro</u> metabolism of HMM at substrate concentrations above 0.5 mM. The appearance of the N-demethylated metabolite (PMM) was estimated by GC. Initial velocities (v) are plotted against substrate concentration (S). Experimental points are the means of three determinations.

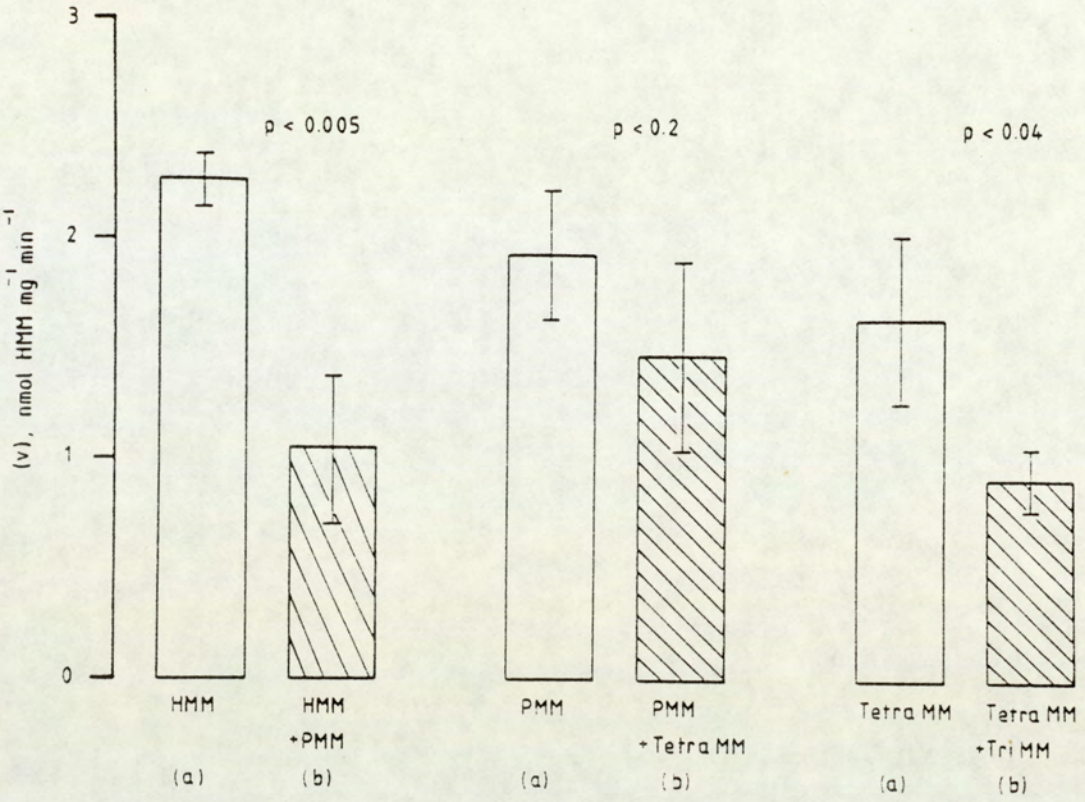


Fig. 17 The effect of addition of desmethyl derivatives on the <u>in-vitro</u> metabolism of HMM, PMM and TetraMM in liver microsomal preparations. (a) (open bars) indicate control values and (b) (hatched bars) indicate values obtained after addition of the second substrate. Initial velocity values (v) were determined by measuring substrate disappearance by GC and represent the means ($\pm$ s.d.) of three experiments. All substrate concentrations were 50μM.

4.2.3 Effect of Agents which Deplete the Liver of GSH, on the N-demethylation of HMM and Other N-methyl-Containing Xenobiotics

It is probable that metabolism renders the HMM molecule an antineoplastic species (see section 1.5), and there may be a specific difference between the N-demethylation of cytotoxic N-methyl-containing agents as compared to the N-demethylation of non-cytoxic N-methyl-containing xenobiotics such as AP.

As part of an effort to test the validity of this hypothesis, the effect of the depletion of hepatic GSH on the metabolism of various N-methyl-containing compounds was investigated (see section 1.5).

Fig.(18) shows that the administration of 75mg/Kg methyliodide 1 hour before the animals were killed and the livers excised, led to a significant decrease in the metabolic generation of formaldehyde from HMM in-vitro. The N-demethylation of HMM using a 9000g liver preparation was reduced to 32% when livers from methyliodide pretreated mice were used as the enzyme source. The N-demethylation of PMM, and carboxymethylphenyldimethyltriazene (CMPDT) was also inhibited, by 36 and 33% respectively (fig.19).

The in-vitro metabolism of dimethylaniline (fig.19) and AP (fig.18) was however not significantly changed after methyliodide pretreatment nor was this inhibitory effect observed when HMM, PMM and AP were metabolised using isolated liver microsomes (fig.20).

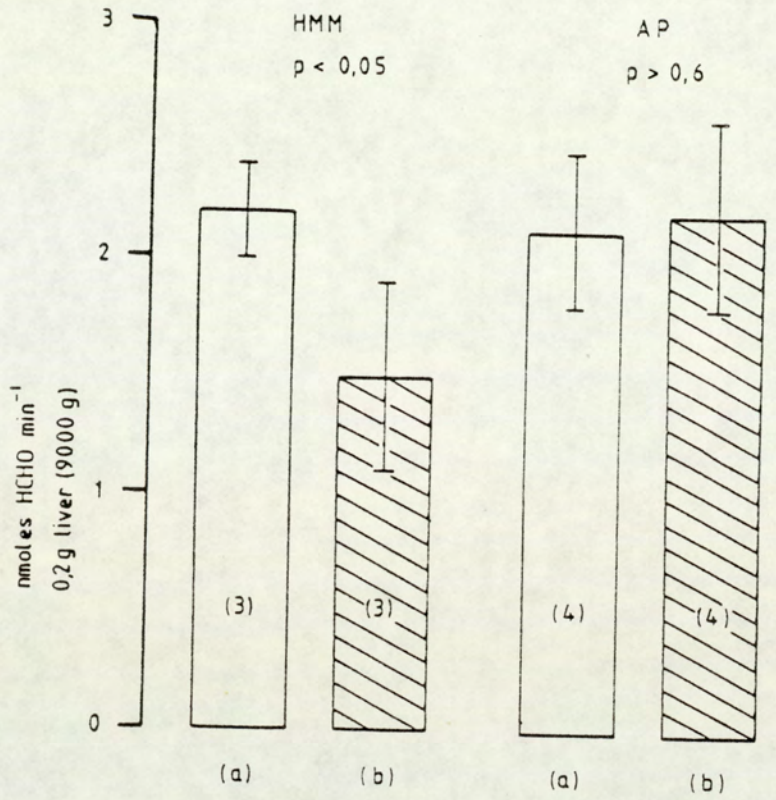


Fig. 18 Effect of administration of methyliodide on the in-vitro metabolism of HMM and AP. (a) (open bars) indicate control values and (b) (hatched bars) indicate values obtained with livers from treated animals. Numbers in brackets represent number of experiments. Colourimetric determination of formaldehyde as a metabolite of HMM and AP was obtained with 9,000g liver supernatent preparations. Only the values of HMM are significantly different from controls.

The concentration of HMM and AP in the 9000g incubation mixture was 2mM and 4mM respectively.

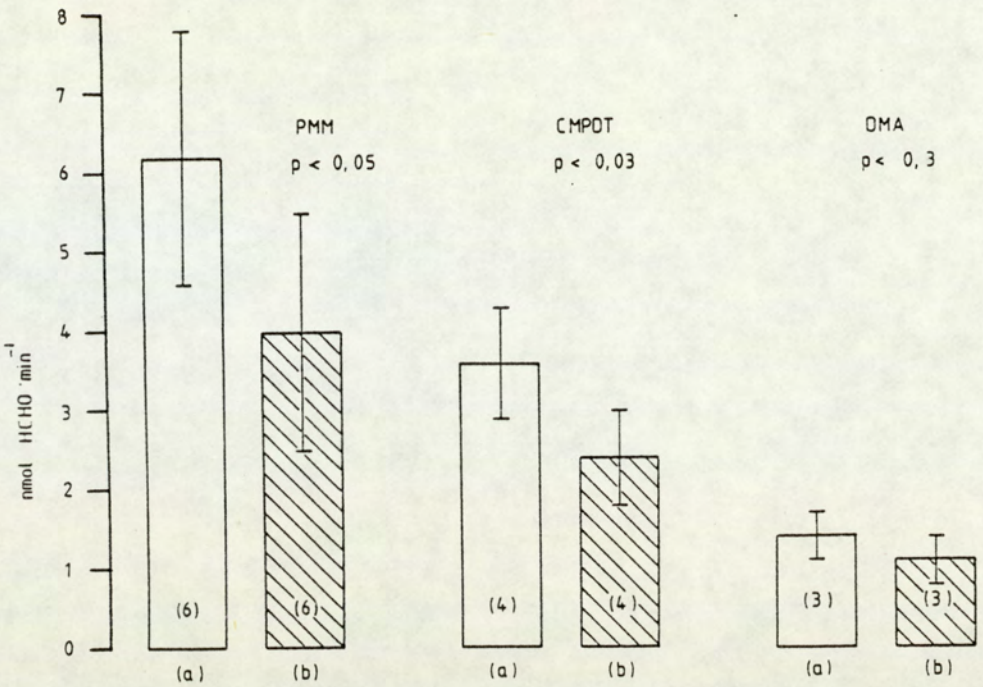


Fig. 19 The effect of administration of methyliodide on the in-vitro metabolism of PMM, 3-(4-Carboxymethylphenyl) -1,1-dimethyltriazene (CMPDT) and dimethylaniline (DMA). (a) (open bars) indicates control values and (b) (hatched bars) values obtained with livers from treated animals. Numbers in brackets represent number of experiments. Colourimetric determination of formaldehyde as a metabolite of PMM, CMPDT and DMA was obtained with 9,000g liver supernatent preparations.

The concentration of PMM, CMPDT and DMA in the 9000g incubation mixture was 0.5mM, 1mM and 2mM respectively.

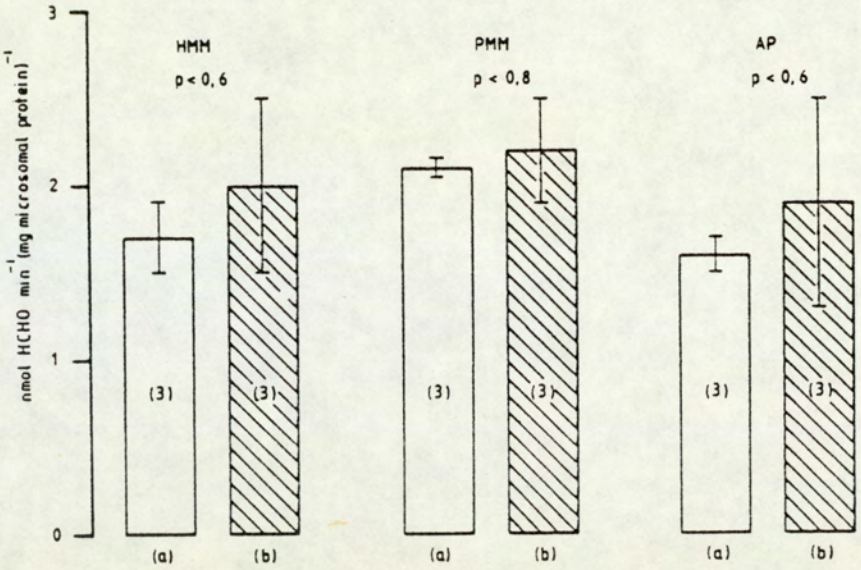


Fig. 20 The effect of administration of methyliodide on the in-vitro metabolism of HMM, PMM and AP. (a) (open bars) indicates control values and (b) (hatched bars) values obtained with livers from treated animals. Colourimetric determination of formaldehyde as a metabolite of HMM, PMM and AP was obtained with liver microsomes. Numbers in brackets represent the number of experiments. The concentration of HMM, PMM and AP in the microsomal metabolising mixture was 2mM, 0.5mM and 4mM respectively.

Adriamycin (Doroshaw et al., 1979) and diethylmaleate (Levine and Finkelstein, 1979) also deplete hepatic GSH pools (see section 1.5); but fig.(21) shows that the rate of N-demethylation of HMM, PMM and AP was not appreciably altered when a 9000g liver preparation from mice pretreated with these GSH-depleting agents was used, and compared with liver preparations derived from mice pretreated with physiological saline.

In order to ascertain that the inhibitory effect of methyliodide on the N-demethylation of HMM was due to the GSH depletion and was not a direct effect of methyliodide, the influence of the addition of methyliodide to the HMM metabolism mixture was studied.

On addition to microsomal incubation mixtures containing 50μM HMM, 100μM methyliodide decreased the rate of the in-vitro metabolism by 36% (fig.22).

It therefore appears likely that the inhibitory effect on N-demethylation by methyliodide is not mediated via GSH. If GSH was capable of modulating the rate of HMM metabolism, its addition to microsomal incubations should have an effect which is contrary to the effect of depleting hepatic GSH levels on the metabolism in 9000g liver fractions. Fig.(23) shows the effect of the addition of different concentrations of GSH to microsomal incubations containing 2mM HMM or AP. After addition of 10 mM GSH, AP demethylation was increased by 31% ($p < 0.02$), whereas HMM metabolism was not significantly changed.

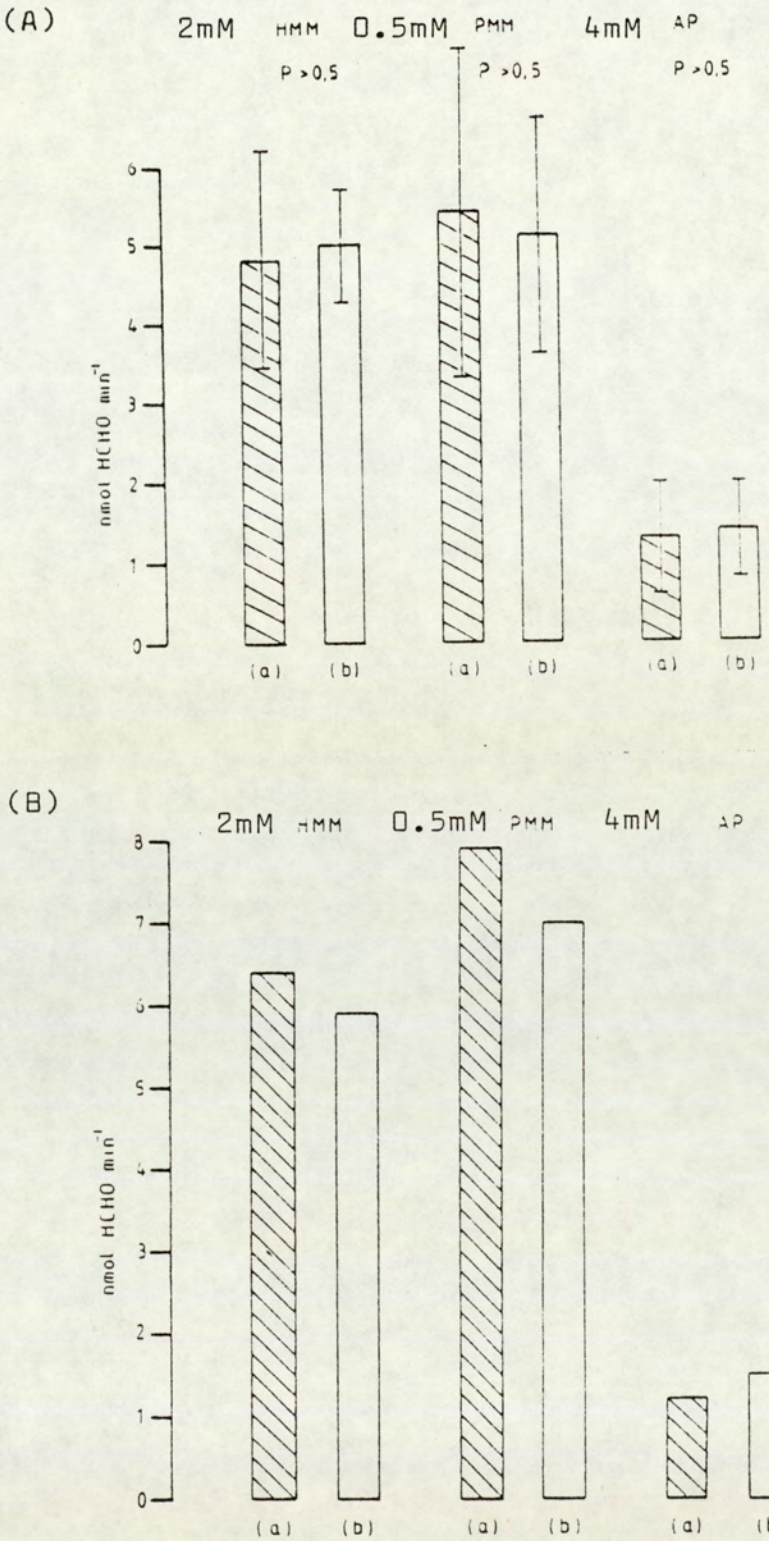


Fig. 21 The effect of administration of adriamycin (A) and diethylmaleate (B) on the in-vitro metabolism of HMM, PMM and AP. (a) (hatched bars) indicate control values and (b) (open bars) values obtained from treated animals. Colourimetric determination of formaldehyde as a metabolite of HMM, PMM and AP was obtained with 9000g supernatent preparations. Values are the mean ($\pm$ s.d.) of three determinations or in the case of values without error bars, the mean of two determinations.

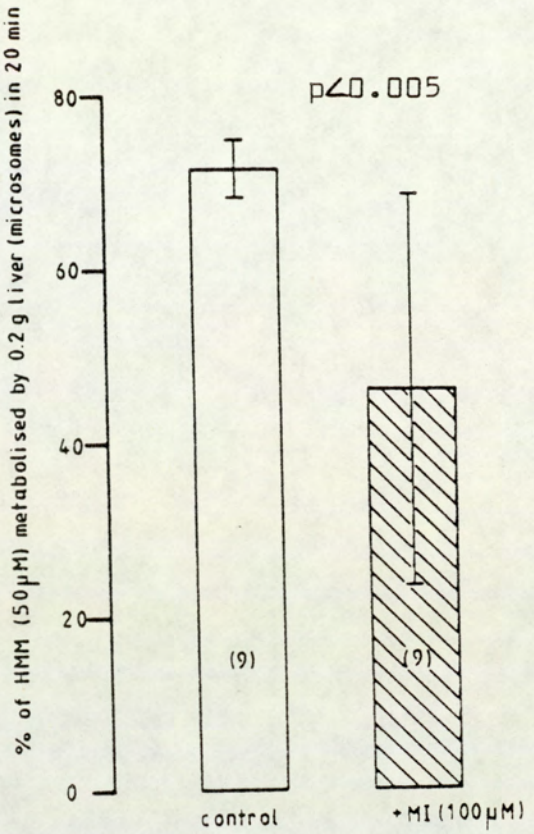


Fig. 22 The effect of addition of methyliodide (100μM) on the <u>in-vitro</u> metabolism of HMM. The open bar indicates control values and the hatched bar values obtained from hepatic microsomal preparations where methyliodide was added. The disappearance of HMM was estimated by GC. Numbers in parenthesis represent the number of experiments.
The concentration of HMM in the microsomal metabolising incubation was 50μM.

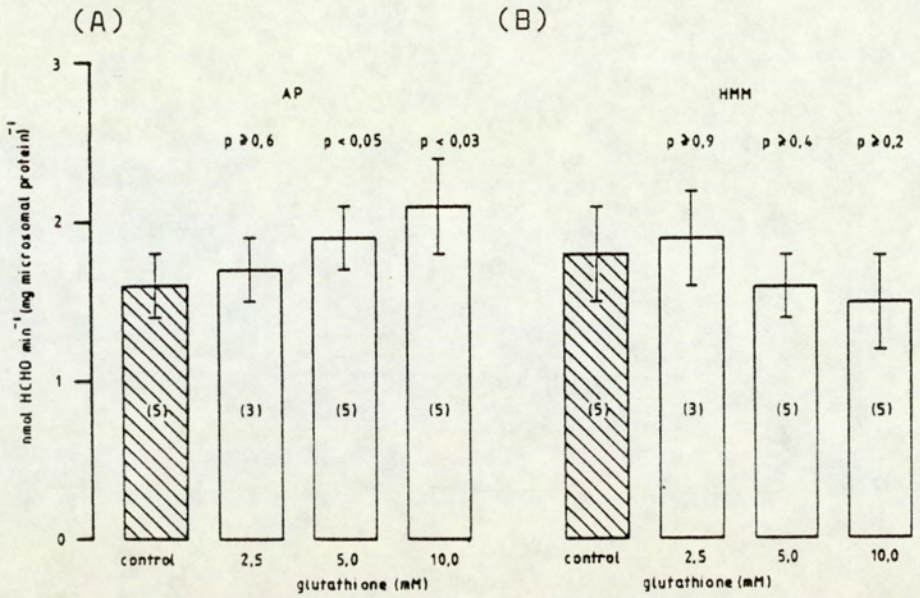


Fig. 23 The effect of addition of GSH on the in-vitro metabolism of AP (A) and HMM (B). Hatched bars indicate control values and open bars values obtained from microsomal preparations where various concentrations of GSH were added. Colourimetric determination of formaldehyde as a metabolite of HMM and AP was performed with liver microsomes. Values in parenthesis represents the number of experiments.

The concentration of both HMM and AP in the microsomal metabolising incubations was 2mM.

4.2.4 Effects of Polar Solvents.

The influence of the organic solvents dioxane and acetone on the metabolism of HMM and AP was investigated to further characterise differences in the metabolism of these N-methyl-containing compounds (see section 1.5). The N-demethylation of HMM, PMM and AP was compared using a liver microsomal metabolising system obtained from mice pretreated with dioxane and acetone and as a control experiment, saline (fig.24).

The metabolism of these compounds was also investigated after addition of the polar solvents to metabolism mixtures where liver microsomes were obtained from untreated mice (fig.25). Fig.(25) revealed no considerable difference in the in-vitro metabolism of the N-methyl-containing compounds under investigation. However, the demethylation of the substrates was enhanced, most markedly for HMM, when microsomes were used from mice pretreated with dioxane and acetone, (fig.24).

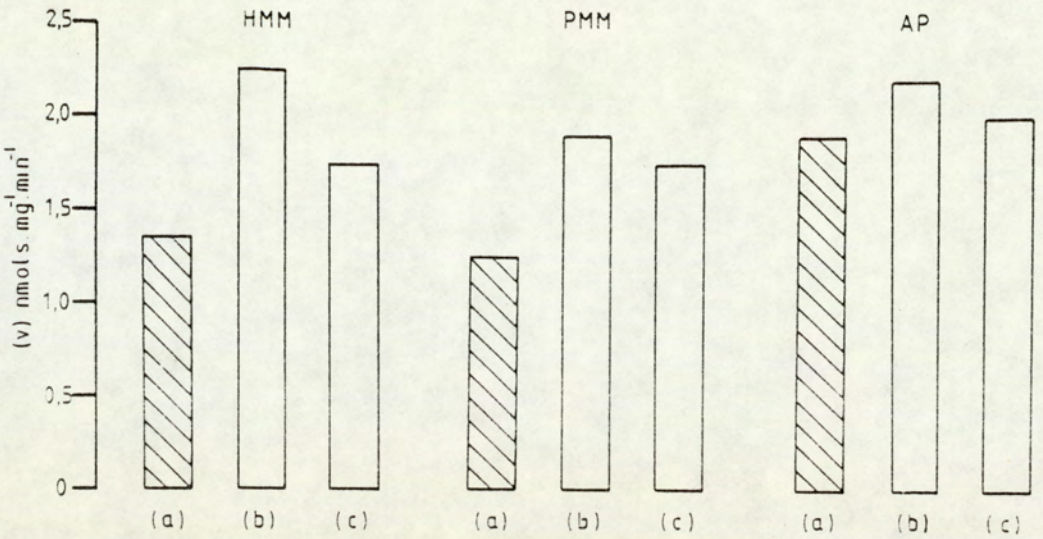


Fig. 24 The effect of administration of polar solvents on the in-vitro metabolism of HMM, PMM and AP. (a) (hatched bars) indicates control values and (b and c) (open bars) indicates values obtained with livers from animals treated with dioxane and acetone respectively. Colourimetric determination of formaldehyde as a metabolite of HMM, PMM and AP was performed with liver microsomes. Values are the means of two experiments.
The concentration of HMM, PMM and AP in the microsomal metabolising incubation was 2mM, 0.5mM and 4mM respectively.

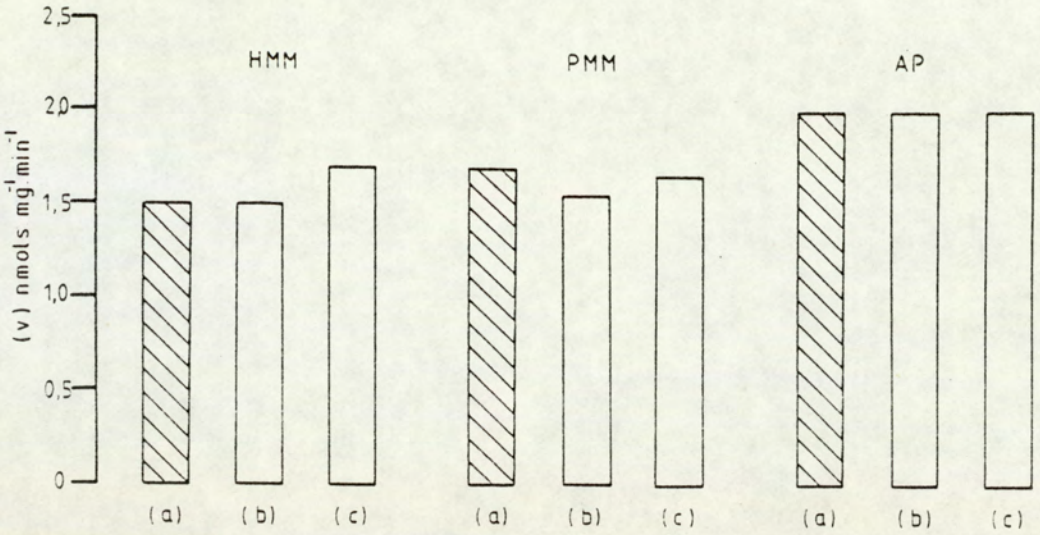


Fig. 25 The effect of addition of polar solvents on the <u>in-vitro</u> metabolism of HMM, PMM and AP. (a) (hatched bars) indicate control values and open bars indicate values obtained from liver preparations where either dioxane (b) or acetone (c) was added. Colourimetric determination of formaldehyde as a metabolite of HMM, PMM and AP was performed with liver microsomes. Values are the means of two experiments.

The concentration of HMM, PMM and AP in the microsomal metabolising incubation was 2mM, 0.5mM and 4mM respectively.

SECTION 5

DISCUSSION

5.1 Factors Influencing the Shape of the Double-Reciprocal Plots

The double reciprocal plots obtained from the kinetic analysis of the metabolism of HMM, its N-demethylated metabolites and AP are curvilinear,concave downwards. This shape has also been reported for other oxidative N-demethylations,e.g. the Lineweaver-Burk plots for the demethylation of AP (Pederson and Aust, 1970; Poland and Nebert, 1972), and ethylmorphine (Hayes, et al., 1973) by rat liver microsomes were shown to be non-linear and revealed two distinct phases from which different Km values were calculated.

In general, a linear double-reciprocal plot is obtained if the enzyme substrate interaction obeys Michaelis-Menten kinetics (Segal, 1975). However, the Michaelis-Menten theory of enzyme kinetics considers only one substrate interacting with one enzyme to form the enzyme-substrate complex (Michaelis and Menten, 1913); and it is most probable that some enzyme-catalysed reactions involve more than one participating enzyme.

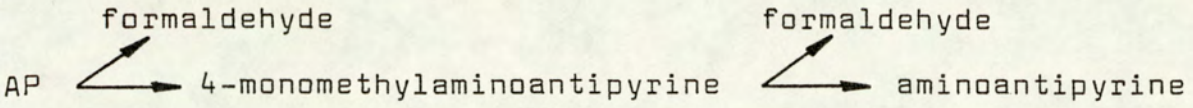
Therefore, one possible reason for obtaining non-linear double-reciprocal plots is the presence of multiple enzymes in the liver preparation which catalyse the demethylation of the substrate. Different forms of cytochrome P-450 are known to be present in the liver microsomes (Kuntzman et al., 1969, Mannering et al., 1969, Nebert and Gielen, 1972) and it is therefore possible that

the observed non-linearity of the Lineweaver-Burk plots is due to the fact that more than one enzyme is involved in catalysing the N-demethylation of HMM and its derivatives. Indeed, the curvature of the Lineweaver-Burk plot is described by Dixon and Webb (1958) as characteristic of a reaction catalysed by two distinct enzymes with different Km values. Therefore, if the enzymes interact with the substrate with different affinities, deviations from Michaelis-Menten kinetics would be expected.

A further explanation for the observed non-linear double-reciprocal plots is that products generated during the N-demethylation reaction may act themselves as substrates for the enzyme(s). Although the desmethyl products of HMM metabolism may interfere with the demethylation of HMM itself, to produce the non-linearity, the curvature of the double-reciprocal plots at high substrate concentrations was explained by most authors as an indication for the presence of a second enzyme acting on the same substrate in the microsomal fraction (Lenk, 1976).

Most drugs are not metabolised by a single specific oxygenation reaction but a variety of distinct oxygenations, each generating a defined derivative of the substrate, and primary oxidative products may be further metabolised.

HMM, its N-demethylated metabolites (see fig. 2) and AP (see fig. 26) are metabolised by successive demethylations, e.g. AP is N-demethylated as shown overleaf:



The colourimetric assay used to quantify the N-demethylation reaction, measures the formation of formaldehyde or N-hyroxymethyl compounds which can also be generated by metabolites of HMM and AP. A more specific analytical technique was also employed; the GC measurement of parent compound, which is metabolised during the reaction.

The appearance of specific demethylated products was also measured by GC, but this is a less appropriate method to be employed in the enzyme-kinetic analysis of the methylmelamines, because these products were further N-demethylated.

The Lineweaver-Burk plots obtained by measuring formaldehyde were non-linear whereas the double-reciprocal plots derived from the GC assay were considered to be linear (figs. 12A and B). This discrepancy can be explained by the fact that the GC analysis was conducted using a smaller range of substrate concentrations; high substrate concentrations could not be used to measure substrate disappearance because the proportion metabolised was too small to quantify accurately, compared with the amount of substrate unmetabolised which was analytically quantified.

It can be assumed that the products of the oxidative metabolism of HMM react with the enzyme at the

same site as the parent compound w;ith different affinities and therefore they may compete with each other for the enzyme site. Indeed, inhibition of substrate demethylation by the product appeared to be occurring during the metabolism of HMM and also to a lesser extent during the metabolism of PMM and TetraMM (fig. 17). It is, of course, possible that HMM inhibited its own metabolism at substrate concentrations above 0.5mM (figs. 15 and 16) by saturating the enzyme site with which it interacts.

In accordance with these findings, Bast and Noordhoek (1981a) have described the competitive inhibition of the metabolism of AP by 4-monomethylaminoantipyrine and aminoantipyrine in-vitro. It has also been shown that HMM inhibited the microsomal O-demethylation of p-nitroanisole (Green and Gescher, 1980).

5.2 Interpretation of the Kinetic Constants

It is worth repeating that formaldehyde is a metabolite not only of HMM, but also its N-demethylated metabolites; PMM, TetraMM and TriMM. Therefore the measurement of formaldehyde as a metabolite of HMM, for example, does not allow for the calculation of specific kinetic constants because the rates of discrete oxidations are not measured, but instead the total product of a series of N-demethylations. However, the present study was not concerned with the calculation of absolute kinetic values but with relative values in order to compare the in-vitro N-demethylation of a series of closely related substances.

The kinetic constants were calculated from three different plotting methods (fig. 13). The Lineweaver-Burk plot (Lineweaver and Burk, 1934) has inherent disadvantages described in section (2.3.1), but it is more sensitive than the Hanes plot (Hanes, 1932) for detecting changes in the shape of the curve. The method of (Hofstee, 1959) gives a more uniform distribution of the points (fig. 13) and where kinetic data reveal deviations from a straight line, the Hofstee procedure of plotting experimental data is superior over the former methods because kinetic constants of more than one participating enzyme can be evaluated more accurately (Lenk, 1976).

Kinetic constants were evaluated from linear portions of the different plotting methods as described in section (2.3.1). In the present investigation sufficient

data was only available to calculate the kinetic parameters from the linear part of the curve at high substrate concentrations (figs. 12A-F).

The present investigation was designed to characterise the N-demethylation of HMM using microsomes from the mouse liver and to compare it with the demethylation of a model drug, AP, by hepatic microsomes from the mouse and other species. The non-linear double-reciprocal plots described for the metabolism of AP in mouse liver microsomes (fig.12E) was also obtained by other investigators using rat liver microsomes (Pederson and Aust, 1970; Poland and Nebert, 1972).

Bast and Noordhoek (1981b) calculated kinetic parameters by estimating formaldehyde formation during the in-vitro demethylation of AP essentially according to the assay conditions described in section (2.1), but using rat liver microsomes. They obtained a linear Lineweaver-Burk plot using substrate concentrations between 0.2mM and 3.33mM. In the present investigation, linear Lineweaver-Burk Hanes and Hofstee plots were obtained using AP concentrations between 0.2 and 4mM. The kinetic constants calculated from mouse and rat liver preparations (Table 4) for AP and those obtained for the N-demethylation of HMM and its demethylated metabolites (Table 2) were of the same order of magnitude.

The kinetic constants evaluated from the colourimetric assay for formaldehyde (Table 2) were also not markedly different from those parameters obtained from the GC assay (Table 3). This is in accordance with the view that HMM and PMM are not metabolised in-vitro by routes

TABLE 4

A Comparison of the Apparent Kinetic Constants Determined for AP Using Liver Microsomes Obtained from the Rat or the Mouse

Liver Microsomal Preparation	Km (mM)	Vmax (nmoles formaldehyde per mg microsomal protein per minute	n[c]
Mouse (see Table 2)	0.84 ± 0.4[a]	2.5 ± 0.7[a]	8
Rat (Bast and Noordhoek, 1981b)	0.491 ± 0.088[b]	7.29 ± 0.95[b]	3

(a) = Km and Vmax (± Standard deviation of the mean)

(b) = Km and Vmax (± Standard error of the Mean)

(c) = number of experiments

other than N-demethylation to the desmethyl metabolites and formaldehyde (Ames, et al., 1979; Worzalla et al., 1974(b); Rutty et al., 1978; Worzalla et al., 1973; Bryan et al., 1968). Bast and Noordhoek (1981b) estimated the kinetic constants for the metabolism of AP by determining formaldehyde production, using a colourimetric assay and also by measuring the formation of metabolites of AP by high pressure liquid chromatography. These authors found that the Vmax value calculated from the formation of monomethylaminoantipyrine and aminoantipyrine was similar to that obtained from the colourimetric assay, but the apparent Km value was appreciably higher. They concluded that the different Km values were obtained because AP was metabolised by routes other than N-demethylation and that the high pressure liquid chromatography assay underestimated the production of formaldehyde. The different metabolic pathways of AP have been well described in rats by Goromaru et al., (1981) and are summarised in fig. (26).

For the reasons outlined above, one would also expect the GC assay to underestimate the production of formaldehyde from HMM and PMM because formaldehyde is also produced from their metabolites. However the Vmax values for HMM and PMM were not lower than those obtained from the colourimetric assay which suggests that formaldehyde, measured colourimetrically, is derived mainly from the N-demethylation of HMM to PMM, with HMM as substrate, and PMM to TetraMM when PMM is used as the substrate; rather

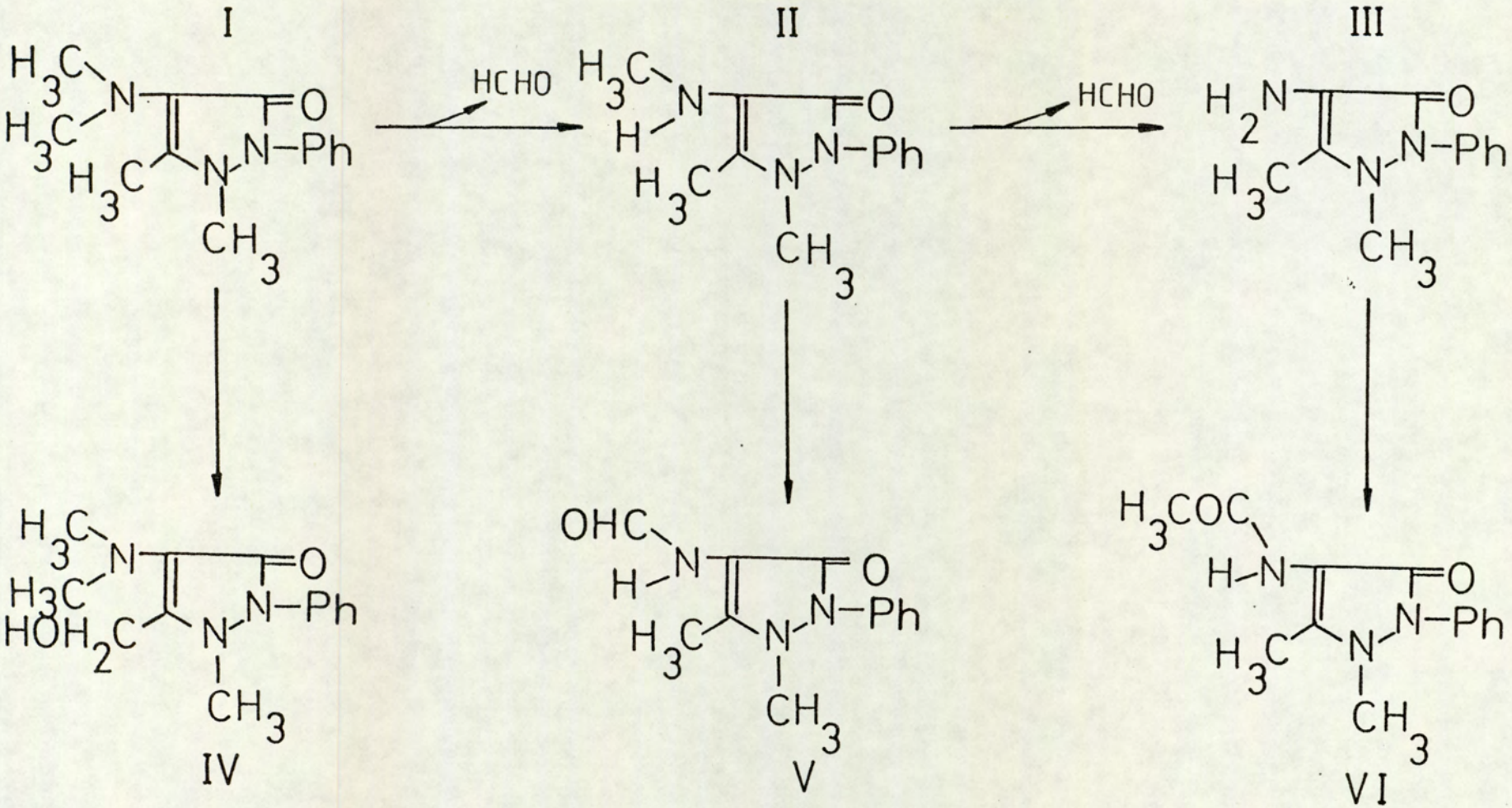


Fig. 26 Metabolic pathways of AP (I) in the rat. Chemical structures, (II), (III), (IV), (V) and (VI) represent; 4-monomethyaminoantipyrine, 4-aminoantipyrine, 3-hydroxymethyl-4, 4-dimethylaminoantipyri 4-formylaminoantipyrine and 4-acetylaminoantipyrine. The chemical scheme is taken from Goromaru et al., (1981).

than from further metabolism of the respective metabolites.

A reduction in the number of methyl groups in the melamine molecule lead to a decreased affinity of the demethylating enzymes for the substrate (section 4.2.1). Rutty and Connors (1977) also observed a relationship between the degree of demethylation, measured as the amount of formaldehyde produced in one hour, in rat liver microsomes and the number of methyl moieties in the melamine ring. They also found a correlation between the ability of the methylmelamines to undergo demethylation and their antitumour activities against the ADJ/PC6A plasmacytoma in mice.

The toxicity of HMM and its N-demethylated metabolites to the ADJ/PC6A plasmacytoma cells in-vitro was also shown to be directly related to the number of methyl groups in the melamine molecule (Rutty and Abel, 1980). However this finding cannot be interpreted in the light of the bioactivation of HMM to antineoplastic species, because one would assume that N-demethylation does not occur under these assay conditions, i.e. there was probably no metabolism of the substrates by the tumour cells. The same authors found no correlation between in-vitro cytotoxicity and antitumour activity, e.g. HMM, HEM and HeptaMM were equally toxic to ADJ/PC6A cells in-vitro but only HMM was effective in inhibiting the growth of the tumour in-vivo (Rutty and Abel, 1980).

If one assumes that the HMM oxidising enzymes in

the tumour cell are similar in their biochemical characteristics to the hepatic oxygenases investigated in this study, the reduction of antitumour activity of TetraMM and TriMM as compared to HMM and PMM may be due to insufficient rates of metabolic transformation of the methylmelamines to electrophyllic N-hydroxymethyl derivatives or formaldehyde.

However, the accessability of the enzyme for the substrate in-vivo is very likely to be different from that in-vitro and therefore caution must be exercised when kinetic parameters obtained with cell fractions in-vitro are used to discuss the metabolism of a drug in-vivo.

It is also worth noting that plasma levels of HMM and PMM in mice after administration of 'therapeutic' doses of the drug (Broggini et al., 1981), did not approach concentrations equivalent to the Km values obtained in-vitro using mouse liver microsomes (table 2). It should be emphasised, therefore, that the apparent Km values can only be interpreted as indicating a trend in demethylation rates rather than reflecting the substrate concentration at the enzyme site in-vivo.

Ebel et al. (1978) suggested that the binding of a substrate to cytochrome P-450 is related to the solubility of the substrate in the microsomal fraction. The lipophilic nature of a substrate may therefore represent the extent to which it can interact with the lipid enviroment of the enzyme site. It is interesting in this context that

Johnson and Torres (1976) found the in-vitro N-demethylation of certain N-methylmelamines to be higher in molecules with high partition coefficients.

The lipophilicity of the methylmelamines is reduced by removing the non-polar methyl groups, e.g. the octanol: water partition coefficient of PMM is less than one fifth of that of HMM but more than four times that of the coefficient of TMM (Cumber and Ross, 1977).

The reduced affinity of the oxidising enzymes for the methylmelamines containing less than five methyl groups (table 2) appears therefore to be related to the decreased lipophilic nature of the substrates compared to HMM and PMM. Also, the addition of polar substituents to the melamine molecule, e.g. in the case of HeptaMM and the glucose derivative of TMM (see fig. 14), increases the aqueous solubility and reduces the enzyme affinity for these substrates even more (table 2).

Rutty and Connors (1977) observed that in general, the water soluble derivatives of HMM do not readily demethylate and are correspondingly poor antitumour agents. It is probable that N-demethylation and lipophilicity are related to antineoplastic activity in this class of compounds. However HEM has only marginal activity against the ADJ/PC6A plasmacytoma in-vivo (Rutty and Connors, 1977) and yet was metabolised at a similar rate in-vitro compared with HMM (table 3). Substitution of methyl moieties in the HMM molecule with ethyl groups imparts increased

lipophilicity and therefore one would assume that the rate of metabolism would be at least comparable to HMM if lipophilicity is related to de-ethylation in-vitro. However this in contrast with the in-vitro metabolism observed by Rutty and Connors (1977) who found that the molecule undergoes de-alkylation to a much smaller extent than HMM. These authors estimated acetaldehyde formation as an index of HEM metabolism by a colourimetric assay whereas in the present study the disappearance of parent compound was measured by GC. It is possible that HEM is metabolised by liver microsomes to a precursor of acetaldehyde, e.g. N-hydroxyethylpentaethylmelamine, which does not decompose completely under the assay conditions described for this colourimetric determination (Stotz, 1943). It is also possible that the different rates of dealkylation of HEM were observed because of the species from which liver microsomes were obtained, i.e. Rutty and Connors (1977) employed liver microsomes from the rat whereas mouse liver microsomes were used in the present investigation.

Broggini et al. (1981) observed that the rate of demethylation of HMM and PMM were essentially the same whereas that for TetraMM was much slower. Similar findings were observed in the present study where apparent Km values for TetraMM and TriMM were higher than those for HMM and PMM (table 2). This may partly account for the long plasma elimination half-lives of TetraMM and TriMM as metabolites of HMM and PMM (Broggini et al., 1981), e.g. the low affinities

of the mixed function oxidases for TetraMM and TriMM may result in their elimination via hepatic metabolism being much less prominent than that for HMM and PMM.

N^2,N^2,N^4,N^4-Tetramethylmelamine (TMM) could not be detected in the urine of humans, rats (Worzalla et al., 1973) and house flies (Chang et al., 1968) after administration of HMM. This finding supports the view of Worzalla et al. (1973, 1974a) and Johnson and Torres (1976) that N-dimethyl groups in the melamine ring are preferentially demethylated compared to N-monomethyl groups as illustrated in fig. (2). Results from the present investigation suggest that the N-demethylating enzymes exhibit a greater affinity (i.e. lowe Km value; see table 2) for dimethyl-substituted tertiary amines (e.g. TMM, Km = 0.21) than for monomethyl-substituted secondary amines (e.g. TetraMM, Km = 0.91). Also, the Km values for PMM and TMM are similar and they both possess two N-dimethyl moieties; although it should be noted that the Vmax values for the two homologues were signifcantly different ($p<0.0005$). That TMM could not be detected as a metabolite of HMM in the plasma may be attributable to the decreased affinity of the metabolising enzymes for monomethyl substituents compared to dimethyl substituents. However, recent evidence (Columbo et al., 1982) has demonstrated the presence of TMM in the bile, urine and faeces of rats which were given HMM, although TetraMM accounted for a greater proportion of the total dose of HMM administered compared to that for TMM.

5.3 Factors Affecting the In-Vitro Metabolism of HMM

The administration of 75 mg/Kg methyliodide, which depleted mouse livers of glutathione (GSH) to 22% of the normal levels (Brindley et al., 1982b), led to a decrease in the metabolic generation of formaldehyde in a 9,000g liver preparation with HMM, PMM and 3,4-(Carboxymethylphenyl)-1,1-dimethyltriazine (CMPDT) as substrates (figs. 18 and 19). A reduction in HMM metabolism after methyliodide pretreatment was also observed when N-demethylation was assayed by GC (Brindley et al., 1982b). However, this effect was not observed with isolated microsomes (fig. 20) nor when mice were pretreated with diethylmaleate or adriamycin which like methyliodide, deplete hepatic GSH pools (Levine and Finkelstein, 1979; Doroshaw et al., 1979); (fig. 21). There was also no significant effect of methyliodide administration on the in-vivo disposition of HMM (Brindley et al., 1982b)and furthermore, the influence of pretreatment with methyliodide on the in-vitro metabolism of HMM and PMM was not eliminated by protecting animals with cysteine which is a precursor of GSH (Brindley et al., 1982b).

It is therefore unlikely that the depletion of hepatic GSH pools was responsible for the reduction in HMM N-demethylation. Further evidence for this hypothesis was obtained when HMM metabolism was found to be unaffected by the addition of GSH to microsomal incubations (fig. 23) and addition of methyliodide decreased the metabolism of HMM using a similar liver preparation (fig. 22). So it is possible that methyliodide either inhibits HMM demethylation

directly or affects an as yet unidentified cytosolic modulator of HMM metabolism in-vitro.

In contrast with the metabolism of HMM, the pretreatment of mice with methyliodide did not influence the in-vitro N-demethylation of AP (fig. 18). However AP metabolism was increased after the addition of GSH to microsomal incubations (fig. 23). It may therefore be postulated that methyliodide influences a particular feature of the N-demethylation of HMM and not the biotransformation of other N-methyl-containing drugs. One might also speculate that this factor may be associated with the cytotoxic mode of action of HMM.

In order to characterise the activity of the demethylase responsible for the demethylation of another N-methyl agent, dimethylnitrosamine, and to explain its mutagenic and carcinogenic effects, the in-vitro metabolism of dimethylnitrosamine was studied by several authors using liver fractions from mice and rats (table 5). Hepatic dimethylnitrosamine demethylase activity was enhanced in 9,000g and microsomal liver preparations from animals pretreated with dioxane and acetone. HMM, PMM and AP demethylase activities were also increased in liver microsomal preparations obtained from mice pretreated with these polar solvents (fig. 24). The highest increase for all three substrates was obtained with dioxane which is in agreement with Argus et al. (1980) who found that dimethylnitrosamine demethylase activity was enhanced to a

TABLE 5A

The Effect of Polar Solvents on the In-Vitro Metabolism of AP and Dimethylnitrosamine (DMN) by Other Authors

Author	Liver Preparation	Substrate	Effect on (c) Demethylation	Solvent
Kawalek et al. (1980)	9000 g (rat)	AP	-	acetone, (a) dioxane
Argus et al. (1980)	9000 g (rat)	DMN	-	acetone, (a) dioxane
	9000 g (rat)	DMN	+	acetone,(b) dioxane
Haag et al. (1980)	microsomal (mouse)	DMN	+	acetone, (b)
Sipes (1978)	microsomal (mouse)	DMN	+	acetone, (b)

(a) = in-vitro addition of polar solvents to the metabolising incubation

(b) = pretreatment of animals with polar solvents prior to excision of livers

(c) = induction (+) or repression (-)

TABLE 5B

The Effect of Polar Solvents on the In-vitro Metabolism of HMM, PMM and AP in Liver Microsomal Preparations

Substrate	Effect on Demethylation (c)	Solvent
HMM	-	acetone, (a)
	-	dioxane
PMM	-	acetone, (a)
	-	dioxane
AP	-	acetone, (a) dioxane
HMM	+	acetone, (b)
	++	dioxane
PMM	+	acetone, (b)
	+	dioxane
AP	+	acetone, (b)
	+	dioxane

(a): in-vitro addition of polar solvents to the metabolising incubation

(b): pretreatment of animals with polar solvents prior to excision of livers

(c): induction (+) or repression (-)

greater extent by dioxane than other solvents. It is therefore possible that acetone and dioxane influence enzymatic pathways responsible for the metabolism of both HMM and dimethylnitrosamine. The molecular mechanisms by which the polar solvents induce microsomal mixed function oxygenases is unknown. However Venkatesan et al. (1971) proposed that the chemical agents may modify the conformation of proteins which regulate the synthesis of enzymes responsible for N-demethylation.

A stimulatory effect of acetone on the microsomal hydroxylation of aniline has been reported by Anders (1969). It was speculated in this case that the enhancement of the hydroxylase by acetone could be brought about by exposing new active sites on the enzyme by modifying the spatial configuration of the resultant microsomes or by interfering with specific functional groups.

Both AP and dimethylnitrosamine metabolism was reduced after addition of these polar solvents to a 9,000g preparation (table 5) and this inhibition of the in-vitro metabolism, after addition of these agents to incubations, may be due to a direct destructive effect on the participating enzymes. However, the in-vitro addition of acetone and dioxane failed to produce a marked effect on the demethylation of HMM and AP (fig. 25) in a liver microsomal preparation.

In conclusion, it appears from the present investigation of the methylmelamines that the major in-vitro metabolites of HMM and PMM are PMM and TetraMM

respectively, since it was concluded that formaldehyde is mainly derived from the first demethylation step of the metabolism of HMM and PMM. Further evidence supported the view that the lipophilicity and rates of demethylation of the methylmelamines are related to antineoplastic activity and that water soluable derivatives of HMM do not readily demethylate and are not effective antitumour agents.

The relatively slow demethylation rates of TetraMM and TriMM as compared to HMM and PMM may explain the long plasma elimination half-lives of TetraMM and TriMM in-vivo.

Differences in the metabolism of HMM compared with AP were only found when the compounds were N-demethylated in 9,000g liver fractions obtained from mice pretreated with methyliodide and when GSH was added to microsomal incubations. Other factors unique to the metabolism of HMM were not found and it is possible that other oxidising systems are present in-vivo which metabolise N-methyl-containing xenobiotics to cytotoxic or alternatively to innocuous chemicals, i.e. HMM and AP may be metabolised by pathways different from the cytochrome P-450 monooxygenases described in section (1.4.2.4).

It has been recently demonstrated (Sivarajah et al., 1982) that the microsomal fraction of ram seminal vesicles, fortified with arachidonic acid, catalysed the dealkylation of various N-methyl compounds including AP and dimethylaniline, at rates comparable to those reported for rat liver microsomes containing NADPH. It appears that oxidising agents are formed during the synthesis of prostaglandins from

arachidonic acid which have the capacity to cause oxidative substrate dealkylation. This also might be a potential mechanism by which the methylmelamines are N-demethylated. This enzyme system does not readily dealkylate S-alkyl and O-alkyl containing compounds and is not inhibited by SKF525A and metyrapone which is in contrast to the cytochrome P-450-dependent monooxygenases. The mechanism by which the protaglandin synthetase-dependent metabolising system catalyses the N-demethylation of N-methyl-containing compounds is therefore likely to be different from that of the cytochrome P-450-dependent system. Further investigations of the type described in the present study, with the latter metabolising system, may reveal differences in the metabolism of HMM on the one hand and AP on the other.

PART TWO

The N-alkylformamides

SECTION 6

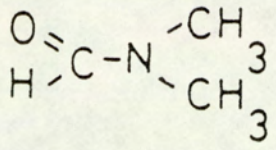
INTRODUCTION

6.1 Antitumour Activity of the N-alkylformamides.

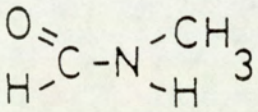
N,N-dimethylformamide (DMF) is used as an organic solvent in industry for the manufacture of polyacrylnitrile fibres. In 1953 a series of formamide derivatives, including DMF, was evaluated for potential antitumour activity against the mouse sarcoma 180 by Clarke et al. (1953). N-methylformamide (NMF) was found to be the most potent inhibitor of tumour growth whilst formamide and diethylformamide showed only marginal activity (Clarke et al., 1953). DMF, N-ethylformamide (NEF) and thioformamide were all found to be inactive (Clarke et al., 1953). The chemical structures of DMF, NMF and NEF are shown in fig.(27). This structure-activity relationship was also described by Furst et al. (1955) who found NMF and to a lesser extent formamide, to be active against Ehrlich ascites cell tumour; whilst DMF revealed only marginal activity.

Recently, NMF was shown to be the only formamide derivative to have significant activity against three murine tumours; namely, mouse sarcoma 180, M5076/A murine reticulum cell sarcoma and TLX5 murine lymphoma (Gescher et al., 1982). Formamide also demonstrated some activity, but not against the TLX5 murine lymphoma, whereas NEF was inactive against all three tumour models (Gescher et al., 1982).

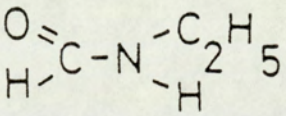
That substitution of an ethyl group in the formamide molecule leads to such a marked reduction in anti-tumour activity is of particular interest for the present



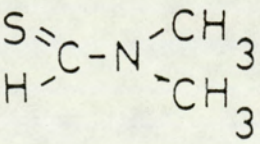
N,N-DIMETHYLFORMAMIDE (DMF)



N-METHYLFORMAMIDE (NMF)



N-ETHYLFORMAMIDE (NEF)



N,N-DIMETHYLTHIOFORMAMIDE (DMSF)

Fig. 27 Chemical structures of the N-alkylformamide derivatives.

investigation. The comparable in-vitro toxicities of NMF and NEF (Gescher et al., 1982) suggests the possibility that NMF may be bioactivated in-vivo to a product or products not formed from NEF, or in insufficient quantities. It was therefore considered relevant to compare the plasma disposition and urinary excretion of NMF and its metabolites with those of other inactive formamide derivatives, including NEF.

6.2 The Hepatotoxicity of NMF

DMF is hepatotoxic in animals (Maxfield et al., 1982; Massman, 1956; Ungar et al., 1976) and in man (Potter, 1973). Both NMF and DMF have produced detectable liver injury in rats (Lundberg et al., 1981); and in a clinical investigation, NMF was found to cause reversible liver damage in all five patients under study (Myers et al., 1956). Toxicity in man was also manifested as nausea and vomiting, and was apparently a systemic effect rather than local irritation (Myers et al., 1956).

Hepatoxicity was considered an unacceptable side effect and interest in the clinical use and pharmacology of NMF disappeared. However, NMF does not appear to be myelosuppressive(Newman et al., 1981, and Langdon et al., unpublished) to mice and dogs, and since it is active against a number of murine tumours, it reemerged as a candidate for further clinical evaluation.

To compliment the phase one clinical trials currently being performed in London and Amsterdam, a pharmacokinetic investigation of NMF was undertaken as part of the present work in order to provide information which would allow the rational use of NMF in the clinical studies.

6.3 Mode of Action of NMF

The mechanism by which NMF exerts its antitumour activity is unknown. NMF and formamide, like amides in general, are relatively stable compounds compared to other antineoplastic agents such as the alkyltriazenes; the possibility of direct chemical reaction with tissue macromolecules would thus seem unlikely for NMF.

Some antitumour agents act as metabolic antagonists e.g. methotrexate, which interferes with C_1-unit metabolism by the inhibition of dihydrofolate reductase. However, NMF does not appear to act by this mechanism since neither folic acid nor citrovorum factor administration was able to block the tumour inhibition or the toxicity of NMF in mice (Clarke et al., 1953).

Early studies suggested that NMF interferes with nucleic acid base synthesis (Clarke et al., 1953; and Skipper et al., 1955) although it also appears to stimulate incorporation of formate into nucleic acid in the liver (Barclay and Garfinkel, 1954).

NMF does not appear to act in the same way as procarbazine and dimethyltriazenes which are also N-methyl-containing antitumour agents. The activity of NMF against a TLX5 lymphoma made resistant to these drugs (TLX5R) was not significantly different from the activity shown against a TLX5 lymphoma which was sensitive to these agents (TLX5S), (Gescher et al., 1982).

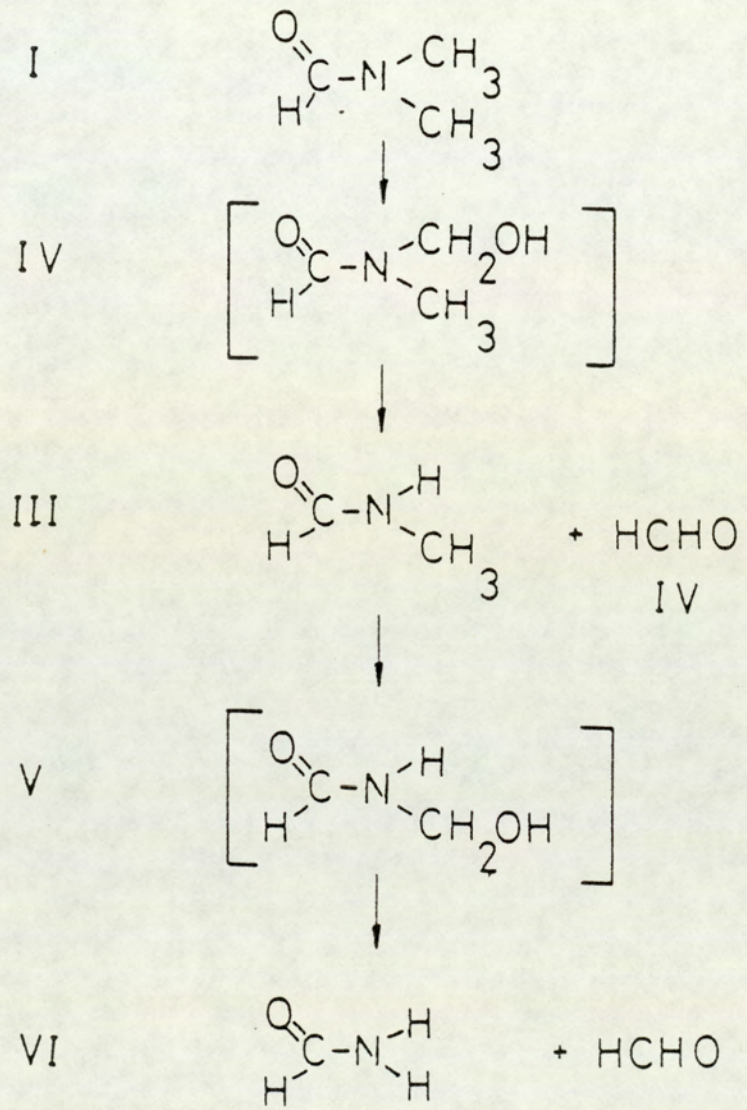


Fig. 28 Suggested metabolic pathway for the metabolism of DMF (I) to NMF (III) and formamide (VI) via the hydroxylated intermediates; N-hydroxymethyl - N-methylformamide (II) and N-hydroxymethylformamide (V). Each demethylation step results in the formation of formaldehyde.

6.4 The Metabolism of NMF

NMF may require metabolic activation to elicit its cytotoxic effect, however evidence to support this hypothesis is lacking. Barnes and Ranta (1972) presented evidence for the in-vitro N-demethylation NMF and DMF by a liver metabolising system. However, recent evidence (Gescher et al., 1982) casts doubt on these findings because the assay procedure employed was thought not to be sensitive enough to measure the low levels of formaldehyde reported in the study of Barnes and Ranta (1972).

DMF appears to be rapidly demethylated in-vivo, since after administration to animals, only small amounts appear unchanged in the urine (Massman, 1956). DMF is metabolised to NMF and formamide in rats and dogs in-vivo (Kimmerle and Eben, 1975) and NMF is metabolised to formamide in mice (Gescher et al., 1982). Whether formamide is still further metabolised to ammonia and formate has yet to be determined.

The presence of formamide (Kimmerle and Eben., 1975) and NMF (Barnes and Ranta, 1972 and Kimmerle and Eben, 1975) in the urine after administration of DMF to rats, and the appearance of formamide (Gescher et al., 1982) and formaldehyde (Brindley et al., in press) in the urine and plasma of mice after administration of NMF, suggests that both DMF and NMF are demethylated in-vivo probably by the mixed function oxidases of the liver as shown in fig. (28).

Scailter et al. (1981) found NMF to be much more toxic to rats than DMF and the latter to be more toxic than formamide. It was therefore considered that the in-vivo production of NMF from DMF was an activation reaction whilst

the metabolism of NMF to formamide was an inactivation reaction. However, this does not explain the difference in the cytotoxicity of these of these agents. The disposition of DMF and NMF in mice was compared in the present investigation, in an attempt to reveal differences in the matabolism and distribution of these molecules. These differences were thought to give some insight into the mechanism by which these agents cause hepatotoxicity.

6.5 Aims and Scope of the Investigation

6.5.1 Aims of Pharmacokinetic Analysis

New drugs can be registered for use in the United Kingdom and United States only if pertinent pharmacokinetic parameters of the drug are submitted. Therefore, a pharmacokinetic investigation of NMF was performed in order to complement the phase one clinical evaluations.

According to Dost (1968) pharmacokinetics are concerned with; "practical information derived from the observation of concentration time curves of a drug and metabolites within the compartments of the body as a whole and, in particular, the blood and urine."

The practical information obtained from such an investigation may be used to evaluate two important aspects of drug distribution within the organism:

(1) Bioavailability: the primary proof of pharmacological or chemotherapeutic availability of a drug at the site of action is the magnitude and duration of the biological response. However, a second indication of availability is the appearance of the drug and its metabolites in the blood, tissues and excreta of the organism. In this investigation the bioavailability of NMF was compared after administration by different routes.

(2) Dose dependency: repeated administration of the drug, or alteration of the dose administered, may influence metabolising and transport mechanisms associated with the disposition of the drug within the body. The plasma

disposition of NMF was investigated in both man and in the mouse after repeated administration of NMF and when NMF was administered at different doses.

6.5.2. Pharmacokinetics in the Mouse

A comparison was made between the active antitumour agent, NMF and the less active or inactive formamide derivatives; DMF, NEF, and dimethylthioformamide (DMSF) (see fig. 27) by investigating the plasma disposition and the urinary appearance of the parent compound and its metabolites after administration of the alkylformamide to mice. This pharmacokinetic study was considered to provide information to explain the marked decrease in antitumour activity brought about by small changes in the formamide molecule.

The pharmacokinetic study was also thought to shed light on the metabolic pathway of the N-alkylformamides. Further information concerning the metabolism of the formamide derivatives may give an insight into the possible mechanisms of cytotoxicity and hepatotoxicity.

In a different project an attempt was also made to follow the distribution of NMF into various tissues in order to locate any accumulation of the drug. These tissue distribution studies were conducted by Mr. T. Barlow as part of his research towards the degree of M.Sc.

6.5.3. Pre-clinical Investigations

In order to obtain information which may facilitate the rational use of NMF in the clinical investigations, several aspects of its disposition in the mouse were studied.

(1) The bioavailability of NMF after intraperitoneal (IP), oral (PO) or intramuscular (IM) administration was investigated because, in the antitumour tests NMF was administered IP whereas in the clinic NMF may be given orally as well as IV or IM. If the systemic availability of NMF after PO administration is not significantly different from that after IV administration, then it may be possible to administer the drug orally which will facilitate patient compliance.

(2) Repeated administration of high doses of NMF was required for optimal antitumour activity in mouse tumour models (Gescher et al., 1982) and it is possible that to be effective in patients, NMF may have to be given in high doses on a multidose schedule. The plasma disposition of NMF after repeated administration of the drug was investigated in order to see any alterations in the pharmacology of NMF, e.g. plasma accumulation.

(3) The presence of tumour may effect the pharmacology of NMF and therefore the plasma disposition of the drug was compared when mice were implanted with TLX5 lymphoma and the M5076/A reticulum cell sarcoma.

6.5.4. Clinical Investigations

As part of the phase one clinical investigations, samples of plasma and urine from patients receiving NMF were assayed for parent drug and metabolites in order to follow the plasma disposition and urinary excretion of the drug in man.

The bioavailability of NMF in man was compared to that of mice.

SECTION 7

MATERIALS

7.1 Substrates and their Derivatives Employed in the In-vivo Investigations

The following compounds were purchased from Aldrich Chemical Co.Ltd., Gillingham, United Kingdom.

N,N-dimethylformamide

N,N-dimethylthioformamide

formamide

N-methylformamide.

N-ethylformamide was prepared according to the method of Saegusa et al. (1969) by Mr. N. E. Gate.

Labelled N, ^{14}C-(Methyl)-formamide was synthesised by Dr. M. D. Threadgill by the reaction of ^{14}C-methylamine hydrochloride with ethylformate in methanol and in the presence of anhydrous sodium carbonate according to a novel synthesis (Threadgill M. D., unpublished).

7.2 Miscellaneous Chemicals

NE 260 Micellar scintillant was purchased from New England Nuclear, West Germany.

^{14}C n-Hexadecane was purchased from Amersham International plc., Amersham, United Kingdom.

Tetramethylurea and Phenobarbitone was obtained from Sigma Co. Ltd., Poole, United Kingdom.

Packing material for the GC columns was obtained from Phase Separations Limited, Queensferry, United Kingdom.

7.3 Animals

Mice were obtained from the same supplier and fed on the same diet as described in section (2.3).

SECTION 8

METHODS

8.1 Drug Administration

Male Cba/ca or female BDF_1 mice (20-25g) were used throughout this in-vivo investigation. Female Cba/ca mice were used only for a comparative investigation. The N-alkylformamides under study were administered to animals, in solution, in 0.9% sterile saline. Gillette, sterile syringes (1ml) fitted with a 26 gauge disposable needle were used for all routes of administration except oral gavage. Immediately prior to drug administration, the animals were weighed and the dosage calculated on a mg/Kg basis. An injection volume of 100µl was used throughout the in-vivo study.

For IV injection, the test animals were kept in an adjustable restraining apparatus, made from a 50ml polypropylene syringe, with openings permitting access to the tail. The site of injection was a caudal vein which was the most dilated after gentle massaging of the tail. The injection was considered to be successful by the following criteria:

(1) ease of plunger movement,

(2) bleeding at the injection site after the needle was removed.

400 mg /Kg (which is one half of the LD_{10} in Balb C mice on a single IP administration), 80 mg/Kg or 14.3 mg/Kg NMF was administered to animals in this way.

Oral administration was achieved using a Gillette, sterile syringe (1 ml) fitted with a feeding needle. The needle was inserted through the animals' mouth, into the oesophagus and downwards towards the stomach where the contents of the syringe were expelled.

400 or 80 mg/Kg NMF was administered in this way.

For intraperitoneal (IP) administration, the drug was injected into the peritoneal cavity of the animals, ensuring that sub-cutaneous administration was avoided. The different N-alkylformamides were administered IP at the following doses:

N-alkylformamide	Dose	
	mg/Kg	nmoles/Kg
DMF	1400	19.2
	496.4	6.8
	99.3	1.36
NMF	400	6.8
	80	1.36
	14.3	0.24
^{14}C-methylformamide	400	6.8
	14	0.24
NEF	591	6.8
DMSF	400	4.5
	200	2.3
	100	1.1

When 400 mg/Kg ^{14}C-NMV was administered IP, the injection solution contained 5 or 15 μ Curies of radioactivity.

8.2 Sampling of Body Fluids

8.2.1. Blood

Single blood samples were collected from animals at least seven times after administration. Initially, samples were taken frequently and as time progressed, samples were taken at longer intervals; sample intervals ranged from 0.04 to 30 hours for the measurement of concentrations of parent drug by GC , and from 0.25 to 192 hours to determine levels of radioactivity after administration of ^{14}C-NMF.

Animals were anaesthetised using a mixture of halothane, nitrous oxide and oxygen distributed by a BOC Boyles apparatus. In control experiments mice were used without anaesthesia.

When blood was obtained by cardiac puncture, animals were anaesthetised and the heart exposed. Gillette, sterile syringes (1ml) fitted with 26 gauge needles, containing approximately 50µl heparin solution, were used to take between 0.5 ml and 1 ml of blood. Blood samples were centrifuged in polypropylene microfuge tubes in a Beckman microfuge for three minutes to separate the plasma fraction.

For blood sampling from the tail,a method was used which was similar to that described by Noney et al. (1975). Animals were anaesthetised and, prior to the first sample collection, the tip of the tail was cut off with a scalpel to allow bleeding. At the sample time the tail was gently stroked in the direction of the apex. 20µl of blood was withdrawn at each sample time using Bilbate heparinsed glass

capillary tubes. The tubes were heat-sealed at one end and centrifuged in a Hawksley haematocrit for three minutes to separate the plasma. (The animals' total blood volume is between 2 ml and 3 ml; together not more than 10% of the total volume was withdrawn in 12 hours).

8.2.2 Urine

Animals were kept in Jencons metabolic cages which were adapted to provide an improved access to drinking water for the animal. The urine was collected in a small flask and removed from this vessel with a pasteur pipette. Then the volume of the urine sample was measured. Samples were taken at 12 hourly intervals up to 96 hours after drug administration.

In order to minimise the risk of evaporative losses of drug and metabolites with the urine, in some cases 10 ml aliquots of distilled water was used to wash the collection funnels of the metabolic cages and thus flush parent drug and metabolites into the collection flask. Urine was collected after these washings at 2 to 4 hour intervals during the first 12 hours and again 24, 48 and 72 hours after drug administration.

In order to investigate the effect of phenobarbitone on the urinary excretion of NMF, mice were administered 50 mg/Kg phenobarbitone in 0.9% saline IP on four consecutive days (Conney et al., 1960). Control animals received saline only.

24 hours after the final administration, the mice then received 400 mg/Kg NMF IP and the urine from these animals was collected as above, up to 24 hours after administration without washing the metabolic cages.

8.3 GC Analysis

8.3.1 Preparation of Samples

100µl of the plasma obtained by cardiac puncture was added to 500ul of acetone containing the internal standard, in polypropylene microfuge tubes. Alternatively, 10ul of the plasma obtained from the tail vein was removed from the collection capillaries using a glass syringe (25µl) and added to 50µl of acetone containing the internal standard. The tubes containing the plasma-acetone mixture were centrifuged in a Beckman microfuge for one minute. 1ul to 2µl of the resultant supernatent was injected into the injection port of the GC using a glass syringe (10µl).

100µl of urine was added to 200µl or 500µl of acetone containing internal standard. The samples were then prepared in the same manner as the plasma-acetone mixtures, described above.

Plasma and urine samples collected from patients were kept frozen and allowed to thaw at room temperature when required. 100µl of the plasma or urine sample was added to 200µl of acetone with the internal standard and centrifuged as in the case of the samples obtained from mice.

8.3.2 Standard Solutions

Tetramethylurea (TMU) was employed as the internal standard throughout the investigations.

Standard curves were prepared from solutions of the alkylformamide under investigation together with plasma or urine collected prior to drug administration. Alkylformamides and TMU were dissolved in acetone and the resultant precipitate was removed by centrifugation in the manner described in section (8.3.1).

To examine the influence of biological fluids on the analytical determination, standards were also prepared where plasma and urine was replaced by acetone. N-alkylformamide concentrations were in the range of 1.25 to 100µg/ml.

The coefficient of variation for the estimation of NMF concentrations in plasma samples was determined in the following manner: A male Cba/ca mouse was administered 400 mg/Kg NMF IP and 1 ml of blood was obtained by cardiac puncture and plasma samples were prepared as described in section (8.3.1). 10 samples of plasma were treated with acetone containing TMU for GC analysis. Peak height ratios were calculated (peak height NMF: peak height TMU) and coefficient of variation calculated from:

$$\frac{\text{standard deviation from the mean}}{\text{mean peak height ratio}} \times 100$$

8.3.3 GC Conditions

The N-alkylformamides and formamide were quantified from plasma and urine samples by a gas chromatographic assay similar to that described by Kimmerle and Eben (1975). Analysis was performed on a Pye-Unicam 204 GC fitted with a nitrogen detector. The column used was a glass tube 1.5 m long and of 3 mm internal diameter packed with 100 to 200 mesh chromasorb W17 AWDMCS and coated with 8% carbowax 20M and 2% KOH. The columns were conditioned for at least 24 hours at 210°C with a carrier gas flow rate of 50ml/minute. The operating temperatures were as follows:

Column oven: 190°C

Injector port: 200°C

Detector port: 250°C

During analysis, nitrogen was used as the carrier gas at a flow rate of 40ml/minute. Air and hydrogen flow rates respectively were 240 and 10 ml/minute.

8.4 Measurement of Labelled Compounds

10µl of plasma, obtained after administration of ^{14}C-NMF, as described for the unlabelled drug, was added to scintillation vials containing 5 ml of NE 260 micellar scintillant. In the case of urine collected from mice after administration of labelled NMF, 1 ml was added to scintillation vials containing 10 ml of NE 260 micellar scintillant.

These samples were shaken using a vortex mixer and radioactivity was counted in a Packard Tricarb 2660 scintillation counter using the external standardization mode.

To prepare a quench curve for this analytical procedure, 5µl of ^{14}C-labelled n-hexadecane of known activity was added to 10 scintillation vials. Increasing volumes (0-800µl) of chloroform, the quenching agent, were then added. Counting efficiency, which is defined as the ratio of the observed counts per minute to the disintegrations per minute, of the prepared samples was determined from its count rate using the calibration (quench) curve (fig.29). External standard ratio (ESR) values were between 0.48 and 0.67 and the counting efficiency was between 85 and 95%.

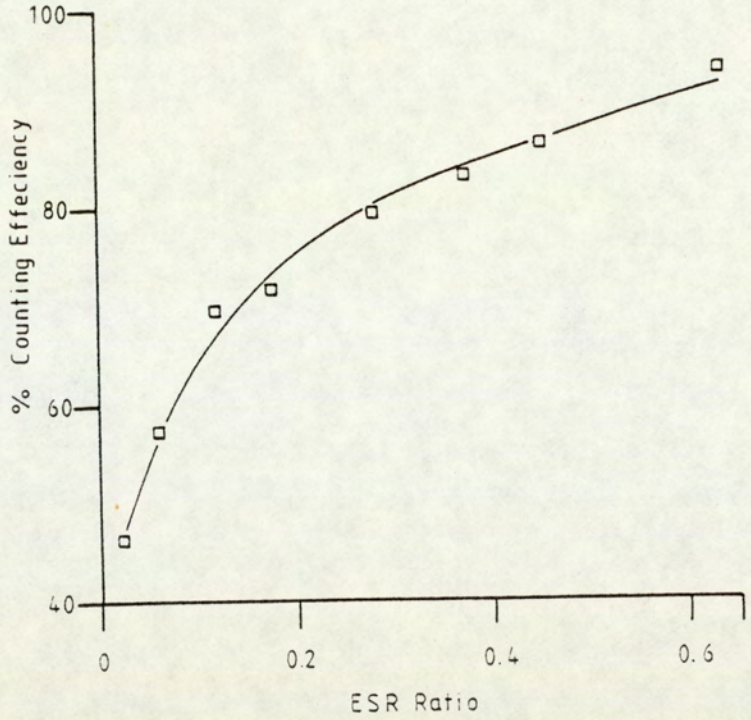


Fig. 29 Correlation curve between the sample counting efficiency and the count rate of the external standard. (External standard ratio; ESR).

8.5 Tumour Implantation

The TLX5 lymphoma and M5076/A reticulum cell sarcoma were maintained by subcutaneous passage in the flanks of male Cba/ca and female BDF_1 mice respectively.

The tumour obtained from these mice was gently homogenised in 0.9% saline to produce a suspension. 10^5 TLX5 lymphoma cells obtained from the routine passage as ascites, were innoculated subcutaneously in the inquinal region of male Cba/ca mice; or 10^6 M5076/A reticulum cell sarcoma cells were implanted into the rear left leg of BDF_1 female mice.

The first dose of NMF was administered 24 hours after implantation.

Tumours were implanted by Mr. S. P. Langdon or Mr. D. Chubb.

8.6 Repeated administration of NMF

400 mg/Kg NMF was administered IP to male Cba/ca mice with or without TLX5 lymphoma, on three consecutive days. At least nine plasma samples were obtained after administration of the third dose.

In the case of the BDF_1 mice bearing the M5076/A reticulum cell sarcoma, altogether 2 g was administered IP either on five consecutive days (400 mg/Kg each dose) or during 17 days, every other day (220 mg/Kg each dose). At least seven plasma samples were obtained after administration of the first and final dose.

8.7 Clinical Study

300, 600 or 1200 mg/m^2 NMF was administered IV as a bolus injection or as an infusion in 0.9% saline over 30 or 60 minutes, or orally, in which case the drug was diluted in fruit juice and was drunk over a period of 15 to 30 minutes.

5 ml blood samples were taken, up to 24 hours after administration and serum or plasma was separated from these samples. Urine was collected every 6 hours, up to a maximum of 120 hours.

300 mg/m^2 NMF was also infused IV repeatedly on five consecutive days. In these patients, plasma samples were obtained immediately after the first and then after the final dose.

All samples were frozen at -20^{o}C and transported with dry ice. This work was supervised by Dr. E. Newlands at the Charing Cross Hospital, London, and Dr. G. McVie at the Netherlands Cancer Institute, Plesmanlaan, Amsterdam.

In order to compare the amount of NMF given to man (mg/m^2) with that which was administered to mice (mg/Kg); 300, 600 and 1200 mg/m^2 is approximately equivalent to 7.2, 14.3 and 28.6 mg/Kg.

For the bioavailability studies, patients received an IV dose of 300 mg/m^2 (A.H., L.G., M.Z. and E.S.), 600 mg/m^2 (M.M. and I.W.) or 1200 mg/m^2 (J.D. and D.C.) NMF. After a rest period of 14 days, these patients then were given the same dose of NMF but orally, rather than IV.

8.8 Estimation of Pharmacokinetic Parameters

Plasma concentration of drug, metabolites or radioactivity was plotted against time after administration. The area under the plasma concentration-time curve (AUC) was estimated by the trapezoidal rule (Gibaldi and Perier, 1975) from the first sampling time and up to 24 hours after drug administration, unless otherwise stated.

Bioavailability was calculated using mean AUC data from the ratio:

$$\frac{\text{AUC IP,PO or IM}}{\text{AUC IV}}$$, described by Wagner (1975).

Apparent elimination half-life ($t_{\frac{1}{2}}$) was calculated from the equation:

$t_{\frac{1}{2}} = \ln.2 / k'$, where k' is the apparent elimination rate constant given by the slope of ln. blood concentration multiplied by time.

Lines of best fit and standard error of the slope of that line were calculated by least squares regression analysis based on a first-order elimination kinetics. Half-lives were only calculated from plasma concentration time profiles when the percentage error of the slope, obtained from a semi-logarithmic plot, was less than 10%.

The clearance of the drug from the plasma was calculated by the following method:

Clearance(c) = D/ AUC (ml. hr^{-1}).

where D is the dose of NMF administered and AUC is the area under the plasma concentration-time profile.

The rate constant of elimination for urinary excretion was determined from:

$\ln.(U_1-U) = \ln.U_1 - k_2 t$ (Gladtke and Hattingberg 1979).

The quantity of NMF excreted, U is substracted in each case from the initial dose, U_1. The quantity, U_1-U remaining in the body is obtained and plotted logarithmically against time, t, on a linear scale. A declining straight line is obtained with a slope of $-k_2$ if the elimination fits a linear kinetic model.

8.9 The <u>In-vitro</u> Metabolism of DMSF

2 mM or 20 mM DMSF was incubated for one hour using a 9,000 g liver metabolising system (described in sections 2.5.1 and 2.5.2).

10µl samples were collected up to one hour after addition of the substrate. At the same time, samples were taken from control incubations containing no liver fraction.

50µl or 100µl of acetone containing internal standard (30µg/ml TMU) was added to these samples which were then centrifuged and the resultant supernatent assayed for DMF as a metabolite of DMSF, by GC.

SECTION 9

RESULTS

9.1 Assay Sensitivity

Calibration curves for the estimation of N-alkylformamides and formamide in body fluids by GC were obtained by plotting peak-height ratios against the concentration of the compounds in standard solutions. The correlation coefficient (r) of the calibration curves used in each assay was greater than 0.99.

The limits of detection for NMF, NEF, DMF, DMSF and formamide were:
1 to 5 µg/ml, 3 to 5 µg/ml, 1 to 2 µg/ml, 5 to 7 µg/ml and 5 to 10 µg/ml respectively.

The reproducibility of the GC assay in the determination of NMF and DMF was examined by analysing peak-height ratios for at least five samples containing either 25 µg/ml NMF or DMF, or 300µg/ml NMF, in plasma. The coefficient of variation for 25µg/ml and 300µg/ml NMF was 4.2 and 3.9% respectively whilst that for DMF was 3.4%.

When radioactivity was counted after administration of 15µCi labelled NMF (equivalent to 400mg/Kg), the limits of detection were between 0.6µg and 1µg NMF equivalents per ml.

After injection of DMF, NMF, formamide and DMSF (all 50µg/ml) with 30µg/ml TMU the chromatogram, as shown in fig. (30) was obtained. Retention times were 1.1, 2.1, 3.1, 4.0 and 1.4 minutes respectively.

Fig.(31) shows the chromatogram of TMU and NEF: the latter had a retention time of 2.0 minutes.

Fig.(32) shows the gas chromatograms of plasma and

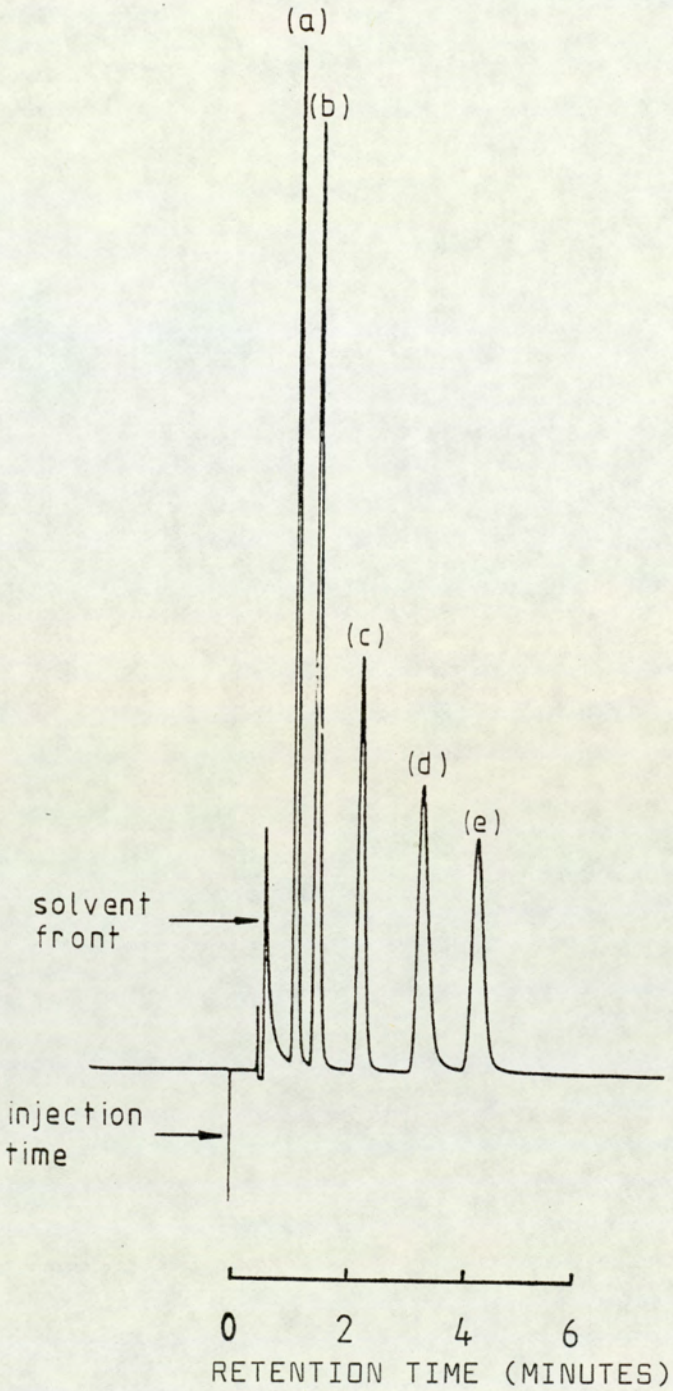


Fig. 30 Gas chromatogram of a standard solution of the N-alkylformamides by nitrogen-phosphorus detection. DMF (a), TMU (b), NMF (c), formamide (d) and DMSF (e) were dissolved in acetone to give a final concentration of 30µg/ml.

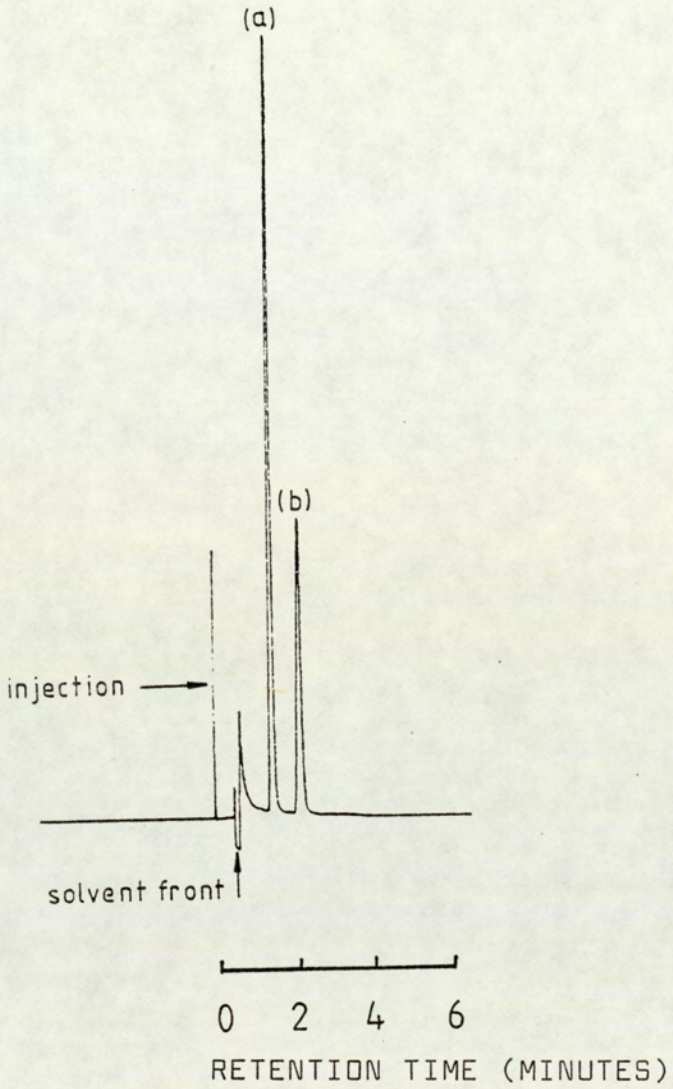


Fig. 31 Gas chromatogram of a standard solution containing 30µg/ml TMU (a) and NEF (b) in acetone.

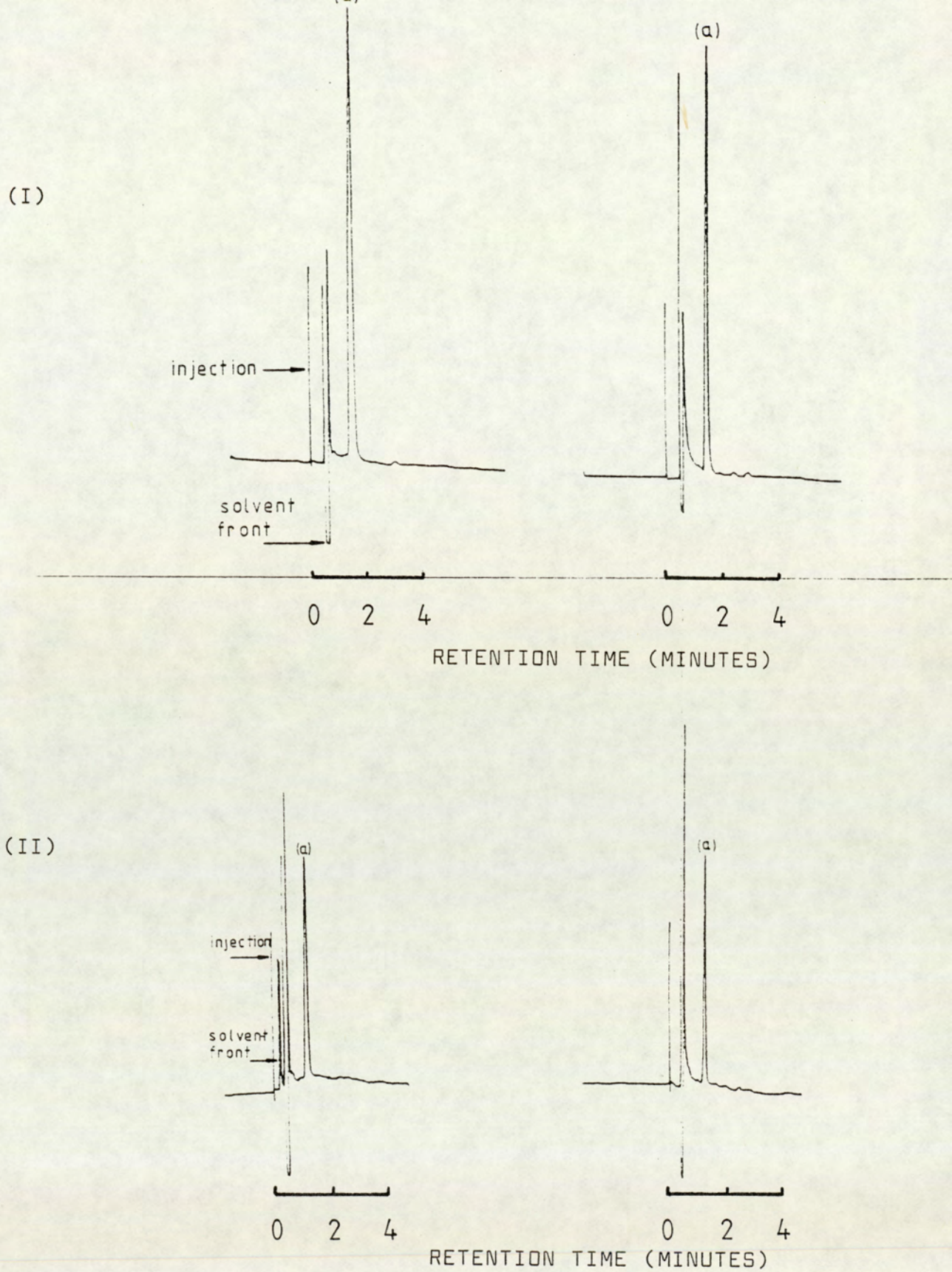


Fig. 32 Gas chromatograms of mouse (I) and patient (C.R.) (II) plasma (on the left) and urine (on the right), before administration of drug. (a) corresponds to the internal standard (TMU). Samples were prepared and treated according to section (8.3.1).

urine samples from a mouse and one patient (C.R.) who had not received any drug and where 30µg/ml and 5µg/ml TMU was added respectively.

Figs.(33A-D) show the gas chromatograms of urine and plasma samples after administration of NMF to one mouse and one patient (C.R.), and after administration of DMF, NEF and DMSF to mice. Retention times of these alkylformamides were identical to those from standard solutions shown in figs.(30) and (31).

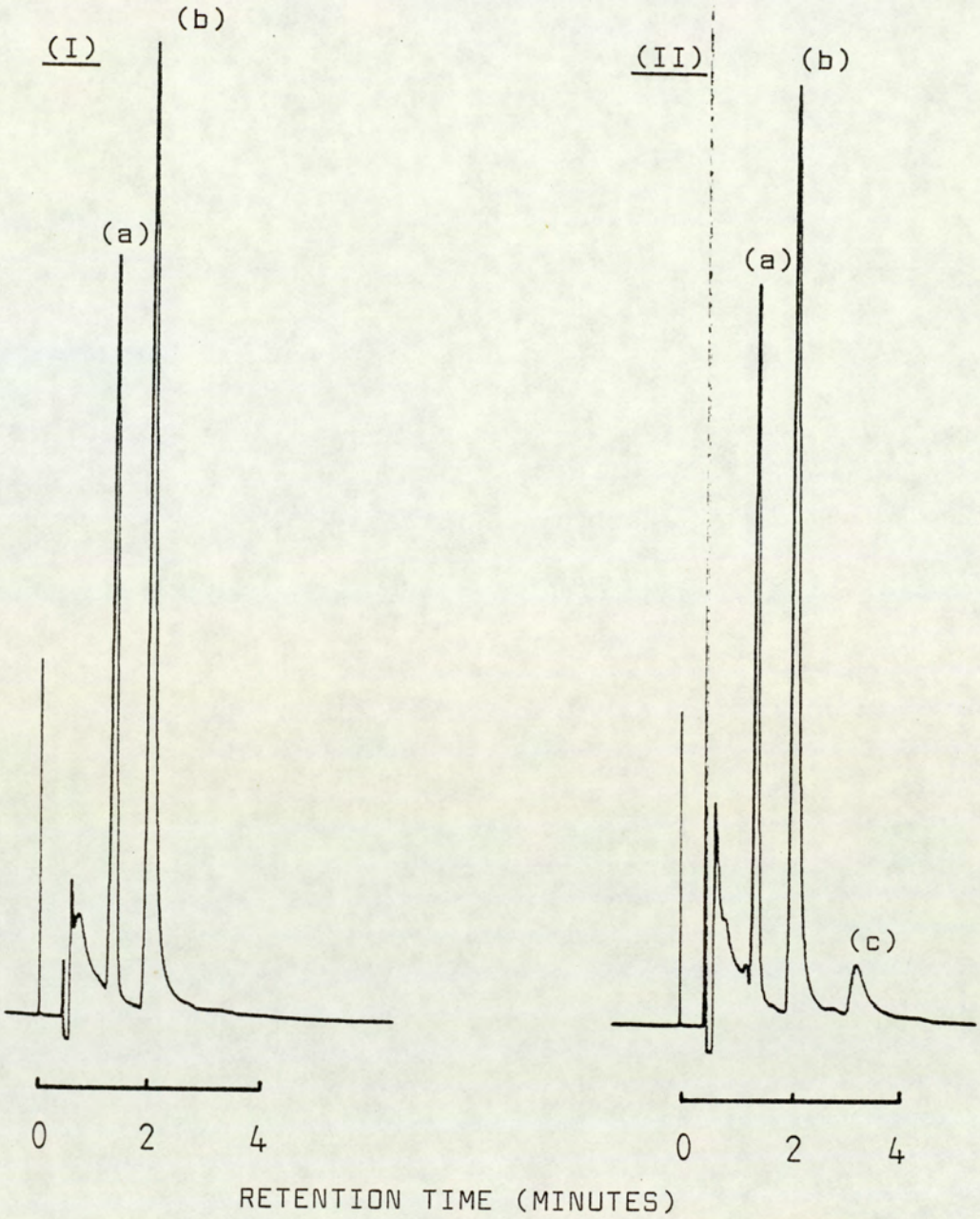


Fig. 33A Gas chromatograms of mouse plasma (I) and urine (II) after IP administration of 400 mg/Kg NMF. Plasma and urine samples were obtained 2 hours and 6 hours after drug administration respectively, and treated according to section (8.3.1). Peaks (a), (b) and (c) correspond to TMU (30 µg/ml), NMF and formamide respectively.

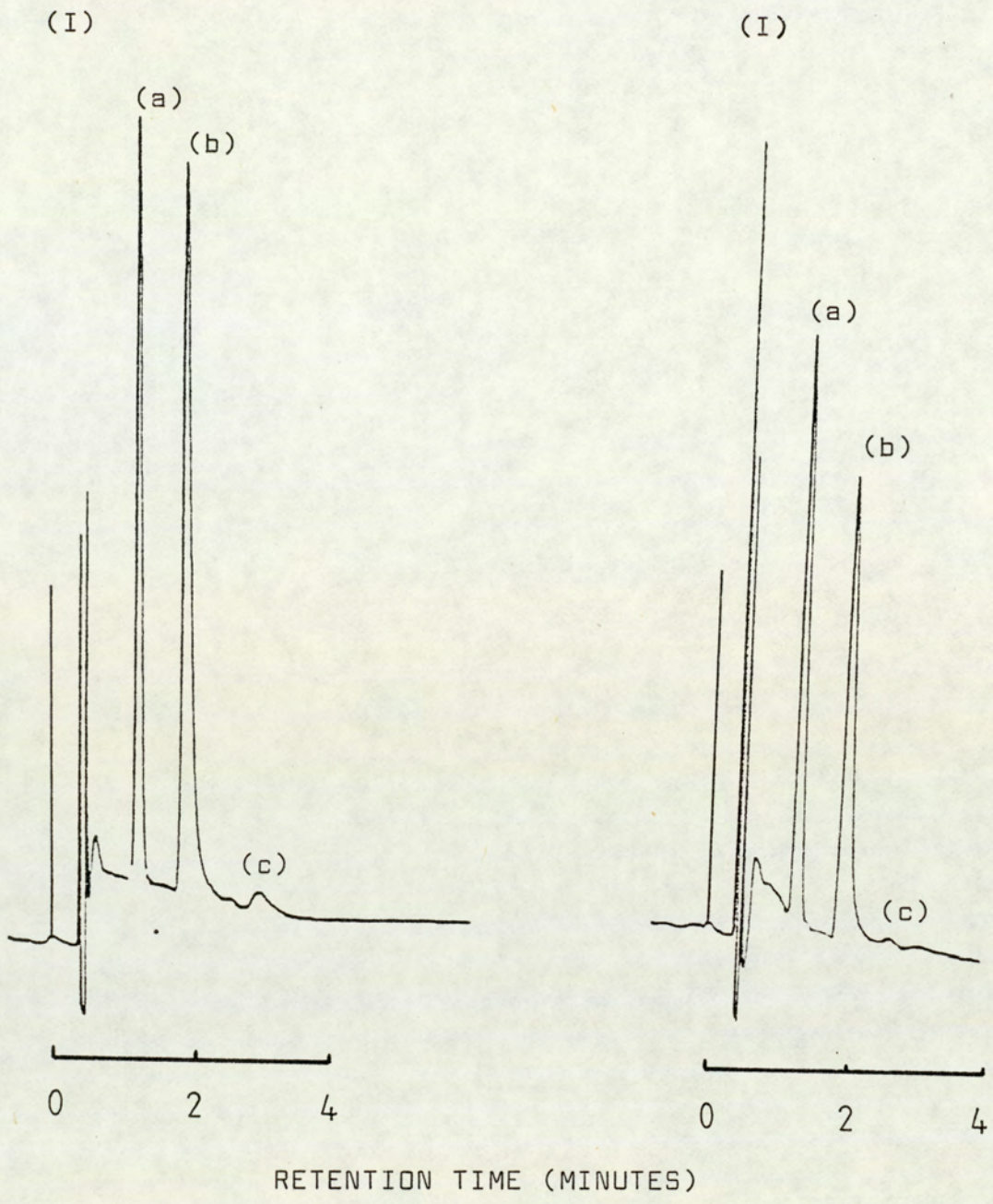


Fig. 338 Gas chromatograms of urine (I) and plasma (II) samples obtained from one patient (C.R.), two hours after IV administration of 600 mg/m^2 NMF. Peaks (a), (b) and (c) correspond to TMU (5 µg/ml), NMF and formamide respectively.

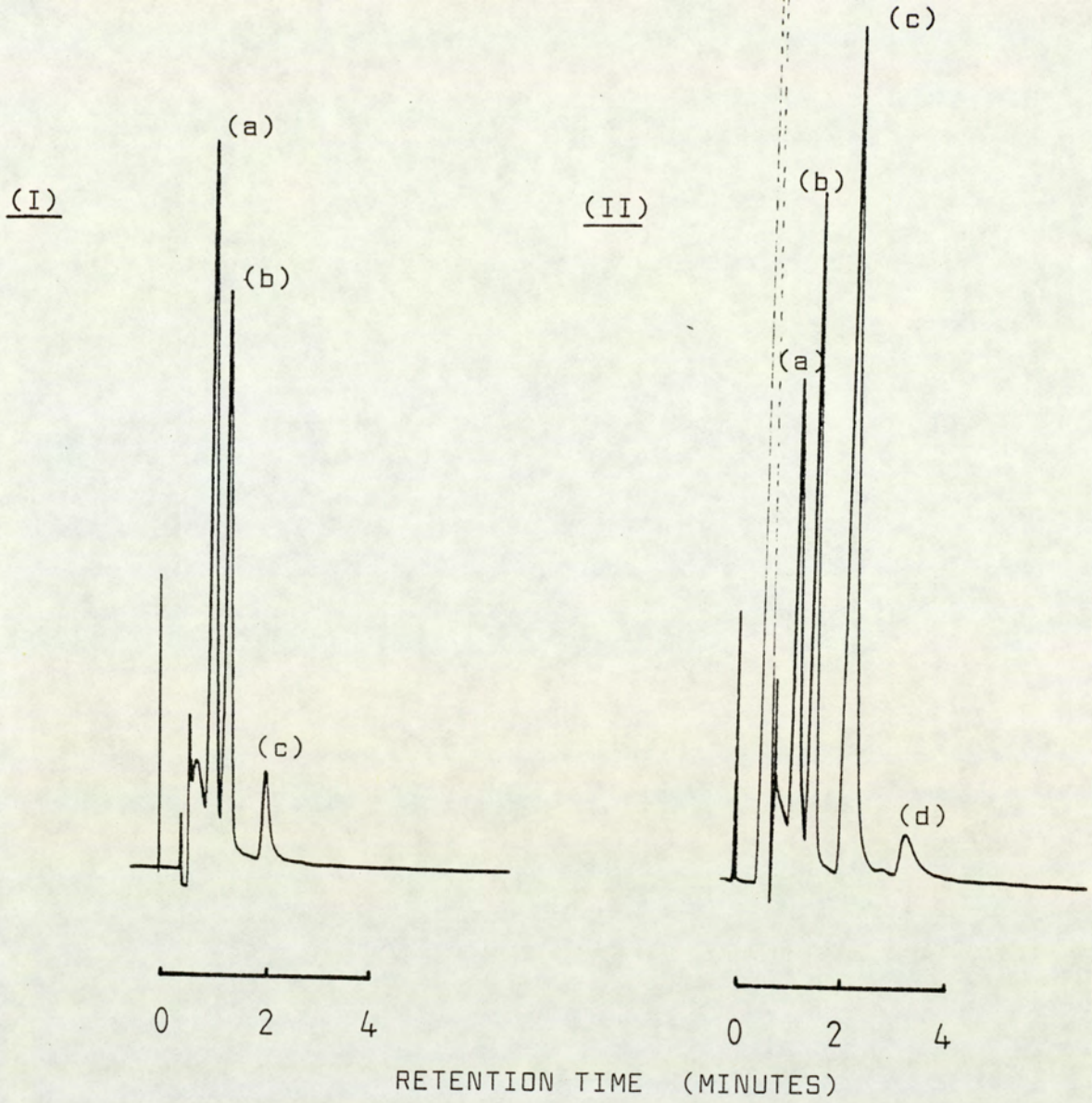


Fig. 33C Gas chromatograms of plasma (I) and urine (II) samples obtained from one mouse after IP administration of 496.4 mg/Kg DMF. Plasma and urine samples were obtained two hours and six hours after drug administration respectively. Peaks (a), (b), (c) and (d) correspond to DMF, TMU (30 μg/ml), NMF and formamide respectively.

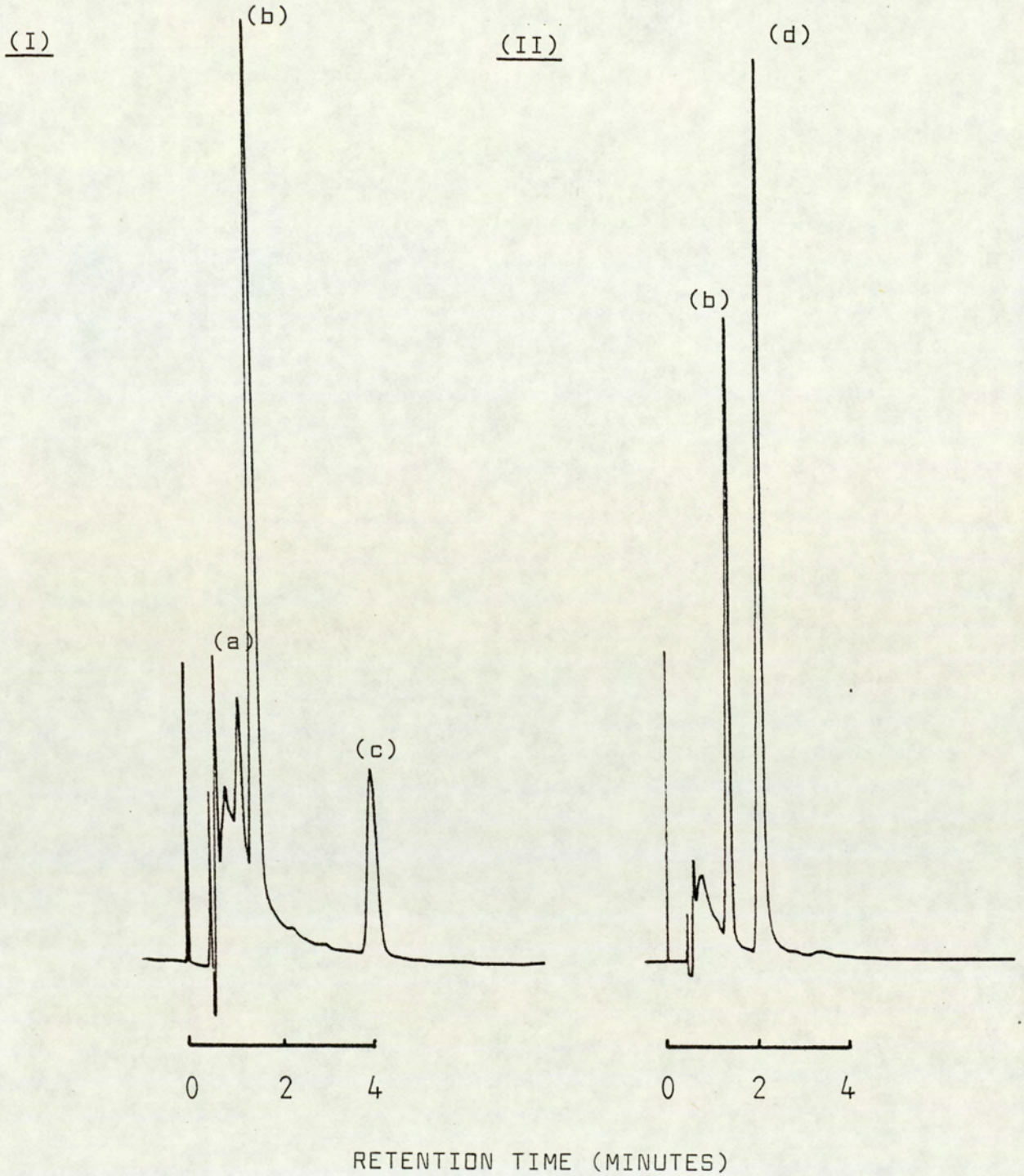


Fig. 33D Gas chromatograms of mouse plasma obtained two hours after IP administration of 100 mg/Kg DMSF (I) and 591 mg/Kg NEF (II). Peaks (a), (b), (c) and (d) correspond to DMF, TMU (30 μg/ml), DMSF and NEF respectively.

9.2 Plasma Disposition of NMF

9.2.1 Bioavailability of NMF

When NMF was administered IV, orally (see fig.34) or IM (see fig. 35), the disappearance of the drug from the plasma did not substantially differ from the plasma disposition after IP administration (see fig.36). A comparison of the plasma concentration-time profiles after administration of NMF by the routes described above is shown in fig. (37).

Area under the plasma concentration-time curves (AUC) was calculated up to 24 hours after administration of 400 mg/Kg NMF (table 6) and were only significantly different when IM was compared to IP administration ($p<0.03$). The bioavailability (systemic availability) of NMF was calculated from the AUC values shown in table (6) and was: 1.01, 1.10 and 1.36 after oral, IP and IM administration respectively (table 14). Bioavailability of NMF after oral administration of 80 mg/Kg was 0.76.

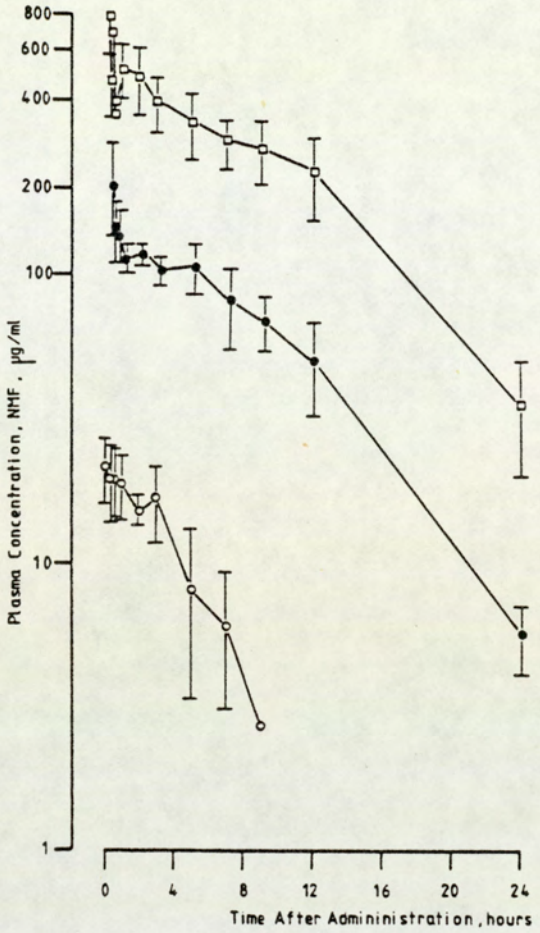


Fig. 34A Effect of dose on the plasma disposition of NMF after IV administration of 400mg/Kg (□), 80mg/Kg (●) or 14.3 mg/Kg (○) NMF. Plasma concentrations are plotted logarithmically against time. Values are the mean (± s.d.) of at least four mice.

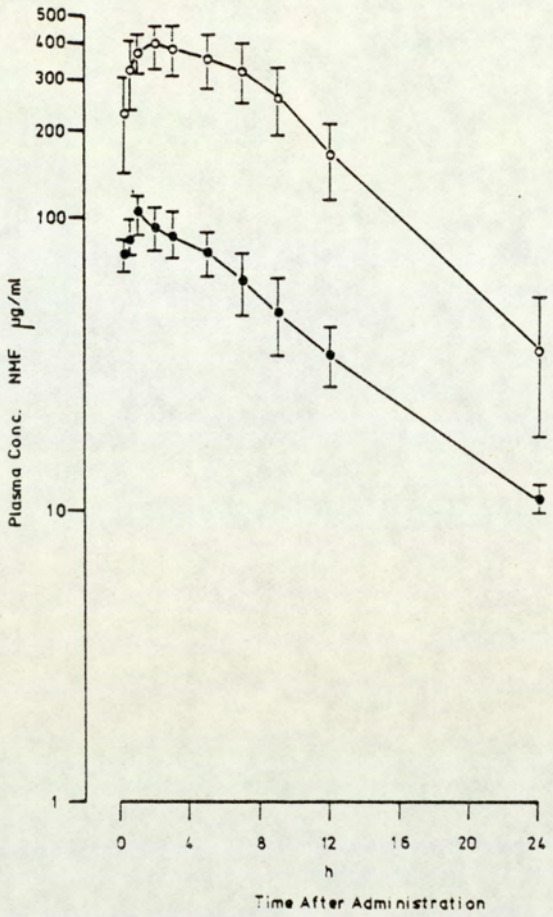


Fig. 34B Effect of dose on the plasma disposition after oral administration of 400 mg/Kg (O) and 80 mg/Kg (■) NMF. Plasma concentrations are plotted logarithmically against time. Values are the mean (± s.d.) of three to eight mice.

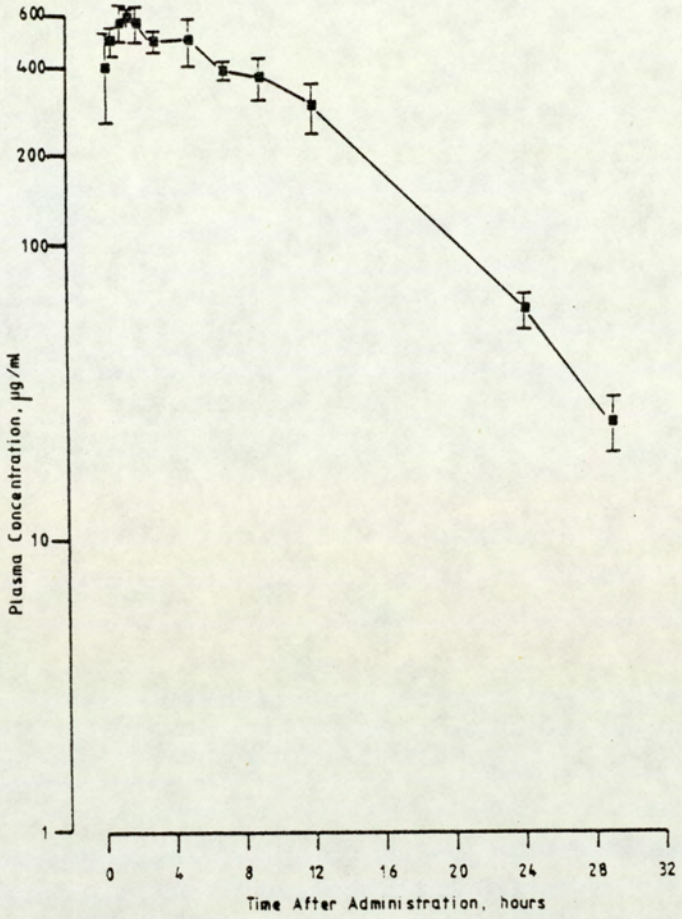


Fig. 35 The plasma disposition of NMF after administration (IM) of 400 mg/Kg NMF. Plasma concentrations are plotted logarithmically against time. Values are the mean ($\pm$ s.d.) of four mice.

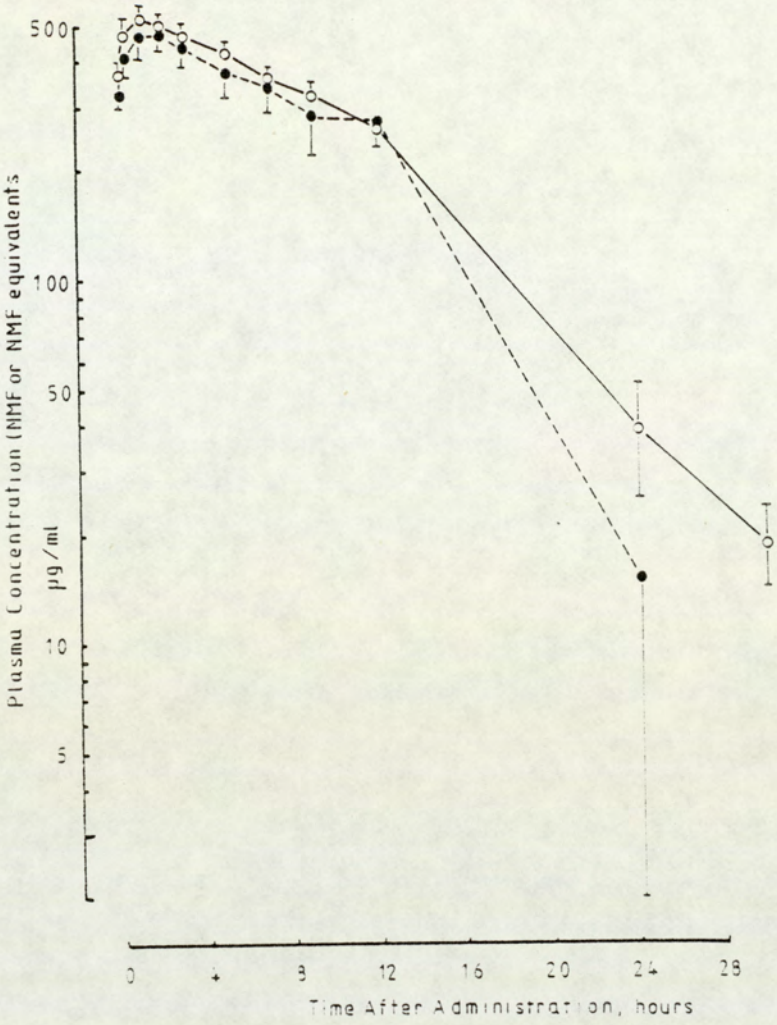


Fig. 36 Plasma disposition of NMF measured by GC (■) and of total radioactivity (○) after administration of 400 mg/Kg NMF or ^{14}C-NMF. Plasma concentrations are plotted logarithmically against time. Concentrations of radioactivity are expressed as NMF equivalents. Values are the mean (± s.d.) of at least five experiments, except for the point without an error bar which is the mean of two experiments.

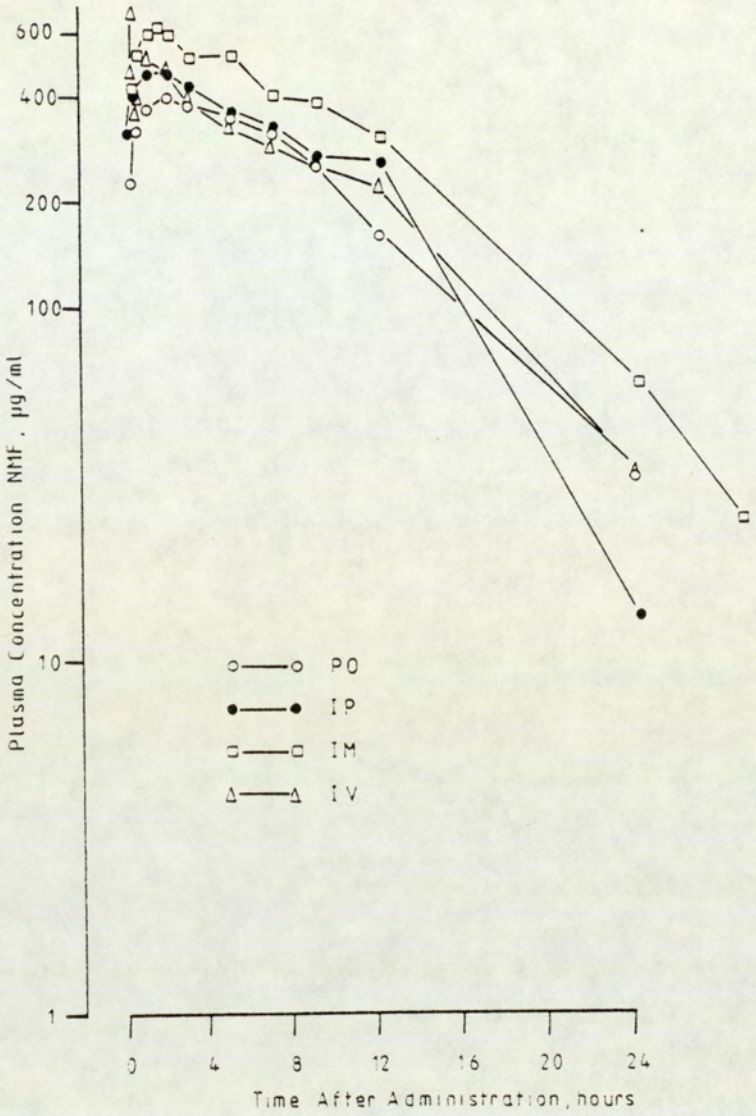


Fig. 37 Effect of route of administration on the plasma disposition of NMF after administration of 400 mg/Kg NMF. Experimental points are identical to mean values shown in figs. (34, 35 and 36).

TABLE 6

AUC Values Calculated up to 24 hours, except where marked*

	Route	dose (mg/Kg)	AUC($\mu g.hr.ml^{-1}$)	n
(a)	NMF			
	IV	400	5325 ± 1270	5
	IV	80	1399 ± 276	4
	IV*	14.3	92.8 ± 30	4
	IP	400	5842 ± 783	5
	PO	400	5360 ± 1117	8
	PO	80	1058 ± 201	3
	IM	400	7254 ± 705	4
	IP (cardiac puncture)	400	2522 ± 749	4

* denotes AUC values calculated up to 9 hours

(b)	^{14}C-NMF			
	IP	400	6339 ± 556	6
	IP**	14	54.2 ± 6.8	6
	IP (non-anaesthetised)	400	5551.5 ± 231.0	3

** denotes AUC values calculated up to 12 hours

(c)	NEF	400	2916 ± 120	4

n = number of experiments

9.2.2 Pharmacokinetic Model Describing NMF Disposition

The plasma disposition of unlabelled NMF as measured by GC was compared to that of ^{14}C(methyl)- labelled NMF (^{14}C-NMF) in order to ascertain how much of the total radioactivity measured could be accounted for as unmetabolised NMF. Fig. (36) shows that the plasma concentration-time profile, up to 24 hours after administration of ^{14}C-NMF is very similar to that of unlabelled NMF. AUC values were not significantly different from each other (see table 6; $p < 0.2$).

GC estimation of NMF in plasma samples, collected more than 24 hours after NMF administration, was unreliable because plasma levels were near or below the detection limit of the assay. However radioactive species derived from ^{14}C-NMF were measurable in the plasma for 192 hours after drug administration (fig. 38).

The disposition of radioactivity beyond 24 hours can be described by a curve which appears to exhibit first-order kinetics with at least two exponential components (fig. 38). The decline of radioactivity in the plasma with time after 60 hours after drug administration was considered to be linear ($r > 0.99$) and may well represent the elimination of NMF and/or its metabolites. The elimination half-life ($t_{\frac{1}{2}}$) was determined to be 71.1 ± 8.8 hours ($K_{el.} = 0.0091 \pm 0.0012\ hr^{-1}$).

Fig. (39) shows the plasma radioactivity concentration-time profile after IP administration of a low dose of

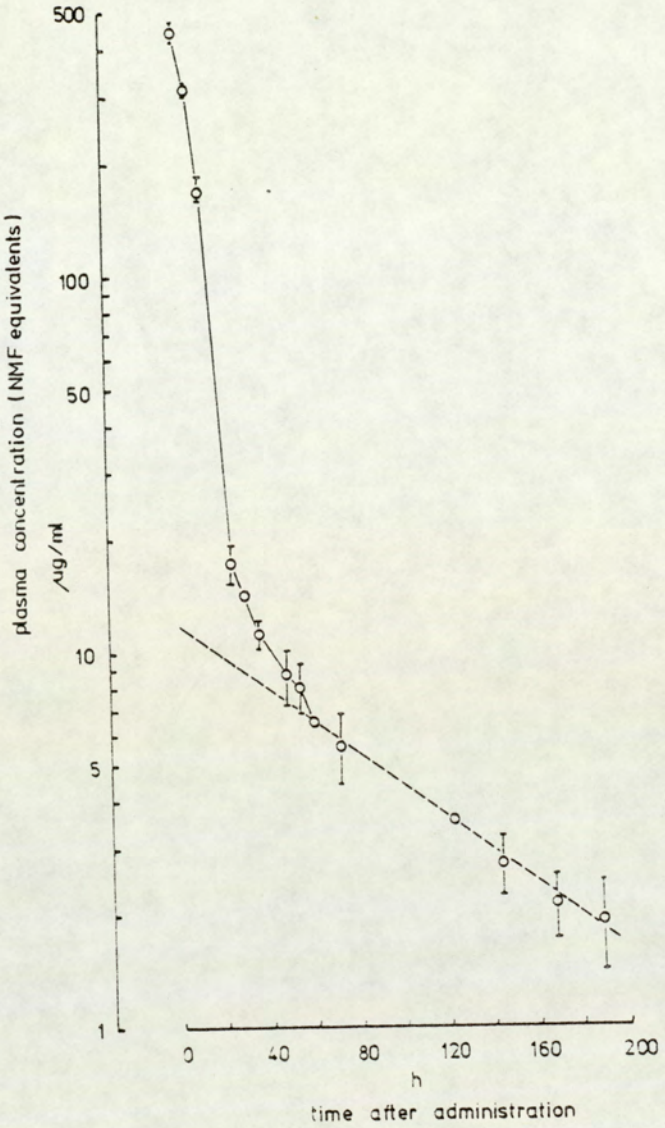


Fig. 38 Plasma disposition of radioactivity after IP administration of 400 mg/Kg ^{14}C-NMF. Plasma concentrations are plotted logarithmically against time. Values are the mean ($\pm$ s.d.) of six experiments; the values without error bars are the mean of three experiments. (-----) indicates the line obtained by linear regression analysis of points beyond 60 hours after drug accumulation, ($t\frac{1}{2}$ = 71.1 hours).

^{14}C-NMF (14mg/Kg). The initial concave-downward curvature exhibited at 400mg/Kg (fig. 36) is not apparent at this dose. The decline in radioactivity (expressed as equivalents of NMF) in the plasma after the peak concentration was reached (one hour after administration) and up to 9 hours after administration, was considered to be linear ($r > 0.99$). The elimination half-life computed for this line was 2.1 ± 0.2 hours and the elimination constant, Kel. was $0.34 \pm 0.04 hr^{-1}$.

Plasma radioactivity after 12 hours after administration of 14mg/Kg ^{14}C-NMF could not be measured reliably since levels were close to the detection limits. The plasma concentration of radioactivity shown at 25 hours after administration in fig. (39), was determined from a sample obtained by cardiac puncture; where a larger sample volume could be analysed (see section 8.2).

The graphs obtained by plotting plasma NMF concentration against time after IV, IM and oral administration of 400 mg/Kg exhibit an apparent concave-downward curvature (figs. 34 and 35) similar to that shown in fig. (36) after IP administration. However this curvature was less noticable when NMF was administered IP and plasma samples were collected by cardiac puncture (fig. 40); the AUC value calculated from this curve was also significantly different from that value obtained by the tail-bleeding method (see table 6; $p < 0.001$). The method of collecting blood samples from mice therefore seemed to influence the model describing the plasma

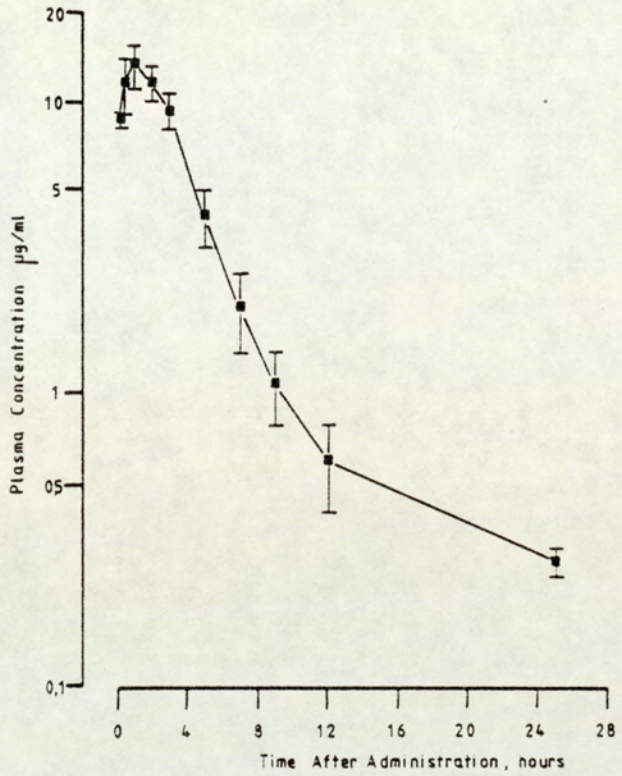


Fig. 39 Plasma disposition of radioactivity after IP administration of 14 mg/Kg ^{14}C-NMF. Plasma concentrations are plotted logarithmically against time. Concentrations of radioactivity are expressed as NMF equivalents. Values are the mean ($\pm$ s.d.) of six experiments except the point measured after 25 hours which is the mean of three mice, ($t_{\frac{1}{2}}$ within 12 hours = 2.1 hours).

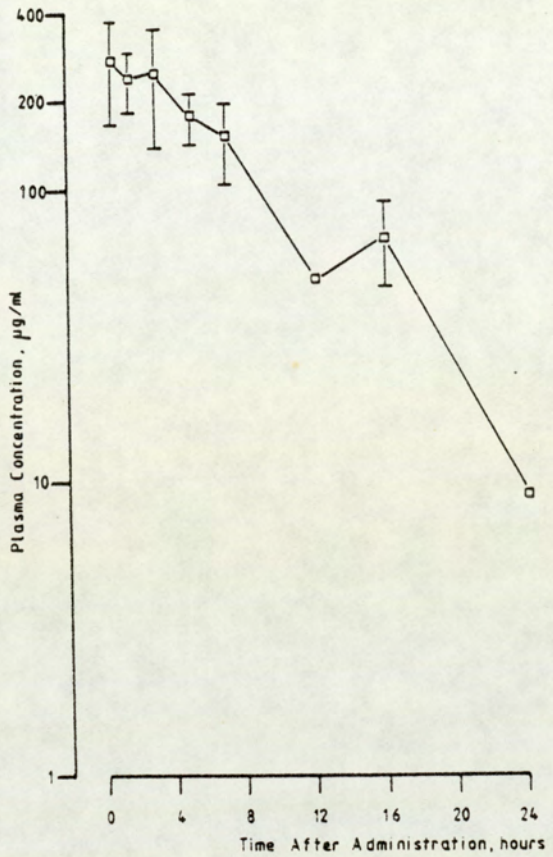


Fig. 40 Plasma disposition of NMF after IP administration of 400 mg/Kg NMF and where plasma samples were obtained by the cardiac puncture technique (see section 8.2.1). Plasma concentrations are plotted logarithmically against time. Values are the mean (± s.d.) of three experiments; points without error bars are the mean of duplicate determinations from one determination.

disposition profile of NMF.

The calculation of half-lives from these curves within 24 hours after drug administration would seem inappropriate since true elimination half-lives may only be estimated when linear kinetics prevail. The time required for plasma levels to decline from peak levels to 50% after oral, IM and IP administration of 400 mg/Kg NMF were 11, 12 and 10 hours respectively and 9 hours after oral administration of 80 mg/Kg (table 7).

Plasma clearance may be calculated irrespective of the model describing the kinetics of NMF (section 8.8) and values are given in table (8). The plasma clearance of NMF is significantly higher after administration of 14.3 mg/Kg NMF (3.3 ± 1.4 ml/hr; $p<0.03$) compared to the values obtained for 400 and 80 mg/Kg NMF (1.51 ± 0.29 ml/hr and 1.54 ± 0.15 ml/hr respectively).

The plasma concentration-time profile obtained from mice which were not anaesthetised (fig. 41) is not significantly different from that obtained from anaesthetised mice (table 6) which demonstrates that the mechanisms which govern the plasma disposition of NMF are not affected by the constituents of the anaesthetic. It should be noted that halothane is known to be hepatotoxic in man (Bruce, 1972) and mice are exposed to it at each time point.

The plasma concentration-time profile obtained from female Cba/ca mice was virtually superimposable on that obtained from male mice after administration of 400 mg/Kg NMF IP, and the AUC values (table 6) were not significantly different ($p > 0.1$), (see figs. 36 and 68).

TABLE 7

The Decline from Peak Concentration Values to 50 per cent of that Value

Route of Administration	Dose (mg/Kg)	Time to decline (hours)
Oral	400	11
IM	400	12
IP	400	10
Oral	80	9

TABLE 8

Plasma Clearance of NMF (Administered IV)

Dose (mg/Kg)	Clearance (ml/hr.)
400	1.51 ± 0.29
80	1.54 ± 0.15
14.3	3.30 ± 1.40

Clearance values were calculated according to section (8.8).

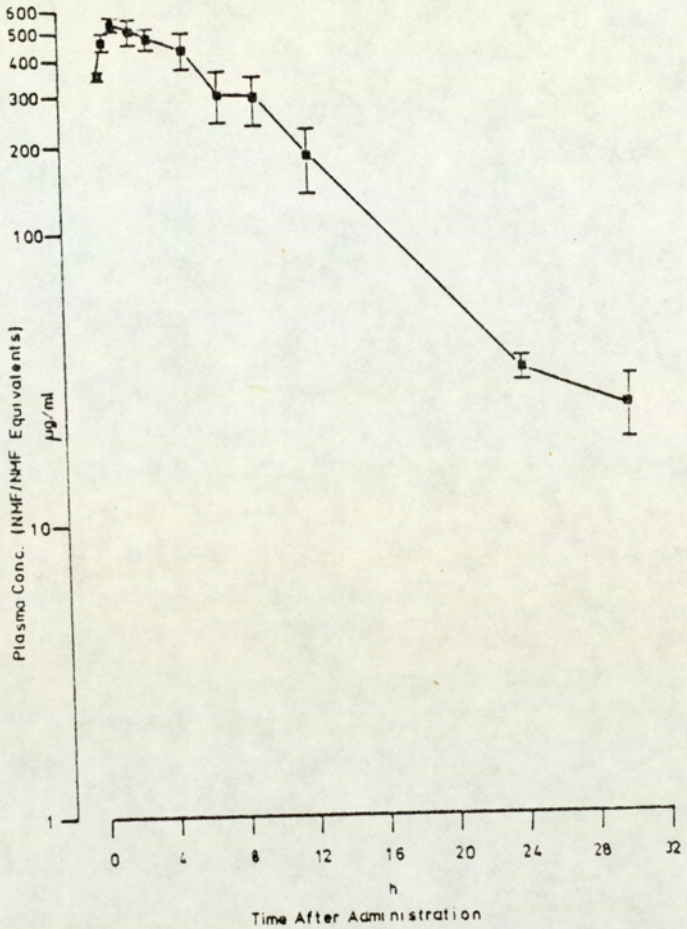


Fig. 41 Plasma disposition of radioactivity after IP administration of 400 mg/Kg ^{14}C-NMF to mice where samples were obtained without anaesthetic. Plasma concentrations are plotted logarithmically against time. Concentrations of radioactivity are expressed as NMF equivalents. Values are the mean ($\pm$ s.d.) of three experiments.

9.2.3 Influence of Repeated Administration on the Plasma Disposition of NMF

In order to maintain a plasma concentration of NMF which was effective against murine tumours sensitive to NMF, it was necessary to administer the drug repeatedly (Gescher et al., 1982). Repeated administration of NMF may influence its disposition. Multiple dosing could for example result in induction or inhibition of its own metabolism.

In order to evaluate such changes in the disposition, 400 mg/Kg NMF was administered IP on three consecutive days and the plasma concentration-time profile was obtained after the final injection, (fig. 42). The shape of the curve was similar to that obtained after a single IP injection of 400 mg/Kg NMF (fig. 36) and there is no significant difference between their AUC values (table 9)($p > 0.2$).

A similar experiment was performed using mice implanted with the TLX5 lymphoma and the resultant plasma concentration-time profile is shown in fig. (43). AUC values (table 9) calculated from figs. (42) and (43) were virtually identical.

The presence of tumour does not therefore appear to influence the plasma disposition of NMF and an additional experiment was carried out in order to verify this hypothesis. Tumour-bearing mice were administered with a single dose of 400 mg/Kg NMF and the plasma concentration-time profile obtained (fig. 44) was virtually superimposable on that in fig. (36), up to 12 hours after administration. A comparison of AUC values up to 24 hours after administration (tables 5, 6 and 9) reveal no significant difference ($p > 0.5$).

TABLE 9

Repeated Administration of NMF

AUC values calculated up to 24 hours except *

Route	Tumour type	Schedule	Dose (mg/Kg)	AUC	n	Day plasma sample taken
IP	(1)	Q01DX03	400	7097 ± 1464	6	3
IP	No tumour	Q01DX03	400	6921 ± 2100	9	3
IP	(1)	Q01DX01	400	6595 ± 564	6	1
IP*	(2)	Q01DX05	400	1802 ± 293	4	1
IP*	(2)	Q01DX05	400	1940 ± 304	4	5
IP*	(2)	Q02DX09	220	939 ± 117	4	1
IP*	(2)	Q02DX09	220	1282 ± 83	4	17

* denotes AUC values calculated up to 7 hours

n = number of experiments

(1) = TLX5 lymphoma

(2) = M5076/A reticulum cell sarcoma

Q01DX03 = NMF administered every day for three consecutive days

Q01DX05 = NMF administered every day for five consecutive days

Q02DX09 = NMF administered every other day for 17 days

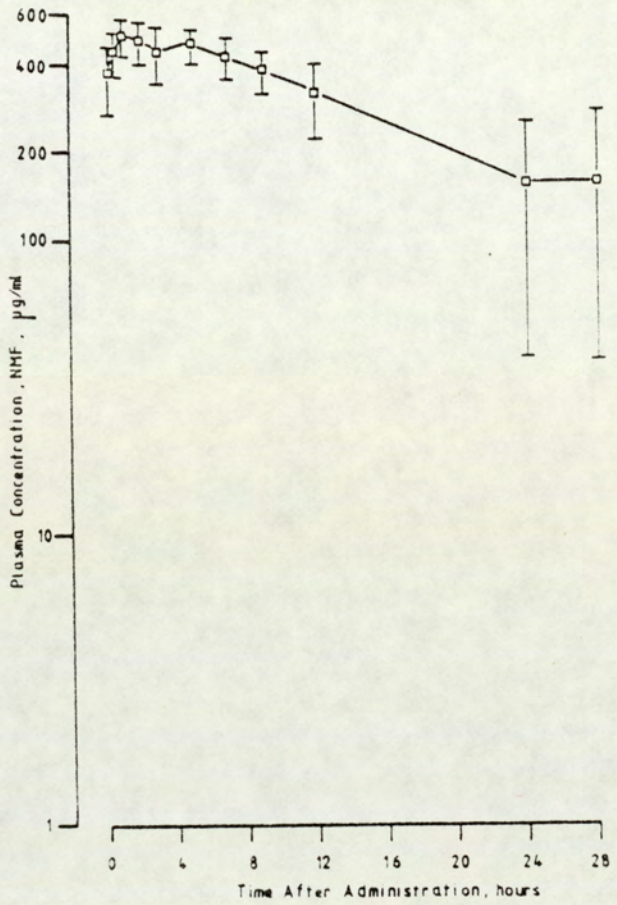


Fig. 42 Plasma disposition of NMF after IP administration of three daily consecutive doses of 400 mg/Kg NMF. Plasma samples were obtained from mice after the final injection. Plasma concentrations are plotted logarithmically against time. Values are the mean ($\pm$ s.d.) of at least six mice.

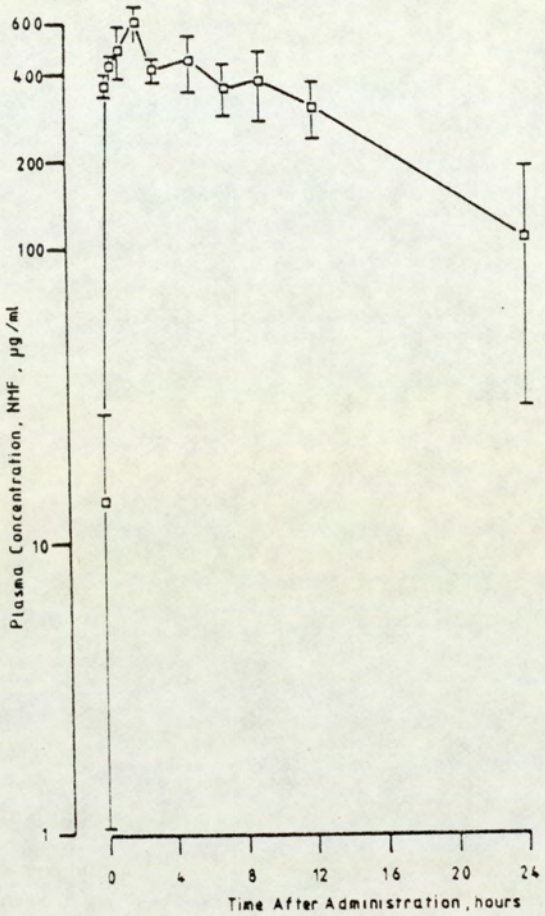


Fig. 43 Plasma disposition of NMF after IP administration of three consecutive daily doses of 400 mg/Kg NMF to mice implanted with the TLX5 lymphoma (10^5 cells). Plasma samples were obtained from mice immediately before, and up to 24 hours after the final injection. Plasma concentrations are plotted logarithmically against time. Values are the mean ($\pm$ s.d.) of three to six mice.

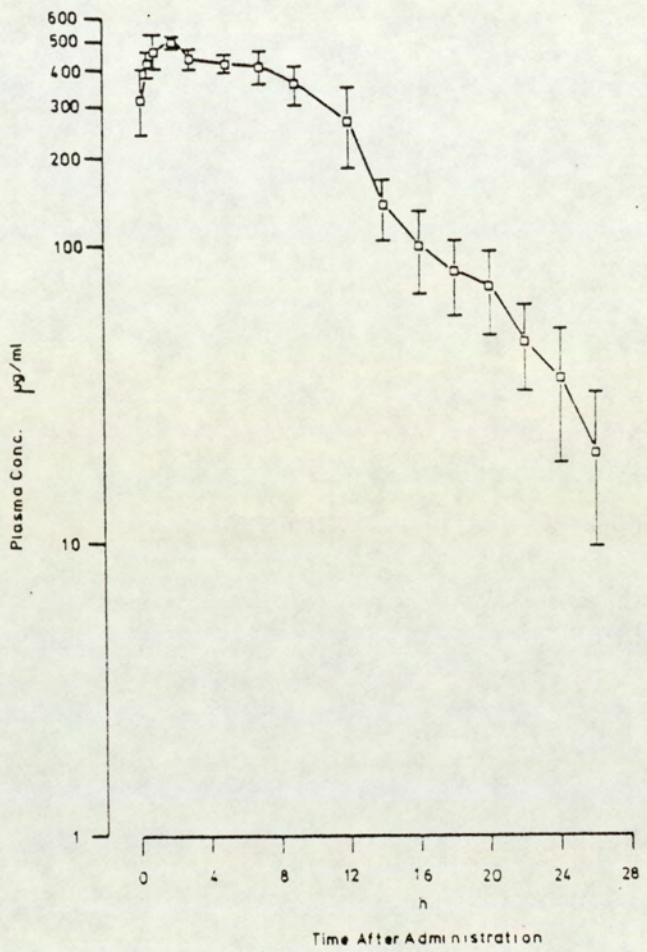


Fig. 44 Plasma disposition of NMF after administration of a single IP dose of 400 mg/Kg to mice implanted with the TLX5 lymphoma (10^5 cells). Plasma concentrations are plotted logarithmically against time. Values are the mean ($\pm$ s.d.) of at least six mice except those values obtained from mice, in a separate experiment, between 14 hours and 22 hours, which are the mean ($\pm$ s.d.) of three mice.

Repeated administration of NMF resulted in a more variable plasma concentration of the drug than that obtained after a single dose, at the 24 hour sample-time, (figs. 42 and 43). This was speculated to be due in part, to the known hepatotoxity of NMF (Lundberg, et al., 1981 and Myers, et al., 1956) which may cause a change in the ability of the liver to clear the drug. Therefore livers were removed from these mice and inspected for gross manifestations of damage. The appearance of the liver surface of some animals which had received multiple dosing was discoloured, which may be an indication of hepatic necrosis.

In a further attempt to evaluate the effect of repeated administration of NMF, mice were implanted with the M5076/A reticulum cell sarcoma and received 400 mg/Kg NMF IP on five consecutive days. Another group of mice was administered 220 mg/Kg NMF every other day for 17 days which was more effective against the tumour than the former schedule, (Langdon, S. P., unpublished). A comparison of the two schedules was made by obtaining plasma concentration-time profiles up to seven hours after administration of the first and final injections (fig. 45).

When the low dose was given over a longer period, the AUC values calculated from the plasma concentration-time profiles after the first and final administration, were significantly different (table 9; $p<0.002$). However AUC values obtained after administration of the high dose on day 1 were not significantly different from those obtained

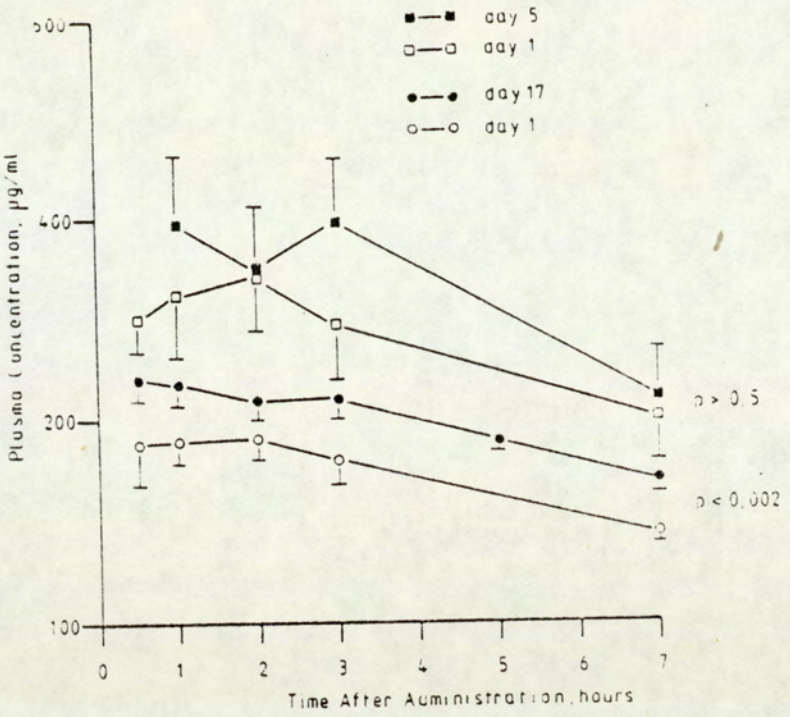


Fig. 45 Effect of repeated administration of NMF on the plasma disposition of NMF. 2 g NMF was administered IP over five consecutive days (square symbols) or 17 alternate days (circle symbols). Plasma samples were obtained from female BDF_1 mice implanted with the M5076/A reticulum cell sarcoma (10^6 cells), after the first (open symbols) and final administration (closed symbols). Values are the mean ($\pm$ s.d.) of at least four mice.

after the final day (table 9; $p > 0.5$).

It is therefore possible that the significant plasma accumulation of NMF is responsible for the increased efficacy of the low dose schedule.

9.3 Plasma Disposition of NEF

A number of murine tumours are sensitive to NMF whereas NEF is without activity against these experimental tumour models (see section 6.1). To see whether there is a pharmacokinetic basis for this difference between the two agents which may account for the structural requirement for activity, the plasma disposition of NEF was studied.

Fig. (46) shows the plasma concentration-time profile of NEF, up to 24 hours after administration. The apparent concave-downward curvature, previously described after NMF administration(fig. 36) was also observed for NEF. However a comparison of AUC values (table 6) reveals a marked difference between the profiles of the two agents and this difference is statistically significant ($p < 0.0005$). The peak plasma levels of both NMF and NEF were reached after two hours but the peak concentration of NEF was less than half that of NMF.

This difference in systemic availability between NMF and NEF might indicate a pharmacokinetic explanation for the requirement of N-methyl moieties in the molecule for antitumour activity: i.e., it is possible that NEF is not an antineoplastic agent because it does not reach the tumour site at sufficient concentrations.

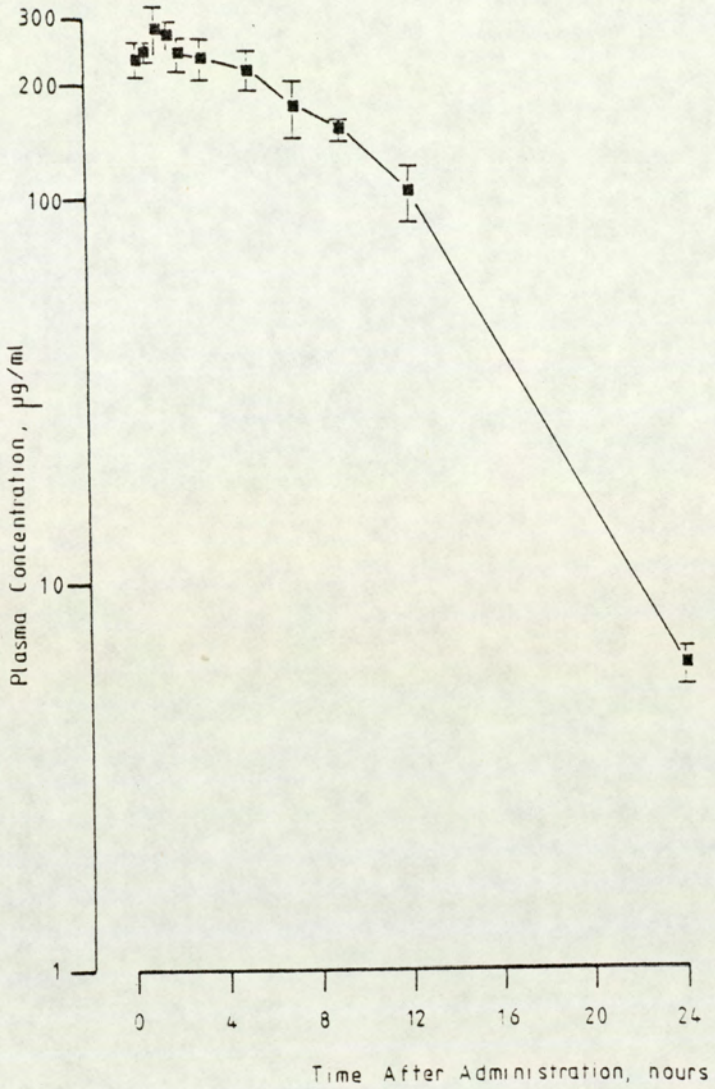


Fig. 46 Plasma disposition of NEF after IP administration of 591 mg/Kg NEF. Plasma concentrations are plotted logarithmically against time. Values are the mean (± s.d.) of four mice.

A comparison of the plasma disposition of NMF and NEF at equimolar doses is shown in fig. (67).

9.4 Plasma Disposition of DMF

DMF is only marginally active against some tumour models which are sensitive to NMF (see section 6.1). The plasma disposition of DMF was compared to that of NMF for the same reasons outlined for NEF (section 9.3).

Plasma concentration-time profiles were obtained for DMF after IP administration of 1400, 496 and 99 mg/Kg. These lower doses are comparable with doses given in the case of NMF, i.e. equimolar (see section 8.1).

Plasma concentrations of DMF could be reliably measured only up to 12 and 5.5 hours after administration of 496 and 99 mg/Kg respectively, whereas it could be quantified up to 16 hours after administration of 1400 mg/Kg.

The apparent concave-downward shape of the curve observed for the plasma concentration-time profile after NMF administration (fig. 36) was particularly marked for the two high doses of DMF (fig. 47).

The plasma concentration of the DMF metabolite NMF, was also measured after administration of the three DMF doses and the resultant plasma concentration-time curves are shown in fig. (48). A comparison of these curves up to 12 hours after administration reveals that the profiles for the two higher doses are similar whereas that of the low dose is appreciably smaller.

The AUC values calculated from the NMF profiles in fig. (48) after administration of the two high doses of DMF (table 10) are not significantly different ($p > 0.5$) which

TABLE 10

AUC Values After Administration of DMF

	Dose (mg/Kg)	AUC ($\mu g.hr.ml^{-1}$)	n	Calculated up to (hours)
DMF appearance	1400	9197 $\pm$ 1604	3	12
NMF appearance	1400	813 $\pm$ 110	3	12
NMF appearance	1400	1423 $\pm$ 100	3	24
DMF appearance	496.4	2434 $\pm$ 361	4	12
NMF appearance	496.4	709 $\pm$ 76	4	12
DMF appearance	99.3	178.7 $\pm$ 9.4	4	5.5
NMF appearance	99.3	175.7 $\pm$ 23.7	4	9

n = number of experiments

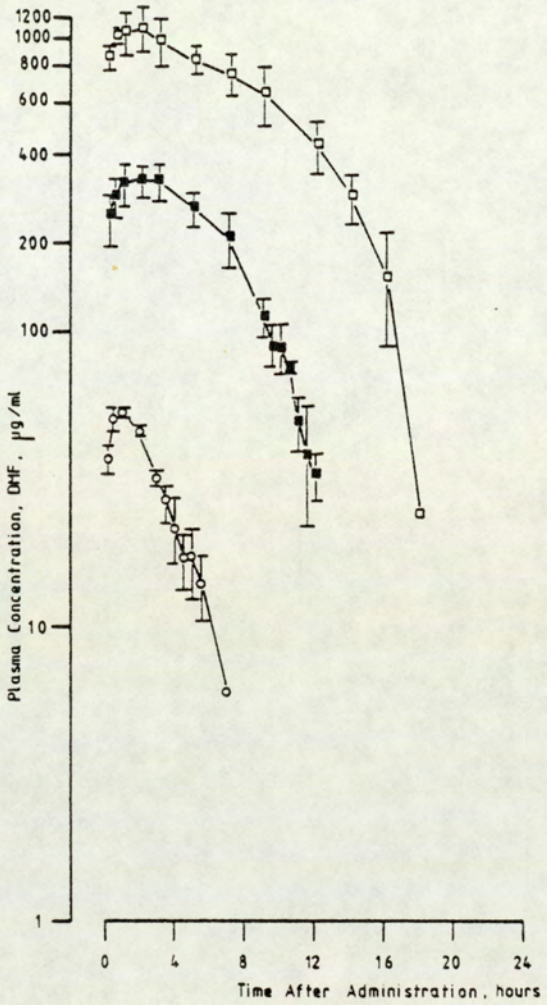


Fig. 47 Effect of dose on the plasma disposition of DMF after IP administration of 1400 mg/Kg (□), 496.4 mg/Kg (■) or 99.3 mg/Kg DMF (○). Plasma concentrations are plotted logarithmically against time. Values are the mean (± s.d.) of at least three mice or in the case of points without error bars, the concentration from one determination.

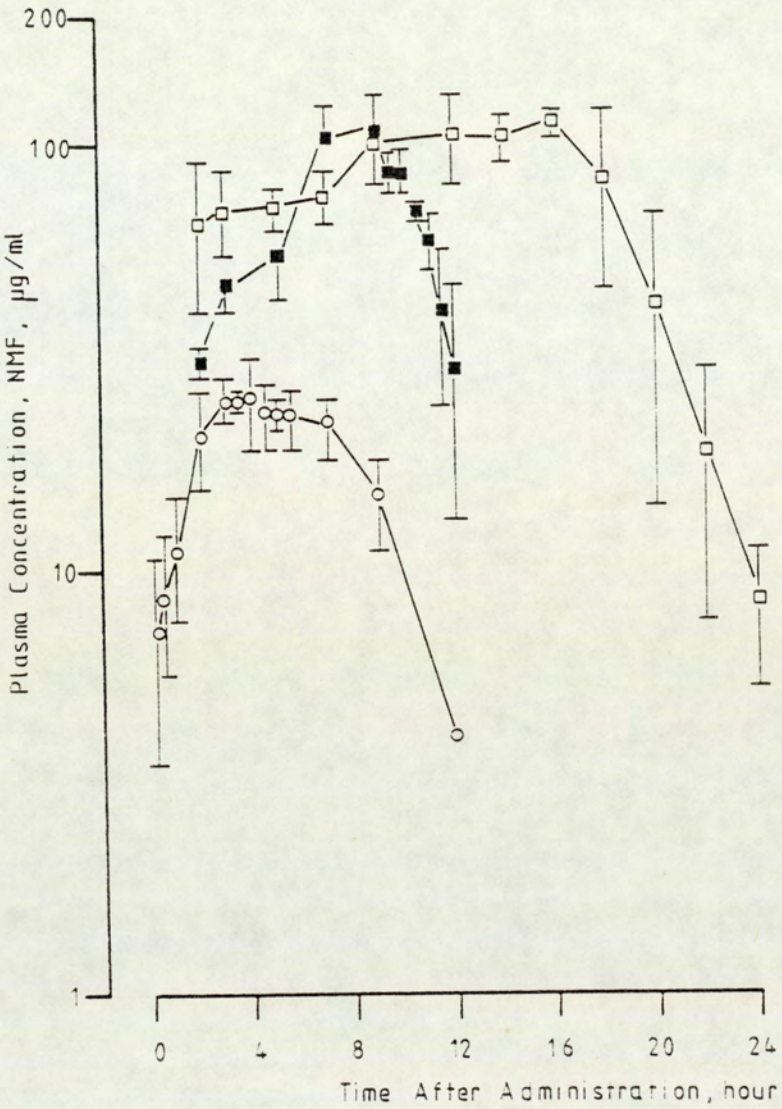


Fig. 48 Effect of dose on the plasma disposition of NMF as a metabolite of DMF after administration of 1400 mg/Kg (□), 496.4 mg/Kg (■) or 99.3 mg/Kg (○) DMF (see fig. 47). Values are the mean (± s.d.) of at least three mice or in the case of points without error bars the concentration from one determination.

indicates that the concentration of NMF influences the mechanisms by which it is removed from the plasma.

9.5 Plasma Disposition of DMSF

DMF and its thio derivative dimethylthioformamide (DMSF), fig. (27) are both inactive against the M5076/A reticulum cell sarcoma at a dose of 400 mg/Kg but DMSF is considerably more toxic. The LD_{50} for DMF and DMSF is 1220 and 260 mg/Kg/day respectively, after IP administration to BDF_1 female mice every other day for 17 days. (Langdon et al. unpublished).

The plasma disposition of DMSF was compared to that of DMF in order to test the hypothesis that there is a pharmacokinetic basis for the difference in toxicity between these two closely related agents.

Plasma concentration-time profiles of DMSF are shown in fig. (49) after IP administration of 400, 200 and 100 mg/Kg DMSF.

As stated above DMSF proved to be appreciably more toxic than DMF and all animals died between 12 and 24 hours after administration of 400 mg/Kg DMSF.

Plasma concentrations of DMSF and its metabolite DMF, could be reliably quantified up to 24 and 8 hours after administration of 200 and 100 mg/Kg DMSF respectively.

The appearance of DMF as a metabolite in the plasma after administration of DMSF (fig. 50) is most probably the consequence of a metabolic desulphuration (Gorrod, 1978) and the toxicological effect observed with DMSF may be produced via a release of a reactive form of sulphur.

In order to see whether DMSF was metabolised in-vitro it was incubated with a liver microsomal metabolising system and the resultant supernatent was assayed for DMF

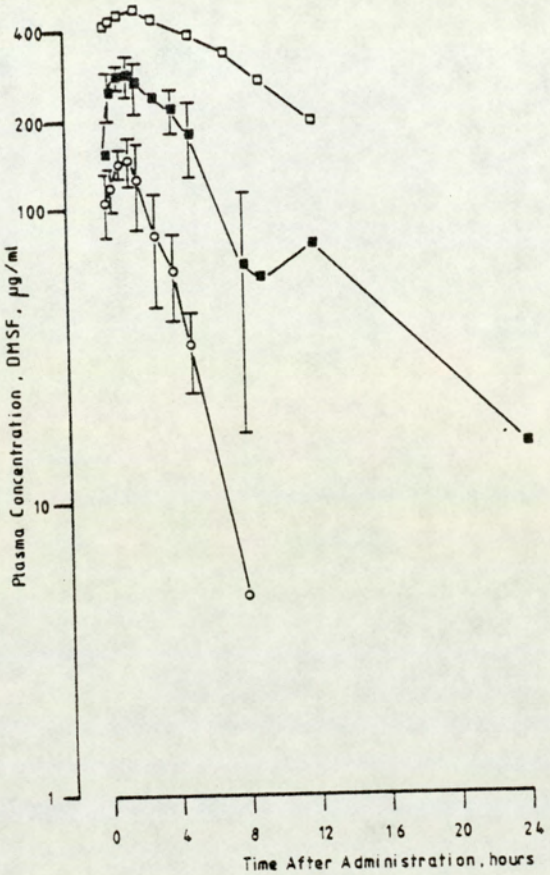


Fig. 49 Effect of dose on the plasma disposition of DMSF after administration of 400 mg/Kg (□), 200 mg/Kg (■) or 100 mg/Kg (○) DMSF. Plasma concentrations are plotted logarithmically against time. Values are the mean (± s.d.) of at least four mice or in the case of points without error bars the mean of two determinations.

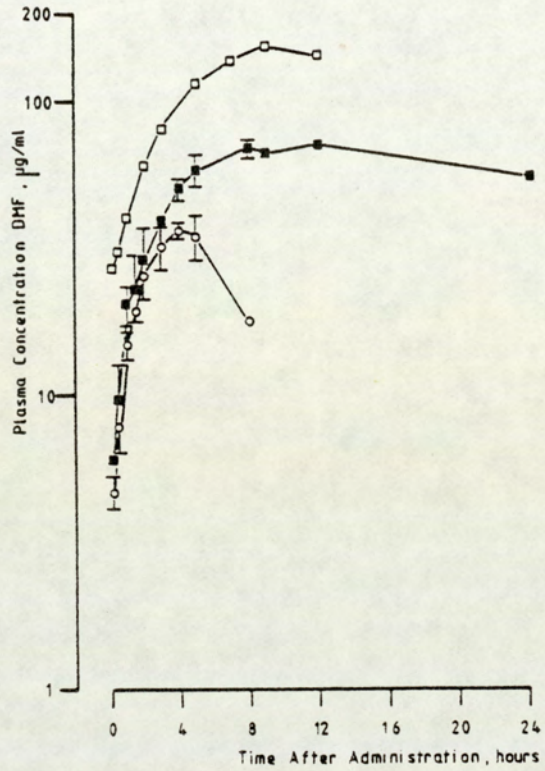


Fig. 50 Effect of dose on the plasma disposition of DMF as a metabolite of DMSF, after administration of 400 mg/Kg (□), 200 mg/Kg (■), or 100 mg/Kg (○) DMSF (see fig. 49). Values are the mean (± s.d.) of at least four mice or in the case of points without error bars, the mean of two experiments.

by GC (see section 8.9). The presence of DMF as a metabolite of DMSF was detected, but there was no appreciable difference from control samples.

9.6 Excretion of NMF and its Metabolite Formamide, into the Urine.

In order to study the elimination of NMF from the animals under investigation , parent drug and its metabolite formamide were assayed in urine samples which had been collected, including frequent washing of the metabolic cages, as described in section (8.2.2). Fig.(51) shows that 75.2 $\pm$ 7.2% of the radioactivity injected with NMF was excreted into the urine within 24 hours.

Only 26.4 $\pm$ 3.8% of the dose appeared in the urine as unchanged NMF and less than 2% as formamide (not shown in fig.51) as measured by GC.

Within the same time-span only 12.6 $\pm$ 5.0% of the dose was recovered as unchanged NMF from urine obtained without washing of the metabolic cages (see section 8.2.2), and less than 2% as formamide (fig.52).

What was identified as formamide by chromatography may be N-hydroxymethylformamide (see fig.28) which decomposed to formamide under the conditions of the GC analysis, (Ross, D., personal communication).

53.0 $\pm$ 4.7% of the total radioactivity was excreted into the urine within 12 hours (fig. 53) and the elimination within this period appears to be exponential ($P > 0.99$). The rate constant of elimination, K_2 was obtained from mean values of the quantity of NMF excreted (as described in section 8.8) and it was 0.4 $mg.hr^{-1}$.

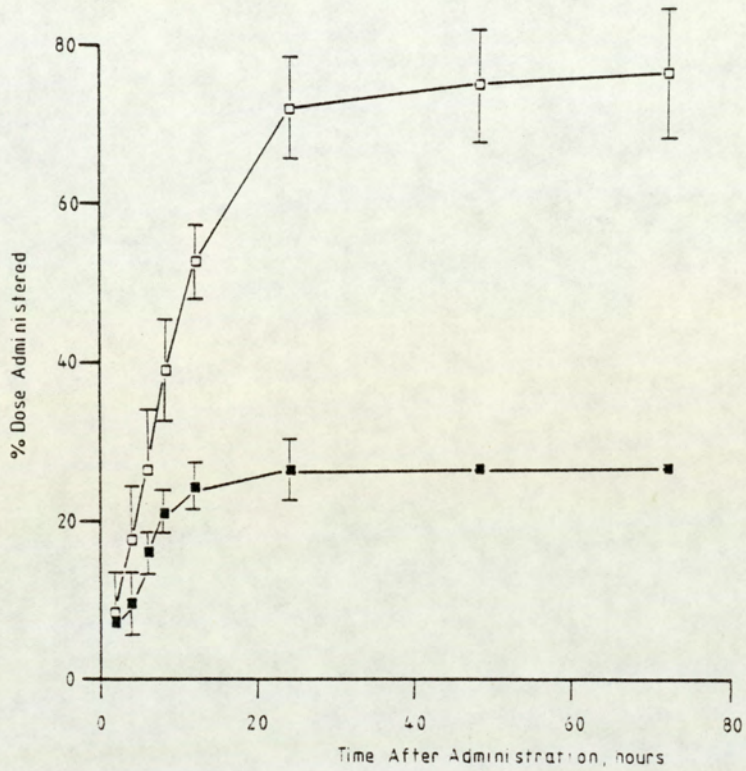


Fig. 51 Cumulative urinary excretion of NMF measured by GC (■) and of total radioactivity (□) after IP administration of 400 mg/Kg NMF or ^{14}C-NMF. Values are expressed as % of the dose administered and are the mean (± s.d.) of at least five experiments. There were no measurable levels of unchanged NMF in the urine after 24 hours.

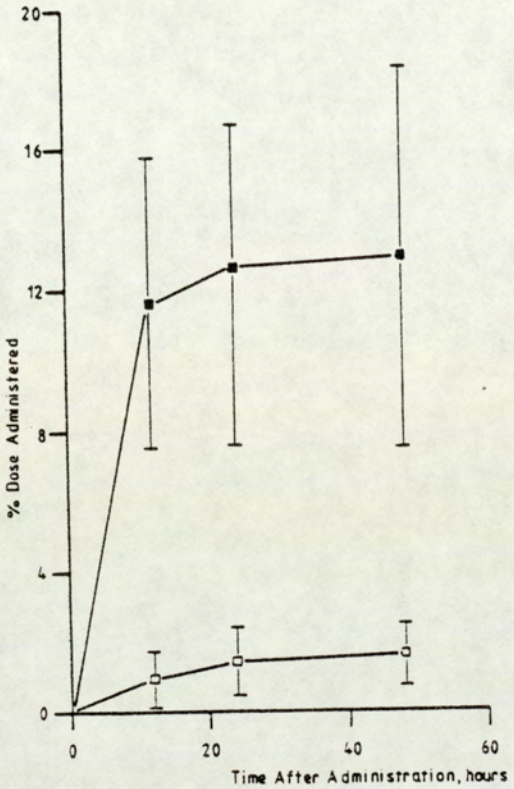


Fig. 52 Cumulative urinary excretion of NMF (■) and formamide (□) after IP administration of 400 mg/Kg NMF where samples were obtained without washing the metabolic cages. Values are expressed as % of the dose administered and are the mean (± s.d.) of three determinations. There are no measurable levels of unchanged NMF or formamide after 48 hours.

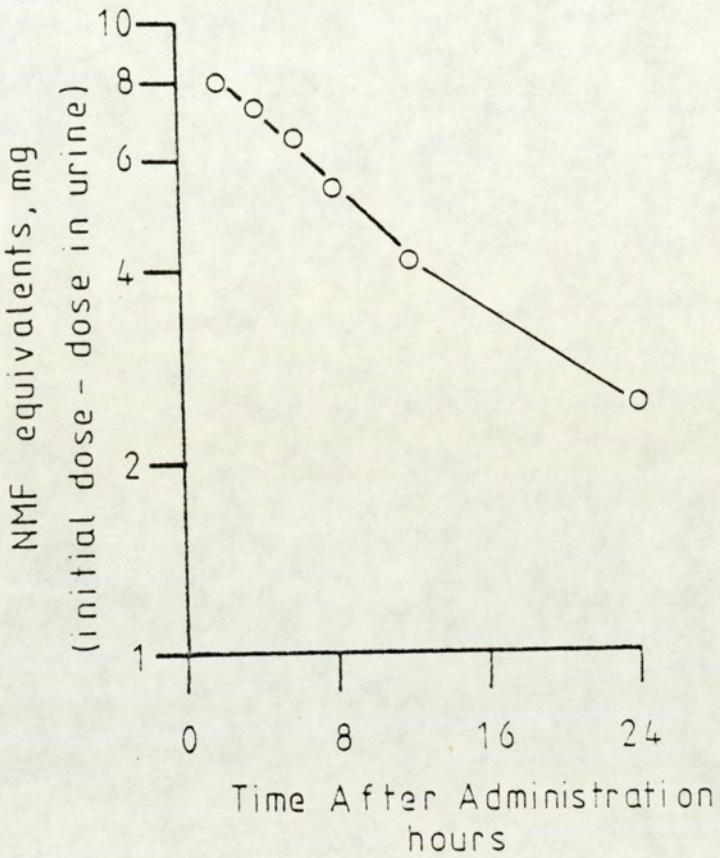


Fig. 53 Determination of the rate constant of elimination from the urinary excretion of total radioactivity after administration of 400 mg/Kg ^{14}C-NMF. The quantity (initial dose-dose in urine) represents radioactivity remaining in the body and is plotted logarithmically against time. The declining straight line has a slope of $-k_2$ which represents the urinary elimination constant (0.4 mg/hr).

Phenobarbitone is known to influence the in-vivo and in-vitro metabolism of certain N-methyl-containing drugs (Testa and Jenner, 1976) e.g. HMM (see section 1.4.2.4).

In order to see whether the metabolism of NMF was affected by phenobarbitone in-vivo, mice were pretreated with the agent (see section 8.2.2) and the urinary excretion of NMF, and its metabolite formamide was assayed by GC from urine samples obtained without washing the metabolic cages.

Table (11) shows the amount of unchanged NMF and formamide excreted into the urine within 24 hours after administration of 400 mg/Kg NMF to mice pretreated with phenobarbitone and control animals which received saline. A comparison of treated and control animals did not reveal a marked difference in the recovery of NMF or formamide, which indicates that the mechanisms responsible for the in-vivo metabolism of NMF are not affected by phenobarbitone.

TABLE 11

Phenobarbitone-treated Mice (urinary excretion)

Percentage of the total dose recovered within 24 hours after administration of NMF (400 mg/Kg)

Control	Treated		Compound assayed
	Mouse 1	Mouse 2	
7.2	7.3	9.8	NMF
2.9	4.0	3.5	Formamide

Each value represents the mean of duplicate determinations from one experiment.

9.7 Excretion of DMF and its Metabolites into the Urine.

DMF, NMF and formamide were assayed by GC from urine samples collected without washing the metabolic cages to minimize evaporative losses. The excretion of DMF and its N-demethylated metabolite NMF, is shown in fig.(54).

DMF appears to be rapidly metabolised since only 2.4% of the initial dose is excreted as unchanged drug within 24 hours, whereas 11.0% was recovered as the metabolite NMF within the same time-span (fig.54). The observation that DMF is rapidly metabolised in-vivo, is in agreement with the findings of Massman (1956). It is worth repeating that the compound which was chromatographically identified as NMF may have been N-hydroxymethyl-N-methylformamide, (see fig. 28) since this decomposed to NMF under the GC conditions, (Ross, D., personal communication).

Less than 1% of the dose was recovered as formamide (or N-hydroxymethylformamide) and this is not shown in fig.(54).

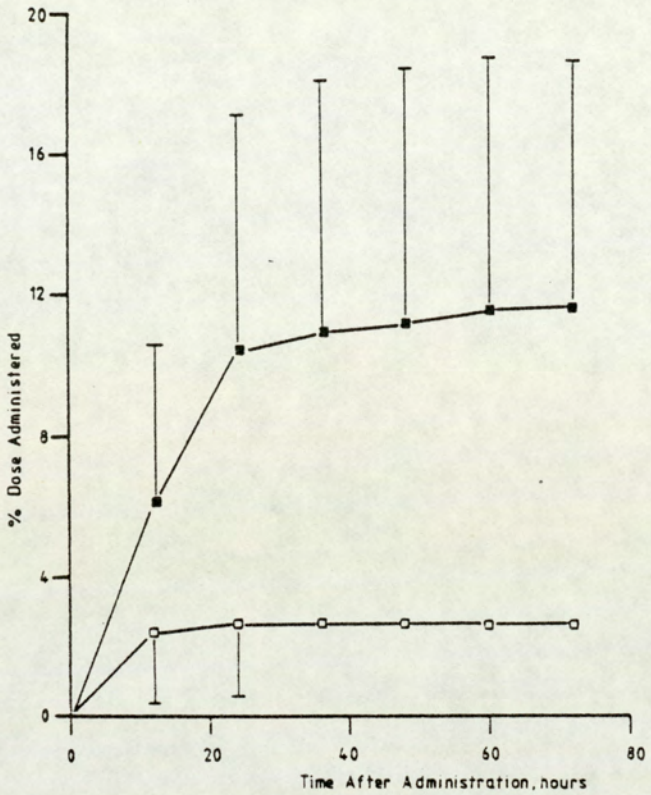


Fig. 54 Cumulative urinary excretion of DMF (□) and NMF (■) after IP administration of 496.4 mg/Kg DMF. Samples were obtained without washing the metabolic cages. Values are expressed as % of the dose administered and are the mean (± s.d.) of two to four experiments. In the case of points without error bars there were no measurable levels of unchanged DMF or NMF.

9.8 Clinical Investigations

Plasma samples were obtained from patients who received 300, 600 or 1200 mg/m^2 NMF as part of a phase I clinical investigation. (see section 8.7 for the equivalent dose given to mice).

Four patients (see table 12) received 300 mg/m^2 NMF, IV on five consecutive days and heparinised plasma samples were collected up to 24 hours after the previous injection and then one hour after each administration of successive days. The plasma concentration of NMF after repeated administration is shown in fig. (55). Within 97 hours, after administration of NMF, the peak plasma levels of unchanged drug, (see fig. 55), were not markedly different from each other. According to D'Incalci (1982) who conducted analogous investigations with etoposide, this indicates that plasma accumulation of NMF is not occurring over this five day period.

These patients also received the same dose orally (see table 12), one week after the final IV administration and a comparison of the plasma concentration-time profiles is shown in fig.(56). The oral bioavailability, calculated from the AUC values in table (13) was 1.3.

Unlike those patients receiving the low dose, (300 mg/m^2), only two of the patients who were given 600 mg/m^2 and 1200 mg/m^2 (table 12) IV, were subsequently administered the drug orally.

However, bioavailability was calculated from plasma samples obtained from these patients, and mean AUC values are shown in table (13). Mean bioavailability was 1.6 and 1.2 after oral administration of 600 and 1200 mg/m^2 respectively.

TABLE 12

Details of Patients who Received NMF During the Phase I Clinical Investigations

Dose (mg/m^2)	Patient Initials	Clinic	Protocol	Patient Sex
300	E.S.	N	2	female
	L.G.	N	2	female
	A.H.	N	2	male
	M.Z.	N	2	female
600	M.M.	C	6	female
	I.W.	C	6	male
	J.L.	N	3	female
	A.V.	N	3	female
	C.R.	N	3	female
	G.H.	N	4	female
	G.T.	N	5	female
1200	J.D.	C	6	male
	D.C.	C	6	male
	W.P.	N	1	female
	V.K.	C	6	female

N: Netherlands Cancer Institute, Amsterdam, (Plasma samples)
C: Charing Cross Hospital, London, (Serum samples)

1: Single dose IV
2: Five consecutive daily IV doses, 14 day rest period, then single PO treatment
3: Five consecutive daily IV treatments
4: Single oral dose
5: Five consecutive daily oral treatments
6: Single IV dose followed by a single oral dose after a 14 day rest period

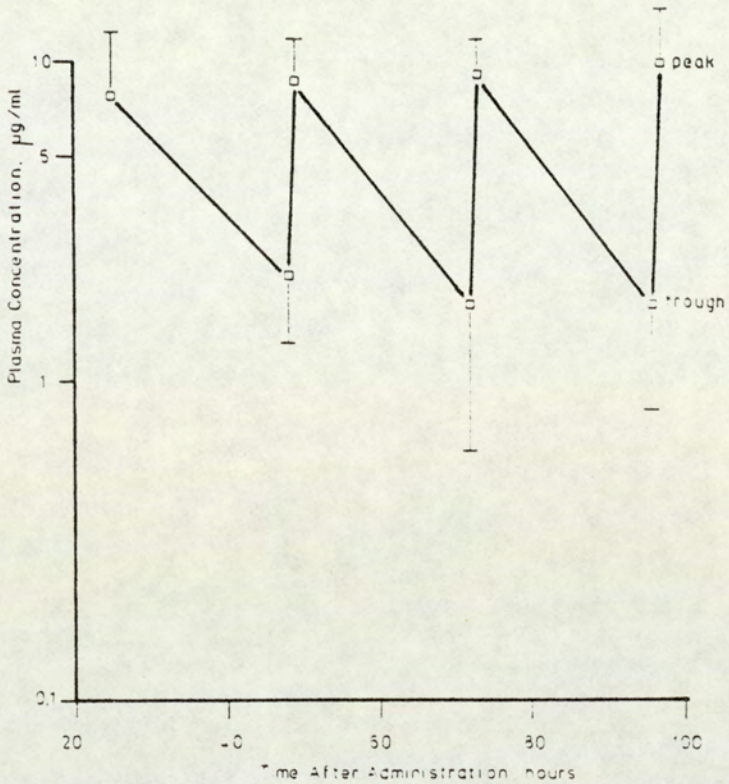


Fig. 55 Effect of IV administration of five consecutive daily doses of 300 mg/m^2 NMF in patients. Plasma samples were obtained one hour (peak) and 24 hours (trough) after injection. Plasma concentrations from the first dose are shown in fig. (56). Plasma concentrations are plotted logarithmically against time. Values are the mean (± s.d.) of four patients (E.S., L.G., A.H., and M.Z.) (see table 12).

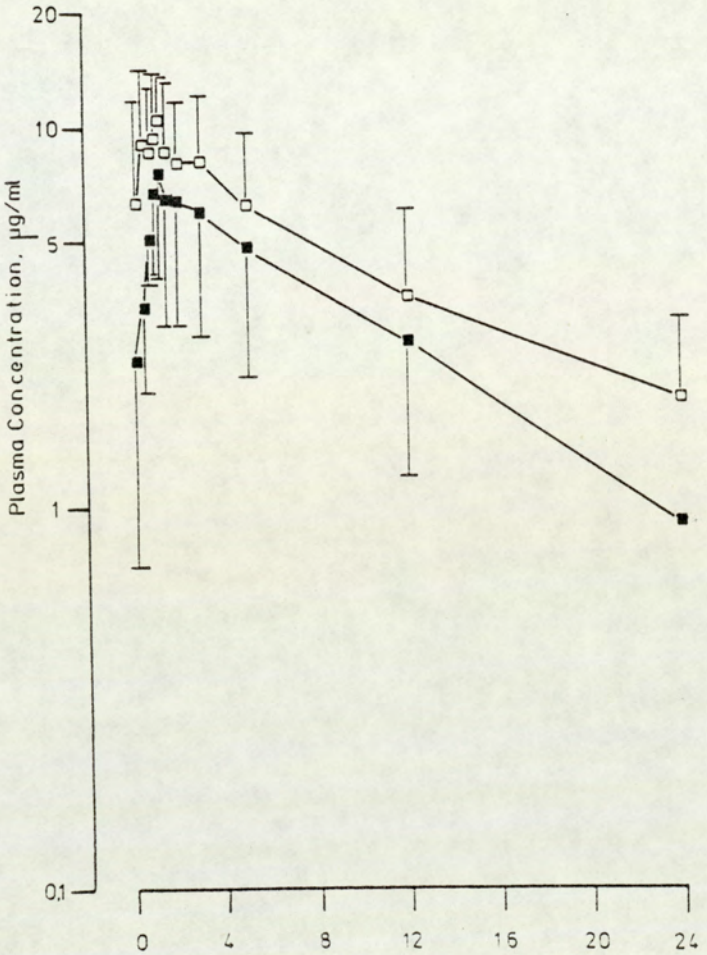


Fig. 56 Effect of route of administration on the plasma disposition of 300 mg/Kg NMF in patients. Plasma samples were obtained after a single IV (■) (30-60 minute infusion) or PO (□) (ingested over 15-30 minutes) administration from the same patient. Values are the mean (± s.d.) of four patients (E.S., L.G., A.H. and M.Z.) or in the case of the point without an error bar, the concentration from one patient.

TABLE 13

Pharmacokinetic Parameters of NMF in Patients

Patient	Dose (mg/m^2)	Route	AUC ($\mu g.hr.ml^{-1}$)	Bioavail-ability	Half-life (hours)
E.S.	300	IV	53.6	0.85	
	300	PO	45.4		
L.G.	300	IV	30.9	1.39	
	300	PO	42.9		
A.H.	300	IV	117.4	1.03	6.93
	300	PO	120.8		5.69
M.Z.	300	IV	87.0	1.99	5.92
	300	PO	173.4		10.50
M.M.	600	IV	120.9	0.77	
	600	PO	93.0		
I.W.	600	IV	63.5	2.4	
	600	PO	154.9		
J.D.	1200	IV	356.1	1.81	
	1200	PO	643.6		
D.C.	1200	IV	163.3	0.62	
	1200	PO	101.1		
J.L.	600	IV	201.8		13.33
A.V.	600	IV	293.7		7.30
C.R.	600	IV	248.3		
G.H.	600	PO	276.9		7.49
W.P.	1200	IV	325.7		7.00
V.K.	1200	IV	359.4		

The oral bioavailability at all dose administered in this investigation was therefore similar to that observed in mice (see section 9.2.1).

An attempt was made to derive the elimination half-life of NMF from the plasma concentration-time profiles of individual patients, based on first-order elimination kinetics (see section 8.8). Peak plasma concentrations of NMF were reached between one and two hours after IV or PO administration (fig. 57) of the drug, and the disappearance of NMF from the plasma, after reaching peak levels, in some patients, was consistent with a linear kinetic model. Elimination half-lives of NMF were between 6.9 and 13.3 hours (table 13).

600 mg/m^2 NMF was given orally to one patient (see table 12) on five consecutive days, and plasma samples were taken up to three hours after administration of the first and after the final dose; from which plasma concentration-time profiles were obtained. Fig.(58) shows the virtually identical NMF plasma levels after the first and fifth daily, oral doses of the five day course; indicating no drug accumulation in the plasma (D'Incalci et al., 1982). AUC values within three hours after administration of the first and final dose were not significantly different (21.9 and 21.2 $\mu g.hr.ml^{-1}$ respectively).

After IV administration of 600 mg/m^2 NMF to three patients (J.L., A.V. and C.R.) only 1.6 $\pm$ 1.2% of the total dose was recovered from the urine as unchanged drug within

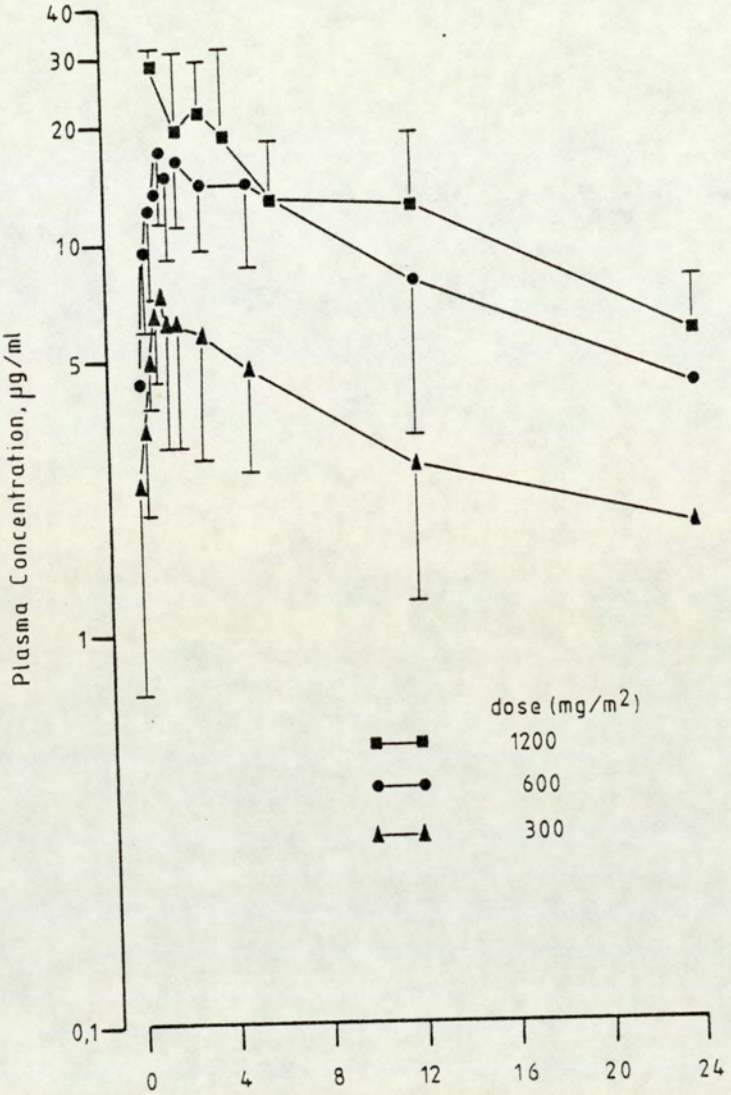


Fig. 57 Effect of dose on the plasma disposition of NMF after IV administration (30-60 minute infusion) of 300 mg/m^2 (▲), 600 mg/m^2 (●) or 1200 mg/m^2 NMF in patients. Plasma concentrations are plotted logarithmically against time. Mean (± s.d.) values were obtained from four patients (E.S., L.G., A.H. and M.Z.) in the case of (▲), four patients (J.D., D.C., W.P. and V.K.) in the case of (■), and five patients (M.M., I.W., J.L., A.V. and C.R.) in the case of (●).

24 hours (fig.59). Formamide was detectable as a metabolite of NMF, but it could not be accurately measured because urine concentrations of the drug were near or below the detection limits of formaldehyde in the GC assay.

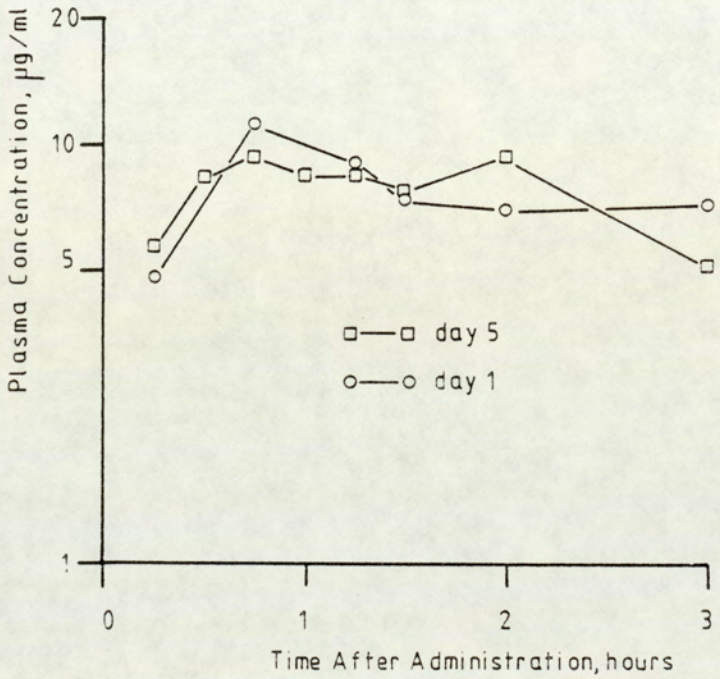


Fig. 58 Effect of oral administration of five consecutive daily doses of 600 mg/m^2 NMF to one patient (G.T.). Plasma samples were obtained after the first (○) and final (□) administration. Plasma concentrations are plotted logarithmically against time.

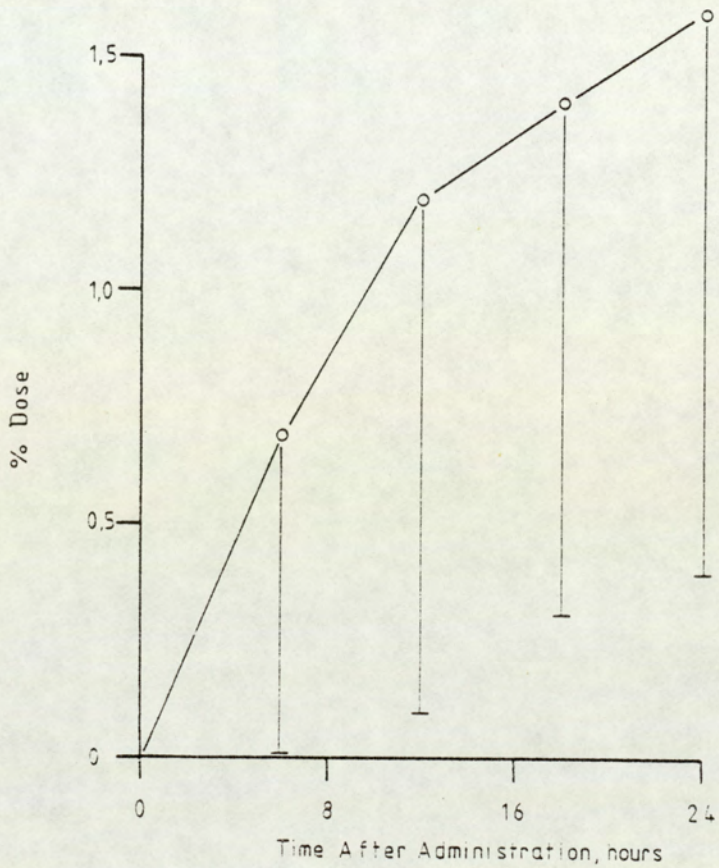


Fig. 59 Cumulative urinary excretion of NMF after IV administration of 600 mg/m^2 NMF to patients. Mean (only half s.d. values shown) values were determined from three patients (J.L, A.V. and C.R.) (table 12).

SECTION 10

DISCUSSION

10.1 Bioavailability of NMF

For a drug to be active in a certain tissue it must reach that tissue in amounts which are able to elicit a pharmacological response. Studies of the bioavailability of a drug are usually performed to discover the quantity of it reaching the biophase, i.e. the biological environment of the drug target, after different modes of administration. The bioavailability of NMF was determined with this concept in mind.

It is important to understand that the relevance of such a study, used for the interpretation of the anti-tumour activity of drugs, is dependent on two assumptions:
(1) The chemotherapeutic activity of the drug is related to the amount of drug at the tumour site. The application of this concept has been reviewed by Wagner (1975).
(2) The amount of NMF at the target site is proportional to the concentration of the drug in the plasma.

The high water and lipid solubility of NMF (Mukundrai, 1981) presumably renders this agent able to fulfill the second assumption; there is also preliminary evidence that radioactivity injected with ^{14}C-NMF distributes rapidly and efficiently into tissues (Barlow T., 1982).

The results of the investigation of the systemic availability of NMF presented in section (9.2.1) show that NMF is bioavailable after oral, IP and IM administration of the drug, (table 14). The systemic availability of NMF after different routes of administration is illustrated in fig.(37).

TABLE 14 Bioavailability of NMF in the Mouse

Dose (mg/Kg)	Route of administration	Bioavailability
400	IP	1.10
400	IM	1.36
400	PO	1.01
80	PO	0.76

NMF is also bioavailable after oral administration of the drug to man (section 9.8) which suggests that no appreciable first pass metabolism of NMF occurs in mice or man when administered by this route. Peak plasma concentrations of NMF in four patients (E.S., L.G., A.H., and M.Z.) were not significantly different ($p > 0.5$) when 300 mg/m^2 was administered either as a 30 minute infusion or as an oral dosage form ingested over 15 to 30 minutes; and peak plasma levels were reached within the same time span (1.25 hours) after NMF was administered by these routes (fig.56). This suggests that NMF is rapidly and completely absorbed from the gastrointestinal tract after oral administration.

Patients appear to prefer to take NMF orally (McVie, G.: personal communication) and the oral preparation is easier to formulate compared to the IV dosage form, because it does not require sterilisation.

These factors together with the good bioavailability of the drug, renders the oral administration of NMF in future clinical evaluations possible. One obvious advantage of the oral dosage form is the probability that patients will be able to take the drug at home with a reasonable chance of compliance.

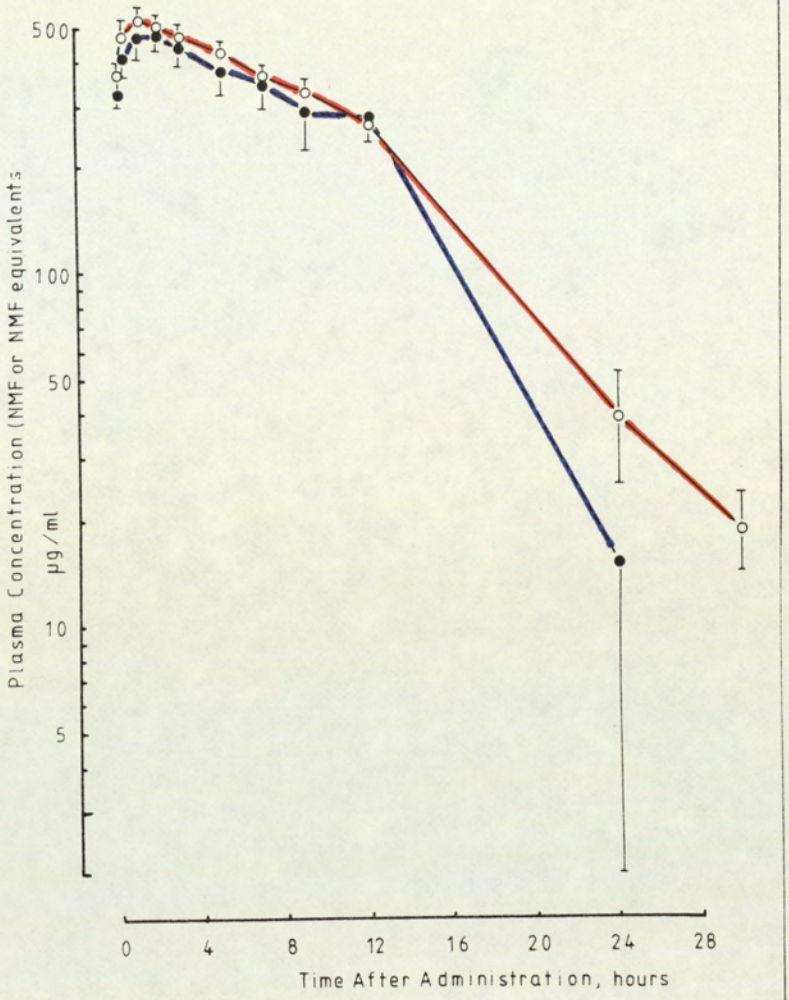
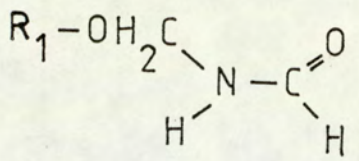


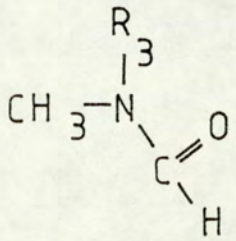
Fig. 60 Plasma disposition of ^{14}C-NMF (O) and NMF (●) after IP administration of 400mg/Kg. Experimental points are identical to mean values shown in Fig.(36)

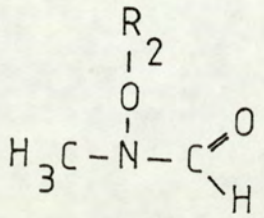
10.2 Pharmacokinetic Model Describing the Plasma Disposition of NMF and DMF

The plasma disposition profile of radioactivity during the first 24 hours after administration of ^{14}C-NMF is virtually superimposable on that of unlabelled NMF (fig.60), which suggests that only a small amount of metabolite(s) is present in the plasma during this time. However, fig (51) shows the presence of a large quantity of, as yet, unidentified metabolite(s) in the urine of mice after NMF administration. This suggests that the metabolites, whatever their chemical nature, are rapidly eliminated from the plasma into the urine after their formation. If this hypothesis is accurate, one could argue that the metabolites of NMF, formed in the liver or extrahepatically, are cleared from the plasma before they reach the tissues in sufficient concentrations to elicit a biological response. Therefore, one might conclude that these metabolites, which appear with the urine, are probably deactivation products of NMF and not its ultimate anti-neoplastic species. However, one cannot discard the possibility that the bioactivation which is important for the antitumour activity of NMF, occurs in the tumour.

The rapid disappearance of the metabolites from the plasma into the urine could mean that they are conjugates of hydroxylated derivatives of NMF with glucuronic acid, sulphate or GSH (see structures below).







R_1 = Glucuronide or sulphate

R_2 = Glucuronide or sulphate

R_3 = Glucuronide or GSH

These possible intermediates of the metabolism of NMF would be extremely polar and therefore easily excreted by the kidney.

The plasma concentration-time curves of unlabelled NMF within 24 hours after administration of IP (fig.60), IV (fig. 34A), oral (fig. 34B) and IM (fig. 35) doses of 400 mg/Kg, exhibit a concave downward curvature when plotted semilogarithmically. Fig. (37) shows that the slope of the plasma disposition profiles obtained on administration of NMF via different routes are very similar. This shape was not obvious when blood samples were collected by cardiac puncture (fig. 40) and was not a result of the anaesthetic employed (fig. 41). When blood samples were collected by the method of cardiac puncture it was necessary to use one animal for each experimental value rather than one mouse providing enough samples for a whole profile. Plasma concentrations obtained from studies using many animals may distort or conceal the actual pharmacokinetic pattern of a drug as compared to when plasma disposition profiles were determined more accurately using one animal.

There are precedents for this in the literature, e.g. Bazare et al. (1981) found that the plasma concentration-time curve for 2,4,5-trichlorophenoxyacetic acid revealed a "cyclic decay pattern" when blood samples were obtained by tail-bleeding of mice whereas the "cyclic pattern" was not observed when repeated sampling from individual animals was employed, and a single straight line fitted the mean data.

Repeated sampling from the same animal presumably provides a more valid indication of the true pharmacokinetic pattern of the drug. It also appears to be a more accurate method because standard deviations of the mean of plasma concentration values obtained by cardiac puncture (fig. 40) were larger than those obtained by tail bleeding at comparable sampling times (fig. 36).

A mathematical treatment of the plasma concentration data seemed to be inappropriate because the plasma disposition of NMF does not appear to fit a linear kinetic model, (see section 9.2.2). In order to verify the non-linearity, the data was treated in the following manner as suggested by Wagner (1973). Plasma concentration values were plotted as a function of time using Cartesian rather than semilogarithmic co-ordinates (figs. 61 and 62), and the NMF concentration during some periods of the disposition appear to fit a straight line. This indicates, according to Wagner (1973), that NMF may be eliminated at a fixed rate independent of its concentration in the plasma, i.e. zero-order kinetics. Such non-linear kinetics can be associated

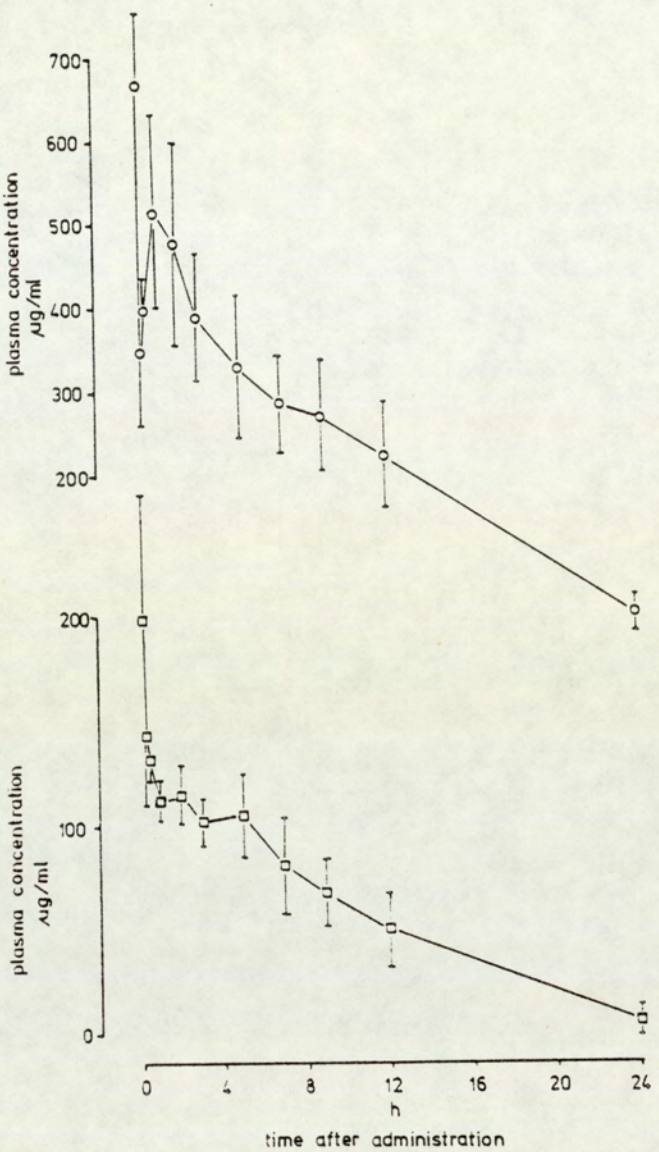


Fig. 61 Plasma disposition of NMF after IV administration of 400 mg/Kg (O) or 80 mg/Kg (□) NMF. Plasma concentrations are plotted linearly against time. Values are identical with the mean values shown in fig. (34A).

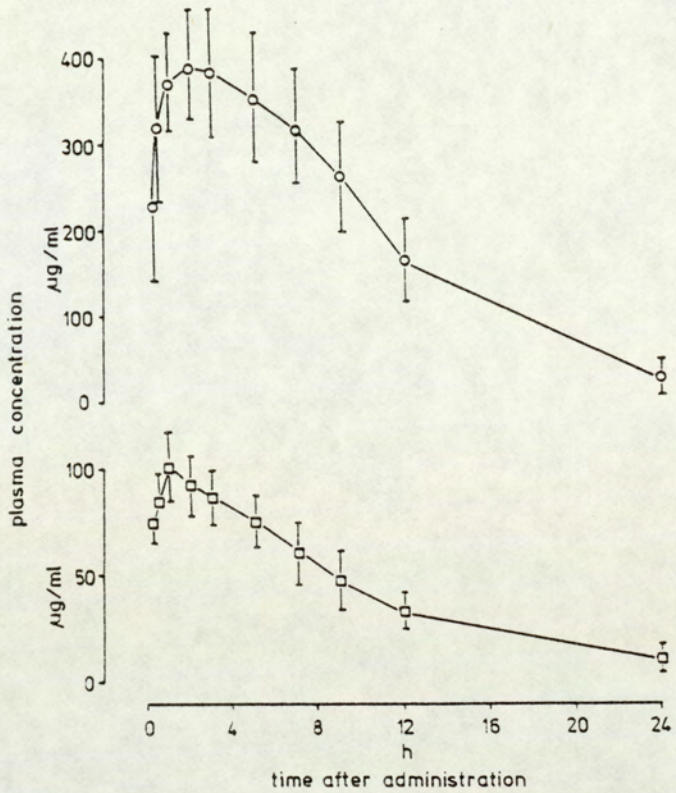


Fig. 62 Plasma disposition of NMF after oral administration of 400 mg/Kg (○) or 80 mg/Kg (□) NMF. Plasma concentrations are plotted linearly against time. Values are identical with the mean values shown in fig. (348).

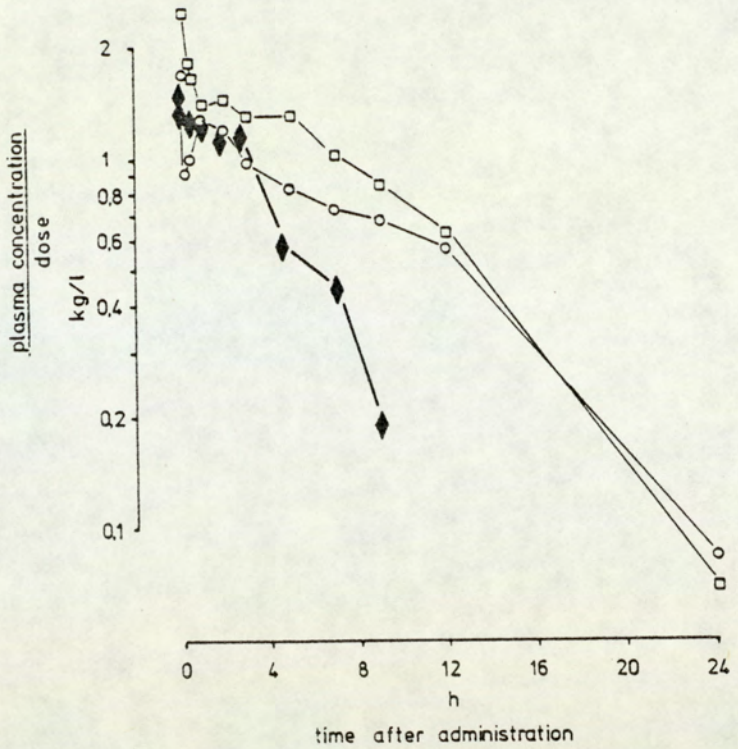
with the saturation of the pathways by which a drug is distributed within or eliminated from the body, e.g. uptake into the tissues or hepatic (or extrahepatic) metabolism (Wagner, 1973).

In a further attempt to characterise the apparent non-linearity of the model describing the disposition of NMF, the plasma concentration values obtained within 24 hours after oral administration of 400 mg/Kg and 80 mg/Kg NMF (fig. 34B) and IV administration of 400 mg/Kg, 80 mg/Kg and 14.3 mg/Kg NMF (fig. 34A) were divided by the dose and the ratios plotted as a function of time (fig. 63).

The profile thus obtained for the low dose of NMF after IV administration was appreciably different from the profiles obtained for the two higher doses. However, genuine non-linearity should lead to a more marked discrepancy of the profiles (Wagner, 1973). Indeed, after IV or oral administration of the two higher doses of NMF, the resultant curves obtained on dividing plasma concentration by the dose are not conspicuously different from each other; a fact which most certainly does not support the evidence of non-linear kinetics governing the plasma disposition of NMF.

The plasma clearance of NMF calculated from the AUC values (see section 8.8) after IV administration of the two high doses of NMF were virtually identical (table 8), i.e. the clearance of NMF was apparently not dependent on the dose. However, after IV administration of the low dose of NMF, the clearance value obtained (table 8) was significantly

(A)



(B)

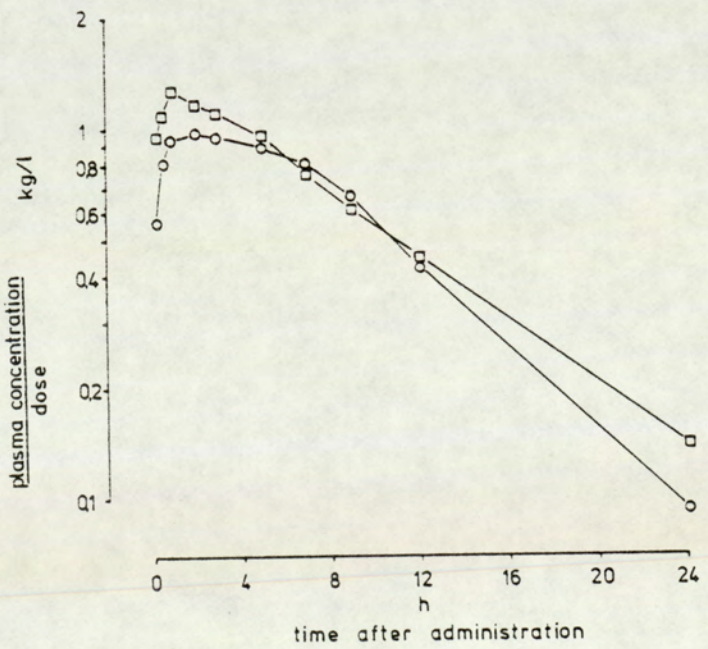


Fig. 63 Plots of NMF plasma concentration divided by the dose against time after IV (A) or oral (B) administration of 400 mg/Kg (○), 80 mg/Kg (□) or 14.3 mg/Kg (◆) NMF. Values are identical with the mean values shown in figs. (62 and 34).

higher compared to the higher doses ($p < 0.03$). Again, these facts do not unequivocally support the evidence for a non-linear, (dose-dependent) kinetic model describing the plasma disposition of NMF.

When a low dose of ^{14}C-NMF was administered IP, a plasma concentration of total radioactivity-time profile was obtained which appeared to be linear within 12 hours after administration, with a half-life of 2.1 hours (see fig.39). The plasma disposition profiles obtained in some patients who received comparable low doses of NMF, were also considered to fit a linear kinetic model, up to 24 hours after administration (fig. 57) and the elimination half-lives were in the order of 8 hours (table 13).

The mean plasma concentration values, obtained after administration of the low doses of NMF to patients, were divided by the dose and plotted as a function of time (fig. 64). The resultant profiles were not markedly different which suggests that the plasma disposition of NMF at these low doses may be independent of the dose administered.

The doses administered to patients (see section 8.7) were far below the tumour effective doses in mice, which revealed apparent non-linear disposition profiles. One might therefore speculate that higher doses of NMF may also lead to concave-downward shaped plasma concentration-time curves in patients. It is relevant in this context that so far, the drug given at these low doses did not appear to elicit an antitumour effect in patients (McVie G. and Newlands E., personal communications).

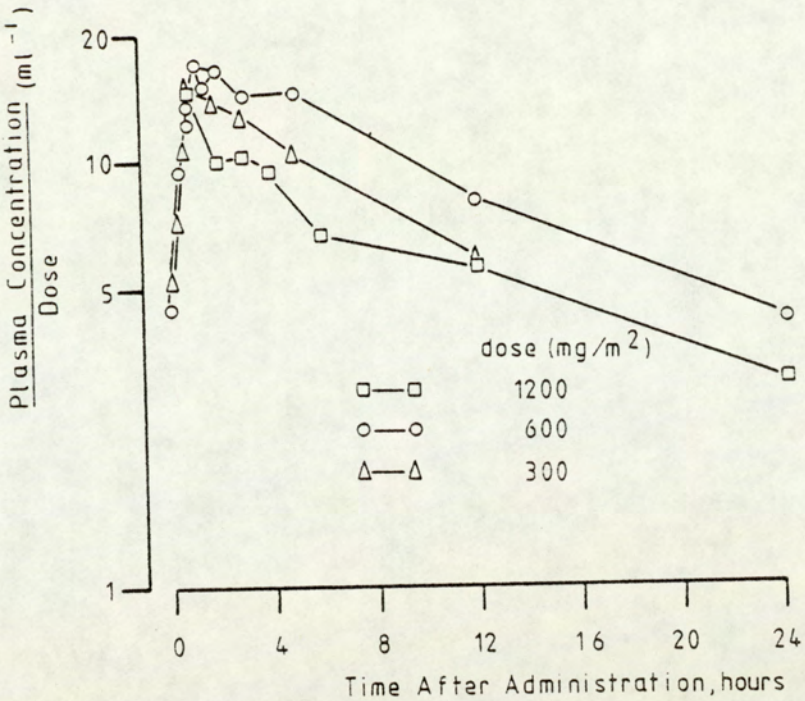


Fig. 64 Plots of NMF plasma concentration divided by the dose against time after IV administration to patients of 300 mg/m^2 (Δ), 600 mg/m^2 (○) or 1200 mg/m^2 (□) NMF. Values are identical with the mean values shown in fig. (57).

So, in summary, the evidence obtained in mice suggests that NMF may be eliminated from the plasma by processes which involve non-linear kinetics; however, further studies are required to unequivocally establish non-linearity of the pharmacokinetics of NMF. E.g., the plasma concentration of NMF has to be determined as a function of time after administration of a large number of different doses and the resultant profiles should give a more clear picture.

The plasma concentration data obtained for DMF was treated in a similar manner to that described for NMF, in order to investigate the kinetic model which describes the plasma concentration-time profile for this methyl anologue of NMF which is devoid of appreciable antitumour activity. Again, the mean plasma concentration-time curves plotted on a Cartesian scale reveal apparently linear sections (fig. 65) which indicates zero-order kinetics most conspicuously at the two high doses.

When the DMF plasma concentration values were divided by the dose and the ratios plotted as a function of time (fig. 66) the resultant curves were clearly not superimposable; indicating that non-linearity is almost certainly involved in the kinetic model which describes the plasma disposition of DMF at these dose levels.

This observation is in contrast with the finding that in the case of the plasma disposition of NMF at equimolar doses (6.8 and 1.36 mmoles/Kg) non-linearity was not unambiguously recognised by treating plasma concentration

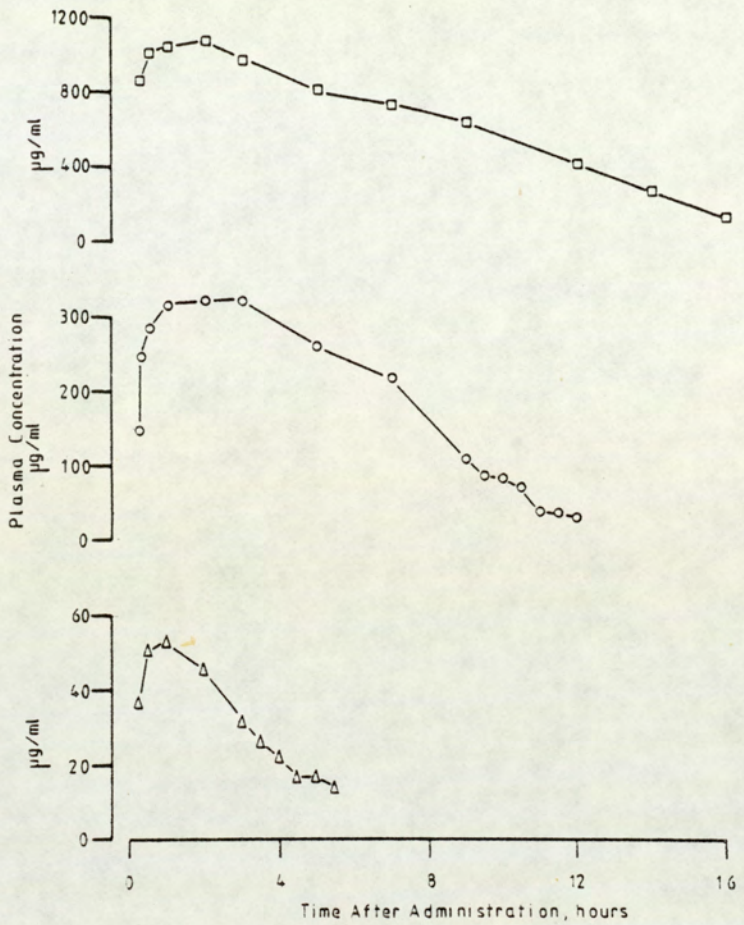


Fig. 65 Plasma disposition of DMF after IP administration of 1400 mg/Kg (□), 496.4 mg/Kg (○) or 99.3 mg/Kg (Δ) DMF. Plasma concentrations are plotted linearly against time. Values are identical with mean values shown in fig. (47).

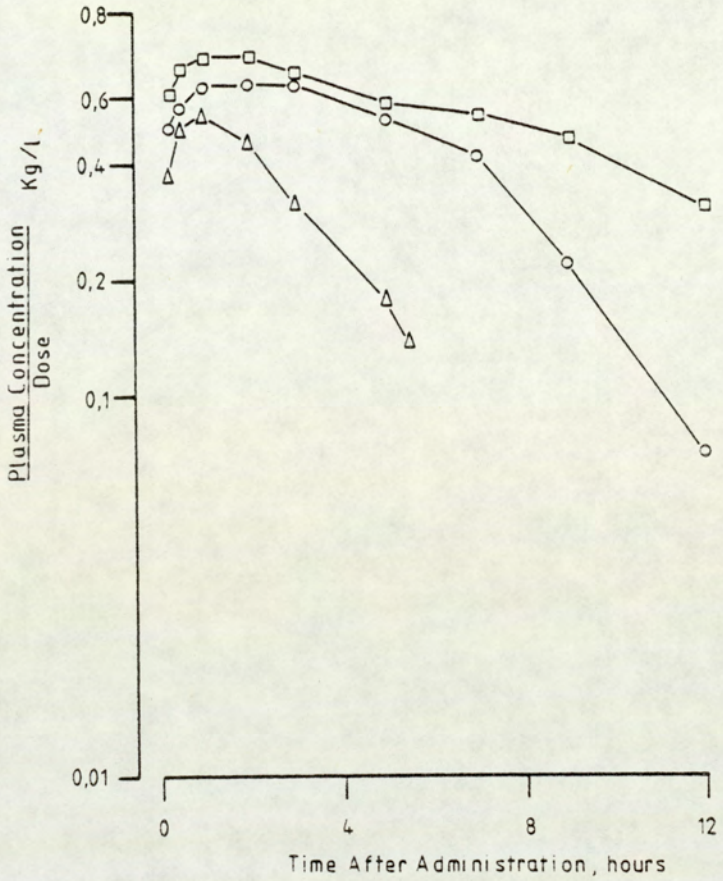


Fig. 66 Plots of DMF plasma concentrations divided by the dose against time after IP administration of 1400 mg/Kg (□), 496.4 mg/Kg (○) or 99.3 mg/Kg (Δ) DMF

data in this way (fig.63). That the plasma disposition of DMF is more justifiably described by a non-linear model than that of NMF may be attributed to the difference in their lipophilicities (Hansch, 1979). In a review of pharmacokinetic data, Wagner (1973) has shown that non-linearities can be due to reabsorption of a drug in the kidney. Since DMF is more lipophilic than NMF (Hansch, 1979) it is possible that the renal clearance of DMF is relatively slower because of an increased reabsorption across the lipoid surface of the kidney tubule. Clearance of DMF would be enhanced by its metabolism to the more polar NMF which is cleared more rapidly. It is interesting in this context to compare the urinary excretion of DMF with that of NMF. The amount of parent drug and respective metabolites recovered in the urine, as a percentage of the total dose administered, within 12 hours after administration of DMF (fig.54) is markedly different from that of NMF (fig.52). However, the cumulative excretion of NMF and DMF and their respective metabolites up to 24 hours after administration of the parent drug was virtually the same.

Therefore, it is conceivable that the different rates of excretion of these drugs, within 12 hours after administration, is involved in the marked non-linearity of the kinetics describing the plasma disposition of DMF, compared to the model in the case of NMF, where the evidence is more equivocal.

The plasma protein binding of NMF and DMF could also theoretically effect their plasma disposition, and

Krüger-Thiemer (1968) has published a mathematical model which describes non-linear kinetics in terms of protein binding with reference to some sulphonamides which show a high degree of binding to plasma proteins.

However preliminary evidence from an in-vitro assay (Gescher A., unpublished) did not demonstrate protein binding of NMF and Meyer and Guttman (1968) have suggested that only in the case of highly bound agents will binding be important. It is probably unlikely that DMF would be bound to plasma proteins sufficiently in order to explain the more marked non-linearity of its plasma disposition compared to that of NMF.

The analysis of the plasma after administration of DMF shows that DMF is metabolised in-vivo to its N-demethylated metabolite, NMF (fig. 48). The plasma disposition of NMF as a metabolite of DMF, was very similar, within 12 hours after administration of 1400 and 496 mg/Kg DMF whereas the plasma concentration of NMF after administration of 99.3 mg/Kg DMF is markedly lower than that of the higher doses. AUC values calculated from the plasma concentration of the metabolite, NMF, versus time curve up to 12 hours after administration of DMF (table 10) were not significantly different in the case of the two high doses and the peak plasma concentrations of NMF were virtually identical (mean values were 107.3 $\pm$ 25.4 µg/ml and 108 $\pm$ 18.6 µg/ml).

The plasma concentration of metabolite clearly indicates a non-proportional dependence on the dose of DMF, i.e. dose-dependency, indicating that an enzymatic or

transport process involved in the disposition of DMF, probably its N-methyl hydroxylation in the liver, becomes saturated at high plasma concentrations of the agent.

However, the kinetic model describing the disposition processes of the metabolite NMF after administration of the low dose of DMF, appears to be linear. A comparison of AUC values obtained after administration of 496 and 99.3 mg/Kg DMF and normalised for the dose, reveal a markedly similarity; they were 709 and 875 ug.hr.ml^{-1} respectively, which indicates that at these low doses of DMF the disposition of the metabolite in the plasma is proportional to the dose.

In order to confirm that the plasma disposition of NMF and DMF is consistent with a model by which metabolic clearance exhibits Michaelis-Menten kinetics, i.e. that the processes involved are saturable at high doses, more plasma concentration studies after several doses of the drug are required. When NMF or DMF are administered at several dose levels and drug and metabolite(s) are measured in the urine, then the amount of metabolite(s) excreted can be plotted as a percentage of the total urinary excretion against the dose. If one obtains a proportional decrease in the amount of metabolite excreted, as a percentage of the total urinary drug species when the dose is increased, then Michaelis-Menten kinetics might be applicable in describing the plasma disposition, (Wagner, 1973).

Further evidence for Michaelis-Menten kinetics can

be obtained by calculating the area under the metabolite concentration-time curve from plasma samples after administration of the drug. AUC values are plotted as a function of the dose and if the resultant plot is non-linear then this would also suggest that the plasma disposition is governed by Michaelis-Menten kinetics, (Workman, 1980a).

10.3 Comparison of the Plasma Disposition of NMF, DMF and NEF with Their Antitumour Activity and Toxicities.

The lack of antitumour activity of DMF and NEF compared to the activity of NMF, may have a pharmacokinetic explanation (see section 9.3). In fig. (67) the plasma concentration-time profiles after IP administration of these alkylformamides at equimolar doses are compared. The AUC values of DMF and NEF are significantly lower than those of NMF.

If one assumes that the concentration of parent drug in the plasma reflects the amount of drug or active metabolites reaching the target site, it may be speculated that NEF and DMF are not antitumour agents because they do not reach sufficient concentrations in the biophase. This hypothesis may be verified by measuring drug levels in the tumour tissue as a function of time. Preliminary studies have indeed shown that levels of radioactivity in the murine TLX5 lymphoma were similar to amounts in other tissues,e.g. kidney, heart and lungs, within 8 hours after administration of 400mg/Kg ^{14}C-NMF; a markedly higher tissue concentration of radioactivity was observed in the liver (Barlow T., 1982).

There is evidence that toxicity in-vivo is related to the pharmacokinetic behaviour of some drugs (e.g. nitroimidazoles), which in turn, is dependent on their lipophilicities (Workman, 1980a). Also, in a series of

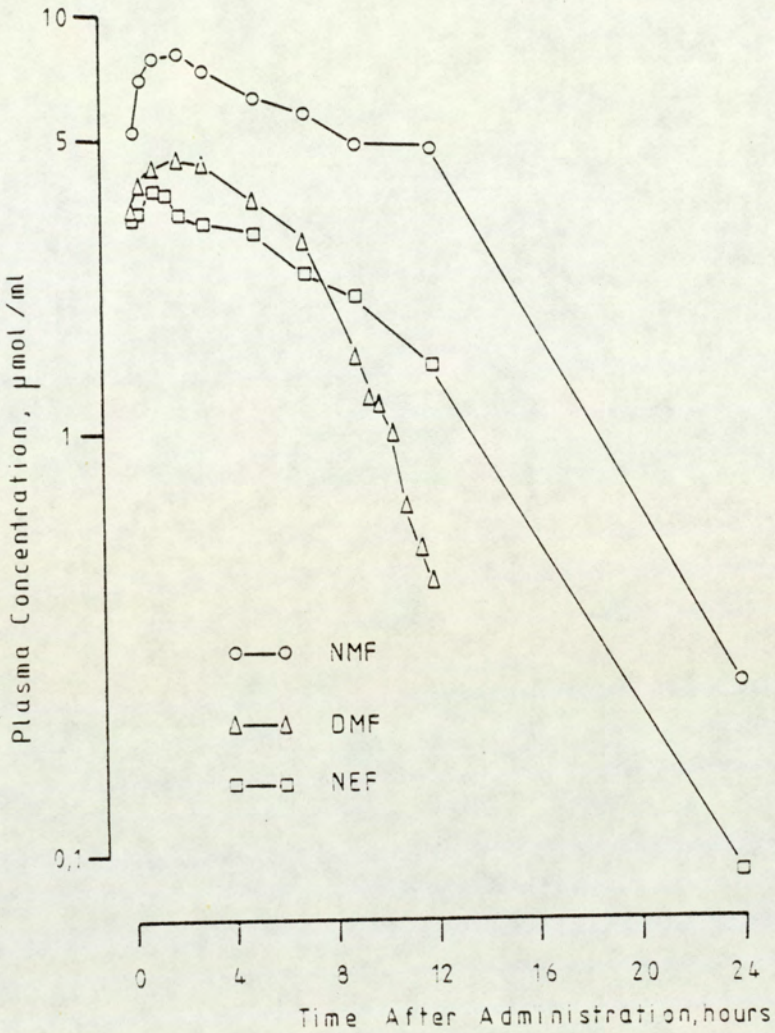


Fig. 67 Comparison of the plasma disposition of NMF (○), NEF (△) and DMF (□) after IP administration of equimolar doses (6.8 mmoles/Kg). Experimental points are identical with mean values shown in figs. (36, 46 and 47).

related drug molecules, different lipophilicities are likely to result in predictably different AUC values (Workman 1980b). The more lipophilic nature of DMF and NEF compared with NMF (Hansch, 1979) may cause their affinities for tissues to be higher than that in the case of NMF, and thereby lead to an increased volume of distribution compared to that obtained with NMF.

Since DMF is metabolised in-vivo to a species which on GC analysis appears to be NMF, one would expect that if a sufficient plasma concentration of NMF, as a metabolite of DMF, could be achieved, then antitumour activity could be elicited which is comparable to that of NMF itself. In order to achieve comparable plasma concentrations of the metabolite NMF, which elicit an antitumour response, a sufficiently high dose of DMF would be required.

The AUC and peak concentration values of the metabolite NMF within 24 hours after administration of 1400 mg/Kg DMF was not dissimilar to the AUC and peak plasma concentration levels of NMF within the same time span, achieved after administration of 80 mg/Kg of genuine NMF (see tables 6 and 10). Therefore, it is possible to speculate that if the plasma concentrations of NMF are related with antitumour activity, the high dose of DMF and the low dose of NMF might have comparable activities against tumour models sensitive to NMF because the plasma levels of NMF are similar.

In a separate series of experiments (Langdon et al., unpublished) an optimal antitumour dose of 1000 mg/Kg

(13.7 mmoles/Kg) DMF showed marginal activity against the M5076/A reticulum cell sarcoma but had no activity against the TLX5 lymphoma. Also, doses as low as 50 mg/Kg (0.9 mmoles/kg) NMF were marginally active against the M5076/A reticulum cell sarcoma but was not active against the TLX5 lymphoma. Therefore, low plasma concentrations of NMF, when produced as a metabolite of DMF seem to manifest an anti-tumour response similar to that of a low dose of genuine NMF in the same tumour model.

However, DMF did not elicit a clear antitumour response and it is possible that the compound analytically determined as NMF, after administration of DMF is really N-hydroxy-N-methylformamide which is stable in a physiological environment but decomposes on the GC column (Ross D., personal communication). This compound is inactive against the tumours described above (Langdon S. P. et al., unpublished) and it is likely to be reasonably stable in the plasma. Therefore the potential active breakdown product NMF, may not reach the target site, i.e. the tumour, in quantities sufficient for activity when N-hydroxymethyl-N-methylformamide is injected into the animal.

If the compound corresponding to NMF on the GC analysis of plasma samples after administration of DMF, is actually the inactive N-hydroxymethyl-N-methylformamide and not genuine NMF, then the marginal activity of DMF may be due to parent compound. It is also possible that the marginal

activity of DMF is mediated via a hitherto undetected metabolite of DMF rather than by NMF.

This hypothesis does not of course, account for the inactivity of DMF against the TLX5 lymphoma.

Alternatively, NMF which is metabolically generated from DMF in-vivo, may reach the tumour in sufficient concentration but the homologue DMF, may competitively occupy the receptor sites for NMF in the tumour tissue. Therefore, if DMF itself is completely without activity, injection of DMF will not elicit an antitumour effect.

It is worth noting in this context that in a toxicological study of DMF, Lundberg et al., (1981, 1982) also suggested that the N-demethylation of DMF to NMF was inhibited at high DMF concentrations.

The hepatotoxicity of NMF has been investigated by a number of authors (see section 6.3). NMF was shown to be more hepatotoxic than DMF in rats and the latter compound was more hepatotoxic than formamide (Lundberg, 1981 and Scailter, 1981). Lundberg et al., (1982) suggested that the hepatotoxicity of DMF is due to metabolically generated NMF rather than the unchanged agent itself. The concentration of radioactivity after injection of ^{14}C-NMF in the liver of mice was three times the concentration achieved in other tissues (Barlow T., 1982) which is congrous with the view of Lundberg (1982).

The mortality in rats was higher after administration of NMF compared to that after administration of DMF and this lethal effect of DMF was greater in male compared to female rats (Scailter, 1981); Eben and Kimmerle (1976) have shown DMF to be more rapidly metabolised in male rats than female rats which supports the hypothesis that DMF is metabolically toxified, especially in male animals.

However, Scailter (1981) found no marked difference in the metabolism of DMF to NMF in either sex of the rat and in the present investigation the plasma disposition of NMF was similar after administration of NMF to male or female mice (figs. 36 and 68; tables 6 and 7).

The argument tentatively put forward relating the difference in antitumour activity between DMF and NMF to plasma concentration could also be adopted to explain the difference in general toxicity and hepatotoxicity exhibited by these agents; a lower tissue exposure of the animals to DMF as compared to NMF on administration of equimolar doses of these alkylformamides may make DMF the apparently less noxious agent even though both compounds may be inherently equitoxic in-vitro.

However, a direct correlation between the antitumour activity of DMF and NMF and their general toxicity cannot be assumed because the therapeutic index of NMF is

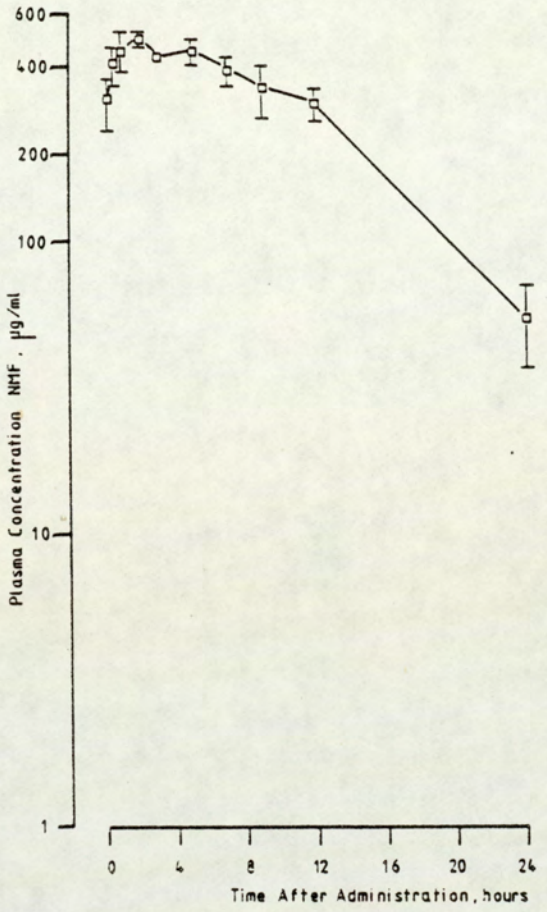


Fig. 68 Plasma disposition of NMF after IP administration of 400 mg/Kg NMF to female Cba/ca mice. Values are the mean of at least three determinations or in the case of the value without an error bar, the mean of two determinations. Plasma concentrations are plotted logarithmically against time.

appreciably larger than that of DMF, i.e. a comparison of the antitumour activity of 1000 mg/Kg DMF and 50 mg/Kg NMF against the M5076/A reticulum cell sarcoma, reveals that NMF is 20 times more effective than DMF in reducing tumour volume whereas NMF (LD_{50} = 300 mg/Kg/day) is only four fold more toxic than DMF (LD_{50} = 1220 mg/Kg/day), (Langdon et al.; unpublished).

10.4 Repeated Administration of NMF

It is not known whether the long elimination half-life of radioactivity which was observed beyond 24 hours after administration of NMF (fig. 38) represents the elimination of either parent drug or of metabolites, because unlabelled NMF could not be measured reliably during this late disposition phase. So, in effect, even though it appears that the radioactivity was attached to metabolites of NMF it is impossible to confirm that NMF was not present in these plasma samples because of the variable detection limit of NMF on GC analysis.

In an attempt to improve the analytical method in this respect, the concentration of NMF in solution was attempted using ion-exchange chromatography but without success as the resin did not retain NMF. The high octanol: water partition coefficient of NMF (Mukundrai, 1981) and its potential metabolites, N-hydroxymethylformamide and formamide, prevents efficient extraction into organic solvents, and freeze-drying of samples seemed to be unsuitable because of the high volatility of NMF and DMF.

The radiolabelled species which was eliminated from the plasma after 24 hours after administration with a long half-life of 71.1 hours, may therefore represent the plasma elimination of NMF or more likely that of its metabolites. Due to this long elimination half-life of radioactivity, repeated administration of NMF at frequent intervals can be assumed to lead to drug or metabolite accumulation.

However, accumulation of NMF, as indicated by a significant increase in AUC values on repeated administration, was not observed after three (figs. 42 and 43) or five (fig. 45) consecutive daily injections of 400 mg/Kg NMF to mice, nor was such accumulation observed in patients receiving five daily doses of 300 mg/m^2 IV (fig. 55) or 600 mg/m^2 orally (fig. 58). Accumulation was however observed in mice after the final dose of a régime consisting of nine injections of 220 mg/Kg every other day for 17 days.

Although NMF did not show accumulation in the plasma on five daily administrations of 400 mg/Kg, measurement of hepatic levels of radioactivity after the fifth of five daily injections of this dose of ^{14}C-NMF appeared to lead to accumulation of radioactive species (Barlow T., 1982).

Repeated administration of high doses of NMF was required for optimal antitumour activity against murine tumour models (Gescher, et al., 1982).Langdon et al., (unpublished) showed that administration of altogether 2g NMF over 17 days, was more effective in reducing tumour volume than administration of the same dose within five days. It is interesting that in the present study only the former but not the latter schedule resulted in plasma accumulation. This finding is puzzling because if the plasma disposition of NMF obeys non-linear kinetics one would expect the high dose régime to result in plasma accumulation of NMF rather than the same total dose given over a longer period. In accordance with this view, Workman (1980a) suggested that the

AUC for a single large dose of misonidazole would considerably exceed that for the same dose given in several smaller fractions because the plasma disposition of misonidazole exhibited saturable (non-linear) kinetics.

One might speculate that prolonged levels of NMF or its metabolite(s) for a certain period of time, are important for achieving optimal antitumour activity,e.g. it is possible that only if NMF reaches a certain plasma level, and reaches this level frequently over a long period, that enough drug or metabolite(s) penetrate the tumour. This would suggest experiments where levels of the drug and metabolite(s) are measured in the tumour after administration of different doses of NMF and thus the critical concentration in the tissue necessary for antineoplastic activity may be derived by relating the dose to its effect in parallel experiments.

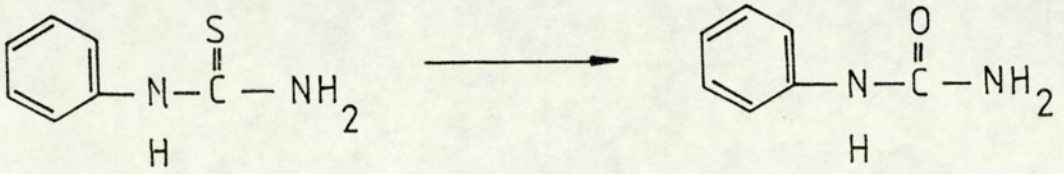
10.5 The Toxicity of DMSF

De Matteis and Seawright (1973) have shown that carbon disulphide is converted to carbon dioxide by the rat in-vivo, indicating that desulphuration takes place as shown below:

$$CS_2 \longrightarrow CO_2 + (S)$$

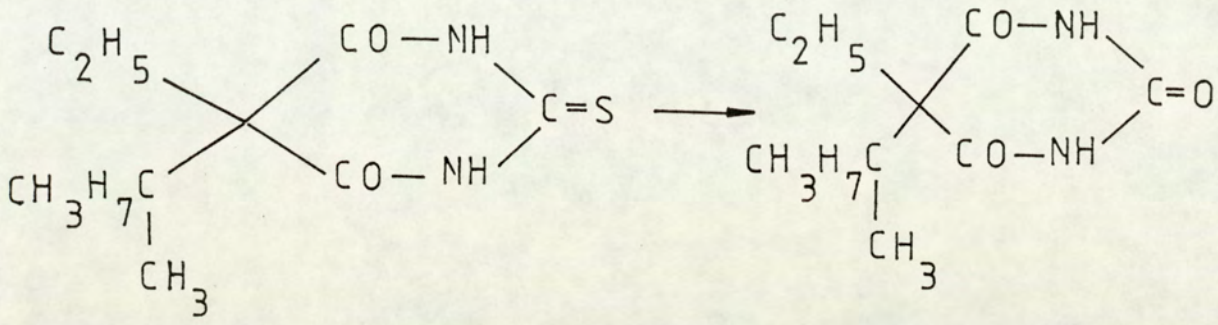
The appearance of DMF in the plasma of mice as a metabolite of DMSF (fig. 50) shows that DMSF also undergoes metabolic desulphuration in-vivo, whereas this metabolic biotransformation reaction could not be detected in-vitro, using a mouse liver microsomal preparation (see section 8.9). Carbon disulphide was however shown to be metabolised in-vitro to a sulphur product which became covalently bound to liver microsomes (De Matteis, 1974). The nature of this bound material was suggested to be hydrodisulphide by Catignani and Neal (1975) and may represent the toxic metabolite of carbon disulphide.

Other metabolic desulphurations are well established, e.g. phenylthiourea is converted to phenylurea in-vivo (Smith and Williams, 1961) and thiopentone is metabolised in-vitro to phenobarbitone (Winters et al., 1955) as shown overleaf:



phenylthiourea

phenylurea



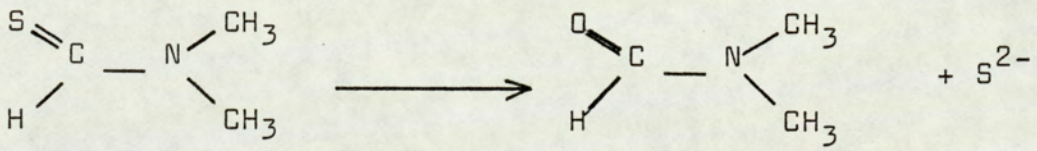
thiopentone

phenobarbitone

However, the details of the enzyme-catalysed reactions shown above have not been fully investigated, nor has the chemical nature of the metabolite containing the sulphur atom been eludidated (Gorrod, 1978).

Some other sulphur-containing compounds are known to have noxious properties, e.g. the toxicity of thiohippuric acid, dithiopiperazine and ethylhippurate seemed to be proportional to the amount of sulphur in these molecules, and the symptoms of intoxication were similar to those described after injection of certain sulphides or hydrogen sulphide inhalation (Supniewski, 1926). It is interesting in this context that hydrogen sulphide can be formed from thioamides by alkaline hydrolysis <u>in-vitro</u> (Hickinbottom, 1957) and therefore

hydrogen sulphide may be produced <u>in-vivo</u> from the desulphuration of DMSF. The toxicity of hydrogen sulphide is thought to be manifested by the release of a sulphide ion (Gorrod, 1978) and it is possible that on hydrolysis of DMSF <u>in-vivo</u>, the oxygenated analogue is formed with the concomitant loss of sulphur in the form of the sulphide ion (S^{2-}). It is thought that the systemic toxicity of the sulphide ion is produced by the inhibition of cytochrome oxidase in the same manner as cyanide elicits its toxic effect (Baselt, 1978). The toxicity of DMSF may therefore be associated with the formation of a sulphide ion as shown below:



DMSF DMF

10.6 Urinary Excretion of NMF and DMF

Of the 75.2% of the radiolabel excreted into the urine within 24 hours after administration of ^{14}C-NMF, only 26.4% was NMF and less than 2% formamide (fig. 51). Less than 2% of the total dose of NMF was excreted as unchanged drug within 24 hours after administration of 600 mg/m^2 NMF to three patients (fig. 59). It is probable that in analogy to the excretion in mice, metabolites of as yet unknown structure were excreted with the urine in these patients.

It is important to identify these urinary metabolites which may give a clue as to the chemical nature of the metabolically activated drug, if indeed NMF requires activation. One urinary metabolite of NMF has been characterised as a stable precursor of formaldehyde, possibly N-hydroxymethylformamide (Brindley, et al., 1982b). However, preliminary studies (Langdon, et al., unpublished) show that this species does not possess the antitumour efficacy of NMF and therefore does not appear to be an active metabolite of NMF.

NMF may be metabolised to N-hydroxymethylformamide and formamide by the following scheme: (see fig. 28)

It is puzzling that N-methyl hydroxylation of NMF was not detected in-vitro (Gescher, et al., 1982) and that phenobarbitone pretreatment did not markedly change the amount of NMF or formamide excreted into the urine (see section 9.6). The evidence above would suggest that a cytochrome P-450-dependent enzyme is not involved in the process determining the rate at which NMF is disposed in the body.

So there is the possibility that NMF is not metabolised by the mixed function oxidase system of the liver which is generally associated with N-demethylation reactions (Testa and Jenner, 1976). Other speculative metabolites which may be formed are briefly described in section (10.2).

Over 50% of NMF excreted with the urine was apparently lost by evaporation in the metabolic cages when they were not washed frequently prior to the collection of urine samples (figs. 51 and 52). After administration of DMF, the urine was assayed for unchanged drug and metabolites without washing the cages and therefore one may assume that similar evaporative losses occurred. The percentage excreted as unchanged drug may well have been around 5-10% of the total dose and the amount of its metabolite NMF (or N-hydroxymethyl-N-methylformamide) around 20-30%; whereas only 11% of NMF and less than 3% of unchanged drug was actually recovered (fig. 54).

The presence of NMF (or N-hydroxymethyl-N-methyl formamide) in the urine, in relatively large amounts

compared with that of unchanged drug, suggests that DMF is metabolised to a much greater extent than NMF is metabolised to formamide (or N-hydroxymethylformamide) which has implications discussed in section (10.2), i.e. the non-linear plasma disposition of NMF and DMF is probably due to the saturation of metabolising-enzymes and therefore the more obvious dose-dependent kinetics exhibited by DMF compared to NMF may be due to the fact that DMF is eliminated by metabolism to a greater extent than NMF.

In summary, there appears to be good bioavailability of NMF on oral administration in both mice and man.

NMF and DMF are eliminated mainly by metabolism and therefore saturation of, as yet unknown, metabolic pathways is the most likely explanation for the non-linear (dose-dependent) kinetics observed with NMF but particularly marked with DMF.

The N-methyl hydroxylation of these agents is probably not catalysed by the mixed function oxygenases of the liver which is in contrast with the hypothesis of Barnes and Ranta (1972) and Kimmerle and Eben (1975).

The lack of antitumour activity of DMF and NEF and the reduced hepatotoxicity of DMF compared to that observed for NMF, is possibly due to differences in plasma levels of these agents which may be related to differences in their relative lipophilicities.

NMF appears to have only a low tendency to accumulate in the plasma after repeated administration of

the drug but it is not impossible that some of its metabolites, perhaps conjugates of N-hydroxymethylformamide, accumulate.

SECTION 11

COMPARISON OF THE METABOLISM AND PHARMACOKINETICS OF N-ALKYLMELAMINES AND N-ALKYLFORMAMIDES

Whereas HMM undergoes N-demethylation in-vitro by liver microsomes (Ames et al., 1981) and tumour cells (Garattini et al., 1981), there is no analytical evidence for the metabolism of NMF in liver cells or liver preparations (Gescher et al., 1982). However both HMM (section 1.4.2.2) and NMF (section 6.4) are known to undergo oxidation on the N-methyl carbon in-vivo. In the case of HMM this leads to N-demethylated metabolites via N-hydroxymethyl intermediates (section 1.4.2.2) and in the case of NMF, metabolism presumably results in the formation of N-hydroxymethylformamide which is identified as formamide on GC analysis (section 10.2).

The major route of elimination of HMM appears to be via metabolism, i.e. HMM is rapidly metabolised in-vivo to demethylated metabolites and no unchanged HMM could be detected in the urine of rats (Worzalla et al., 1973) and only small quantities of HMM (less than 1%) were found in the plasma (Ames et al., 1979) and urine (Ames et al., 1979; Colombo et al., 1982) of man. NMF is also eliminated by metabolism to what appears to be its N-demethylated product, but to a lesser extent than HMM in mice. Over 26% of unchanged NMF was recovered in the urine of mice whereas only 1.6% was recovered as unchanged drug in patients (section 10.6). The rapid metabolism of HMM, presumaby in the liver, may explain the reduced and variable oral bioavailability of HMM in man (D'Incalci et al., 1978) compared to the good oral bioavailability of NMF in both mice and patients (section 10.1).

HMM appears to require oxidative metabolism by the hepatic mixed function oxidases (section 1.4.2.4) or tumour enzymes (Garattini et al., 1981) in order to elicit

its antitumour effect (section 1.4.2.1) whereas it is not known whether NMF requires metabolic activation for antitumour activity and the cytochrome P-450-dependent monooxygenases of the liver may not be responsible for its metabolism (section 10.2). It is possible that NMF undergoes oxidative N-demethylation in extrahepatic tissues or that it is catalysed by as yet unrecognised enzymes.

If the metabolic biotransformation of HMM and NMF is required for antitumour activity it is important to explain why other N-methyl-containing agents, e.g. AP and certain N-methyl derivatives of HMM and NMF, are inactive. The lack of antitumour activity of methylmelamines with four or less methyl groups in the molecule (section 1.4.2.2) may be attributable to the reduced affinity of the demethylating enzymes for these agents (section 4.2.). It was hypothesised in section (1.5) that HMM and AP may undergo demethylation to different products, i.e. relatively stable cytotoxic methylol intermediates in the case of HMM (section 1.4.2.3), and formaldehyde, which is rapidly metabolised to formic acid and carbon dioxide, in the case of AP.

However the above hypothesis does not explain the lack of antitumour activity of DMF compared with that of NMF since this agent is metabolised in-vivo to what appears to be NMF (section 9.4). In an attempt to explain the lack of antitumour activity of DMF, HEM and other agents, compared to NMF and HMM, the plasma disposition of HMM, NMF, NEF, DMF, and HEM was looked at. The AUC value for NMF

(section 9.2.2) and that for HMM (Broggini et al., 1981) are markedly higher compared to those for their inactive derivatives NEF and DMF (section 10.3) and HEM (Brindley et al., 1982b) after administration of equivalent doses. The replacement of N-methyl groups in the molecules of HMM and NMF with ethyl moieties renders the resulting species inactive against murine tumours which are sensitive to HMM (section 1.5) and NMF (section 6.1) respectively. The observed difference in systemic availability suggests a pharmacokinetic basis for the lack of antitumour activity of DMF, NEF and HEM, i.e. these agents may not reach the tumour site at sufficient concentrations necessary to elicit an antitumour response.

The difference in plasma levels of these agents may be due to their relative lipophilicities. Substitution of the methyl moiety in the HMM and NMF molecules with ethyl groups imparts increased lipophilicity (Cumber and Ross, 1977; Threadgill M. D., Personal communication) and addition of a methyl group in the NMF molecule also increases lipophilicity (Hansch, 1979). It is possible that HEM and NEF, and DMF, exhibit an increased tissue affinity compared to HMM and NMF, respectively which results in a greater apparent volume of distribution for the inactive derivatives.

REFERENCES

Ames, M.M., Powis, G., Kovach, J.S. and Eagan, R.T. (1979)
Disposition and Metabolism of Hexamethylmelamine in Rabbits and Humans.
Cancer Res., 39, 5016-5021

Ames, M.M., Sanders, M.E. and Tiede, W.S. (1981)
Metabolic Activation of Hexamethylmelamine and Pentamethylmelamine by Liver Microsomal Preparations.
Life Sciences, 29, 1591-1598

Archakov, A., Karuzine, I., Iveritinov, V. and Kokareva, I.(1974)
Hydroxylation of Aniline and Aminoantipyrine (1-phenyl-2,3-dimethylaminopyrasolon-5).
Derivatives in Liver Endoplasmic Reticulum.
Biochem. Pharmac., 23, 1053-1063

Argus, M.F., Neuberger, B.J., Myers, S.C. and Arcos, J.C.(1980)
Induction of Dimethylnitrosamine-demethylase by Polar Solvents. (40721)
Proc. Soc. Exp. Biol. Medicine, 163, 52-55

Baker, B.R. and Ashton, W.T. (1972)
Irreversible Enzyme Inhibitors. 196. Active-site-directed Irreversible Inhibitors of Dihydrofolate Reductase Derived from 1-(4-Benzyloxy-3-Chlorophenyl)-4,6 diamino-1,2-dihydro-2, 2- dimethyl-S-triazine and Bearing a Terminal Phenyl Sulfonate Group.
J. Med. Chem., 15, 945-947

Barclay, R.K. and Garfinkel, E. (1954)
The Influence of N-methylformamide on Formate ^{14}C Incorporation. I. in Nucleic Acids of Rat Liver.
J. Biol. Chem., 208, 875-882

Barlow, T. (1982)
A Pharmacokinetic Study of the Antitumour Agent N-Methylformamide in Mice, using ^{14}C Radiolabelling.
(MSc. Project), University of Aston in Birmingham, U.K.

Barnes, J.R. and Ranta, K.E. (1972)
The Metabolism of Dimethylformamide and Dimethylacetamide.
Toxicol. App. Pharmac., 23 271-276

Baselt, R.C. (1978)
in: Disposition of Toxic Drugs and Chemicals in Man. Vol. 2. Peripherally-acting Drugs and Common Toxic Chemicals.
Biomedical Publications, Connecticut.

Bast,A. and Noordhoek, J. (1981) (a)
Product Inhibition During the Hepatic Microsomal N-Demethylation of Aminopyrine in the Rat.
Biochem. Pharmac., 30, 19-24

Bast,A. and Noordhoek,J. (1981) (b)
Evaluation and Comparison of Colorimetric, Radiometric and High Performance Liquid Chromatographic Assays for Aminopyrine-N-demethylation by Rat Liver Microsomes.
J. Pharm. Pharmac., 33, 14-18

Bateman,A.E., Peckham,M.J. and Steel G.G. (1979)
Assays of Drug Sensitivity of Cells from Human Tumours: In-Vitro and In-Vivo tests on a Xenografted Tumour.
Br. J. Cancer, 40, 81-88

Bazare,J., Leamons,M.L. and Young,J.F. (1981)
Sampling Methods for Pharmacokinetic Studies in the Mouse.
J. Pharmac. Methods, 5, 99-120

Benvenuto,J.A., Stewart,D.J., Benjamin,R.S. and Ti Li Loo (1981)
Pharmacology of Pentamethylmelamine in Humans.
Cancer Res., 41, 566-569

Bergevin,P.R., Tormey,D.C. and Blom,J. (1973)
Clinical Evaluation of Hexamethylmelamine (NSC 13875).
Cancer Chemother. Rep., 57, 51-58

Bickel,M.H. and Baggiotini,M. (1966)
The Metabolism of Imipramine and its Metabolites by Rat Liver Microsomes.
Biochem. Pharmac., 15, 1155-1169

Bolis,G., D'Incalci,M., Belloni,C. and Mangioni,C. (1979)
Hexamethylmelamine in Ovarian Cancer Resistant to Cyclophosphamide and Adriamycin.
Cancer Treat. Rep., 63, 1375-1377

Bonomi,P.D., Mladineo,J., Morrin,B., Wilbanks,G. and Slayton,R.E. (1979)
Phase II Trial of Hexamethylmelamine in Ovarian Carcinoma Resistant to Alkylating Agents.
Cancer Treat. Rep., 63, 137-138

Borden,E.C., Larson,P., Ansfield,F.J., Bryan,G.T., Johnson,R.O., Ramirez G. and Wilson W.L. (1977)
Hexamethylmelamine Treatment of Sarcoma and Lymphomas.
Med. Ped. Oncol., 3, 401-406

Borkovec,A.B. and DeMilo,A.B. (1967)
Insect Chemosterilants V. Derivatives of Melamine.
J. Med.Chem., 10, 457-461

Brindley, C.J., Gescher, A., Harpur, E.S., Ross, D., Slack, J., Threadgill, M.D. and Whitby, H. (1982) (a)
Studies of the Pharmacokinetics of N-methylformamide (NSC 3051) in Mice.
Cancer Treat. Rep., (in Press)

Brindley, C.J., Gescher, A., Langdon, S.P., Broggini, M., Columbo, T. and D'Incalci, M. (1982) (b)
Studies of the Mode of Action of Antitumour Triazenes and Triazines-III. Metabolism Studies of Hexamethylmelamine
Biochem. Pharmac., 31, 625-631.

Broggini, M., Colombo, T., D'Incalci, M., Donelli, M.G., Gescher, A. and Garattini, S. (1981)
Pharmocokinetics of Hexamethylmelamine and Pentamethylmelamine in Mice.
Cancer Treat. Rep., 65, 669-672

Bruce, D.L. (1972)
What is a "Safe" Interval Between Halothane Exposures?
J. Am. Med. Assoc., 221, 1140-1143

Bryan, G.T., Worzalla, J.F., Gorske, A.L. and Ramirez, G. (1968)
Plasma Levels and Urinary Excretion of Hexamethylmelamine Following Oral Administration to Human Subjects with Cancer.
Clin. Pharmac. Ther., 9, 777-782

Buckley, S.M., Stock, C.C., Crossley, M.L. and Rhoades, C.P.(1952)
Inhibition of the Crocker Mouse Sarcoma 180 by Certain Ethyleneimine Derivatives and Related Compounds.
Cancer Res., 5, 144-152

Casper, E.S., Alcock, N.W., Kelsen, D.P. and Young, C.W.(1980)
Phase I Study of Pentamethylmelamine (PMM) by 24 Hour Infusion.
Proc. Am. Assoc. Cancer Res., 178

Catignani, G.L. and Neal (1975)
Evidence for the Formation of a Protein bound Hydrodisulphide Resulting from the Microsomal Mixed Function Oxidase Catalyse Desulfuration of Carbon Disulfide.
Biochem. Biophys. Res. Commun., 65, 629-636

Chang, S.C., De Milo, A.B., Woods, C.W. and Borkovec, A.B.(1968)
Metabolism of C^{14}-Labelled Hemel in Male House Flies.
J. Econ. Entomol., 61, 1357-1365

Chang, S.C., Terry, P.H. and Borkovec, A.B. (1964)
Insect Chemosterilants with Low Toxicity for Mammals.
Science, 144, 57-58

Chasseaud, L.F. (1976)
in: Glutathione: Metabolism and Function (Arias, I.M. and Jakoby, W.B. eds.)
Raven Press, New York. pp. 77-114

Clark, D.A., Philips, F.S., Sternberg, S.S., Barclay, R. and Stock, C.C. (1953)
Effects of N-methylformamide and Related Compounds in Sarcoma 180.
Proc. Soc. Exp. Biol. Med., 84, 203-207

Clayton, J.W., Barnes, J.R., Hood, D.B. and Schepers, G.W.H. (1963).
The Inhalation Toxicity of Dimethylformamide.
Am. Ind. Hyg. Assoc. J., 24, 144-154

Cleland, W.W. (1967)
The Statistical Analysis of Enzyme Kinetic Data.
Adv. Enzymol., 29, 1-32

Clinical Brochure. Pentamethylmelamine (NSC-118742)
Division of Cancer Treatment National Cancer Institute.
Bethesda Md., (1978)

Columbo, T., Broggini, M., Gescher, A. and D'Incalci, M.
Routes of Elimination of Hexamethylmelamine and Pentamethylmelamine in the Rat.
Xenobiotica, (in press).

Conney, A.H., Davison, C., Gastell, R. and Burns, J.J. (1960)
Adaptive Increases in Drug-Metabolising Enzymes Induced by Phenobarbital and Other Drugs.
J. Pharmac. Exp. Ther., 130, 1-8

Connorts, T.A., Cumber, A.J., Ross, W.C.J., Clarke, S.A. and Mitchley, B.C. (1977)
Regression of Human Lung Xenografts Induced by Water Soluble Analogues of Hexamethylmelamine.
Cancer Treat. Rep., 61, 927-928

Connors, T.A., Goddard, P.M., Merai, K., Ross, W.C.J. and Wilman, D.E.V. (1976)
Tumour-inhibitory Triazenes. Structural Requirements for an Active Metabolite.
Biochem. Pharmac., 21, 241-246

Cumber, A.J. and Ross, W.C.J. (1977)
Analogues of Hexamethylmelamine. The Antineoplastic Activity of Derivatives with Enhanced Water Solubility.
Chem.-Biol. Interac., 17, 349-357

De Matteis, F. (1974)
Covalent Binding of Sulfur to Microsomes and Loss of Cytochrome P-450 During the Oxidative Desulfuration of Several Chemicals.
Molec. Pharmac., 10, 849-854

De Matteis, F. and Seawright, A.A. (1973)
Oxidative Metabolism of Carbon Disulphide by the Rat. Effect of Treatments Which Modify the Liver Toxicity of Carbon Disulphide.
Chem.-Biol. Interac., 7, 375-

Clarke,D.A., Philips,F.S., Sternberg,S.S., Barclay,R. and Stock,C.C. (1953)
Effects of N-methyl formamide and Related Compounds in Sarcoma 180.
Proc. Soc. Exp. Biol. Med., 84, 203-207

Clayton,J.W., Barnes,J.R., Hood,D.B. and Schepers,G.W.H. (1963)
The Inhalation Toxicity of Dimethylformamide.
Am. Ind. Hyg. Assoc. J., 24, 144-154

Cleland,W.W. (1967)
The Statistical Analysis of Enzyme Kinetic Data.
Adv. Enzymol., 29, 1-32

Clinical Brochure. Pentamethylmelamine (NSC-118742)
Division of Cancer Treatment National Cancer Institute.
Bethesda Md., (1978)

Columbo,T., Broggini,M., Gescher,A. and D'Incalci, M.
Routes of Elimination of Hexamethylmelamine and Pentamethylmelamine in the Rat.
Xenobiotica, (in press).

Conney,A.H., Davison,C., Gastell,R. and Burns,J.J. (1960)
Adaptive Increases in Drug-Metabolising Enzymes Induced by Phenobarbital and Other Drugs.
J. Pharmac. Exp. Ther., 130, 1-8

Connors,T.A., Cumber,A.J., Ross,W.C.J., Clarke,S.A. and Mitchley,B.C. (1977)
Regression of Human Lung Xenografts Induced by Water Soluble Analogues of Hexamethylmelamine.
Cancer Treat. Rep., 61, 927-928.

Connors,T. A., Goddard,P.M., Merai, K., Ross, W.C.J. and Wilman, D.E.V. (1976)
Tumour-inhibitory Triazenes. Structural Requirements for an Active Metabolite.
Biochem. Pharmac., 21, 241-246

Cumber,A.J. and Ross,W.C.J. (1977)
Analogues of Hexamethylmelamine. The Antineoplastic Activity of Derivatives with Enhanced Water Solubility.
Chem.-Biol. Interac., 17, 349-357

De Matteis,F. (1974)
Covalent Binding of Sulfur to Microsomes and Loss of Cytochrome P-450 During the Oxidative Desulfuration of Several Chemicals.
Molec. Pharmac., 10, 849-854

De Matteis, F. and Seawright, A.A. (1973)
Oxidative Metabolism of Carbon Disulphide by the Rat.
Effect of Treatments Which Modify the Liver Toxicity of Carbon Disulphide.
Chem.-Biol. Interac., 7, 375-388

DeMilo,A.B. and Borkovec,A.B. (1968)
Insect Chemosterilants VII. Oxidative Degredation of Hexamethylmelamine.
J. Med. Chem., 11, 961-963

D'Incalci,M., Bolis,G., Mangioni,C., Luciano,M. and Garattini,S. (1978)
Variable Absorption of Hexamethylmelamine in Man.
Cancer Treat. Rep., 62, 2117-2119

D'Incalci,M., Erba,E., Balconi,G., Morasca,I. and Garattini,S. (1980)
Time Dependence of the In-Vitro Cytotoxicity of Hexamethylmelamine and Its Metabolites.
Br. J. Cancer, 41, 630-635

D'Incalci,M., Farina,P., Sessa,C., Mangioni,C., Conter,V., Masera,G., Rocchetti,M., Brambilla Pisoni,M., Piazza,E., Beer,M. and Cavalli,F. (1982)
Pharmacokinetics of VP16-213 Given by Different Administration Methods.
Cancer Chemother. Pharmac., 7, 141-145

D'Incalci,M., Morazzoni,P. and Pantarotto,C. (1979)
Gas Chromatographic Determination Of Hexamethylmelamine in Mouse Plasma.
Analyt. Biochem., 99, 441-449

Dixon,M. and Webb,E.C. (1958)
in: Enzymes
Longman's Green, London. p95

Doroshow,J.H., Locker,G.Y., Baldinger,J. and Myers,C.E. (1979)
The Effect of Doxorubicin on Hepatic and Cardiac Glutathione.
Chem. Path. Pharmac., 26

Dost,F.H. (1968)
in: Grundlagen der Pharmakokinetk, Stuttgart: Thieme

Dovlatian,V., Eliazian,K. and Aghajanian,L. (1977)
Synthesis and Conversions of 2-(B-Cyanoethyloxy)-4,6-bis-alkyl(dialkyl)amino-s-triazines.
Armenian Chem. J., 30, 66-69

Dowd,J. and Riggs,D. (1965)
A Comparison of Estimates of Michaelis-Menten Kinetic Constants from Various Linear Transformations.
J. Biol. Chem., 240, 863-869

Earle,W.R. (1943)
Production of Malignancy In-Vitro. IV. The Mouse Fibroblast Cultures and Changes Seen in Living Cells.
J. Nat. Cancer Inst., 4, 165-212

Ebel,E.R., O'Keefe,D.H. and Peterson,J.A. (1978)
Substrate Binding to Hepatic Microsomal Cytochrome P-450. Influence of the Microsomal Membrane.
J. Biol. Chem., 253, 3888-3897

Eben,A. and Kimmerle,G. (1976)
Metabolism Studies of N,N-Dimethylformamide
III Studies About the Influence of Ethanol in Persons and Laboratory Animals.
Int. Arch. Occup. Environ. Hlth., 36, 243-265

Ewig,R.A. and Kohn,K.W. (1977)
DNA Damage and Repair in Mouse Leukaemia L1210 Cells Treated with Nitrogen Mustard 1,3-bis(2-chloroethyl)-1-nitrosourea and Other Nitrosoureas.
Cancer Res., 37, 2114-2122

Fouts,J.R. (1970)
Some In-Vitro Assay Conditions that Effect Detection and Quantitation of Phenobarbital Induced Increases in Hepatic Microsomal Drug-Metabolising Enzyme Activity.
Toxicol. Appl. Pharmac., 16, 48-65

Furst,A., Cutting,W.C. and Gross,H. (1955)
Retardation of Growth of Ehrlich Ascites Tumor by Formamides and Related Compounds.
Cancer Res., 15, 294-299

Garattini,E., Donelli,M.G., Colombo,T., Paesani,R. and Pantarotto,C. (1981)
In-Vivo and In-Vitro Irreversible Binding of Hexamethylmelamine to Liver and Ovarian Tumour Macromolecules of Mice.
Biochem. Pharmac., 30, 1151-1154

Gescher,A., D'Incalci,M., Fanelli,R. and Farina,P. (1980)
N-Hydroxymethylpentamethylmelamine, a Major In-Vitro Metabolite of Hexamethylmelamine.
Life Sciences, 26, 147-154

Gescher,A., Gibson,N.W., Hickman,J.A., Langdon,S.P., Ross,D. and Atassi,G. (1982)
N-methylformamide: Antitumour Activity and Metabolism in Mice.
Br. J. Cancer, 45, 843-850

Gescher,A., Hickman,J.A. and Stevens,M.F.G. (1979)
Oxidative Metabolism of Some N-Methyl-containing Xenobiotics can Lead to Stable Progenitors of Formaldehyde.
Biochem. Pharmac., 28, 3235-3238

Gescher,A. and Raymont,C. (1981)
Studies of the Metabolism of N-Methyl containing antitumour agents. $^{14}CO_2$ Breath Analysis After Administration of ^{14}C-Labelled N-Methyl Drugs, Formaldehyde and Formate in Mice.
Biochem. Pharmac., 30, 1245-1252

Gibaldi,M. and Perrier,D. (1975)
in: Pharmacokinetics, Marcel Decker, New York, pp 293-396

Gilman,A. and Philips,F.S. (1946)
The Biological Actions and Therapeutic Applications of the B-Chloroethyl Amines and Sulphides.
Science, 103, 409-415

Gladtke,E. and Hattingberg,H.M. (1979)
in:Pharmacokinetics. An Introduction.
Springer-Verlag, New York, pp 20-21

Goromaru,T., Furuta,T., Baba,S., Noda,A. and Iguchi,S. (1981)
Metabolic Studies of Aminopyrine in Rat and Man by Using Stable Isotope Tracer Techniques.
Chem. Pharm. Bull, 29, 1724-1729

Gorrod,J.W. (1978)
in: Drug Toxicity, ed. Gorrod,J.W.
Taylor and Francis, London. pp. 20-22

Gram,T.E., Wilson,J.T. and Fouts,J.R. (1968)
Some Characteristics of Hepatic Microsomal Systems which Metabolise Aminopyrine in the Rat and Rabbit.
J. Pharmac. Exp. Ther., 159, 172-181

Green,A.E. and Gescher,A. (1980)
Drug Metabolism Interactions with Anticancer Agents in Mice.
Biochem. Pharmac., 29, 131-136

Haag,S. and Sipes,I. (1980)
Differential Effects of Acetone or Aroclor 1254 Pretreatment on the Microsomal Activation of Dimethylnitosamine to a Mutagen.
Mutat. Res., 74, 431-438

Hahn,D. (1980)
Hexamethylmelamine: A Review.
Drug Intelligence and Clinical Pharmacy, 14, 591-598

Hanes,C.S. (1932)
Studies on Plant Amylases I. The Effect of Starch Concentration Upon the Velocity of Hydrolysis by the Amylase of Germinated Barley.
Biochem. J., 26, 1406-1421

Hansch,C. (1979)
Substituent Constants for Correlation Analysis in Chemistry and Biology.
Wiley-Interscience, New York

Hayes, J., BcModile, M. and Campbell, T. (1973)
Effect of Protein Deficiency on the Inducibility of the Hepatic Microsomal Drug-Metabolising Enzyme System-I. Effect of Substrate Interaction with Cytochrome P-450.
Biochem. Pharmac., 22, 1005

Heere, L.J. and Donnely, S.T. (1971)
Antitumour Activity of Hexamethylmelamine and 4(5)-(3,3-dimethyl-1-triazeno)-imidazole-5(4)-carboxamide
Proc. Am. Assoc. Cancer Res., 12, 101

Hendry, J.A., Rose, F.L. and Walpole, A.L. (1951)
Cytotoxic Agents I. Methylolamides with Tumour Inhibitory Activity and Related Inactive Compounds.
Brit. J. Pharmac., 6, 201-234

Hickinbottom, W.J. (1957)
Reactions of Organic Compounds.
Longmans, London. pp 398-399

Hofstee, B.H. (1959)
Non-Inverted Versus Inverted Plots in Enzyme Kinetics.
Nature, Lond. 184, 1296-1298

Ihde, D.C., Dutcher, J.S., Young, F.C., Cordes, R.S., Barlock, A.L., Hubbard, S.M., Jones, R.B. and Boyd, M.R. (1981)
Phase I Trial of Pentamethylmelamine: A clinical and Pharmacological Study.
Cancer Treat. Rep., 65, 755-762

Ihde, D.C., Young, F.C., Cordes, R.S., Barlock, A.L., Dutcher, J.S., Jones, R.B. and Boyd, M.R. (1980)
Phase I Trial of Pentamethylmelamine (PMM).
Proc. Am. Assoc. Cancer Res., 21, 143

Johnson, B.L., Fisher, R.I., Bender, R.A., De Vita, V., Chabier, B. and Young, R. (1978)
Hexamethylmelamine in Alkylating Agent - resistant Ovarian Carcinoma.
Cancer, 42, 2157-2161

Johnson, B.L. and Torres, E. (1976)
In-vitro N-demethylation of the Isomeric and Homologous Methylmelamines.
Proc. Am. Assoc. Cancer Res., 17, 56

Kaiser, D.W., Thurston, J.T., Dudley, J.R., Schaefer, F.C. and Hechenbleikner, I. (1951)
Cyanuric Chloride Derivatives II. Substituted Melamines.
J. Am. Chem. Soc., 73, 2984-2986

Kawalek,J.C. and Andrews,A.W. (1980)
The Effect of Solvents on Drug Metabolism In-Vitro.
Drug Metabolism and Disposition, 8, 380-382

Kimmerle,G. and Eben,A. (1975)
Metabolism Studies of N,N-Dimethylformamide I. Studies in Rats and Dogs.
Int. Arch. Arbeitsmed., 34, 109-125

Kohn,K.W. (1979)
DNA as Target in Cancer Chemotherapy: Measurement of Macromolecular DNA Damage Produced in Mammalian Cells by Anticancer Agents and Carcinogens.
Methods Cancer Res., 16, 291-345

Krüger-Theimer,E.(1968)
Non-linear Dose-concentration Relationships.
Farmaco, 23, 717-756

Kuntzman,R., Levin,W., Schilling,G. and Alvarea,A. (1969)
The Effects of 3-Methylcholanthrene and Phenobarbital on Liver Microsomal Hemoproteins and on the Hydroxylation of Benzpyrene.
in: Microsomes and Drug Oxidations (Eds. Gillette,J.R., Conney,A.H., Cosmides,G.J., Estabrooke,R.W., Fouts,J.R. and Mannering,G.J.)
Academic Press, New York. pp. 349-363

LaBreque,G.C., Fye,R.L., DeMilo,A.B. and Borkovec,A.B. (1968)
Substituted Melamines as Chemosterilants of House Flies.
J. Econ. Entomol., 61, 1621-1632

Lake,L.M., Grunden,E.E. and Johnson,B.M. (1975)
Toxicity and Antitumour Activity of Hexamethylmelamine and its N-demethylated Metabolites in Mice with Transplantable Tumours.
Cancer Res., 35, 2858-2863

Legha,S.S., Slavik,M. and Carter,S. (1976)
Hexamethylmelamine. An Evaluation of Its Role in the Therapy of Cancer.
Cancer, 38, 27-35

Lenk,W. (1976)
Commentary: Application and Interpretation of Kinetic Analysis from the Microsomal Drug Metabolising Oxygenases.
Biochem. Pharmac., 25, 997-1005

Levine,W.G. and Finkelstein,T.T. (1979)
A Role for Liver Glutathione in the Hepatobiliary Fate of N,N-Dimethyl-4-Aminoazobenzene.
J. Pharmac. Exp. Ther., 208, 309-405

Lichtenfield,J.L., Van Echo,D.A., Aisner,J. and Wiernek,P.H. (1980)
A Phase I Trial of Pentamethylmelamine (NSC 118742, PMM) in Patients with Previously Treated Malignancies.
Proc. Am. Assoc. Cancer Res., 21, 347

Lineweaver,H. and Burk,D. (1934)
The Determination of Enzyme Dissociation Constants.
J. Am. Chem. Soc. 56, 658-666

Lowry,O.R., Rosenbrough,N.J., Farr,A.L. and Randall,R.J. (1951)
Protein Measurement with Folin-phenol Reagent.
J. Biol. Chem., 193, 265-275

Lundberg,I., Lundberg,S. and Kronevi,T. (1981)
Some Observations on Dimethylformamide Hepatotoxicity.
Toxicology, 22, 1-7

Lundberg,I., Pehrsson,A., Lundberg,S. and Kronevi,T. (1982)
Accumulation of Monomethylformamide after Dimethylformamide Inhalation in Rats.
Submitted for publication.

Mannering,G.J., Sladek,N.E., Parli,C.J. and Shoeman,D.W. (1969)
Formation of a New P-450 Hemoprotein after Treatment of Rats with Polycyclic Hydrocarbons.
in: Microsomes and Drug Oxidations (Eds. Gillette,J.R., Conney,A.H., Cosmides,G.J., Estabrook,R.W., Fouts,J.R. and Mannering,G.J.).
Academic Press, New York. pp. 303-330

Massman,W. (1956)
Toxicological Investigations on Dimethylformamide.
Br. J. Ind. Med., 13, 51-54

Maxfield,M., Barnes,J.R., Azar,A. and Trochimowicz,H.T. (1975)
Urinary Excretion of Metabolite Following Experimental Human Exposure to DMF or to DMAC.
J. Occup. Med., 17, 506-511

Meyer,M.C. and Guttman,D.E. (1968)
The Binding of Drugs by Plasma Proteins.
J. Pharm. Sci., 57, 895-918

Michaelis,L. and Menten,M.L. (1913)
Die Kinetic der Invertin wirking.
Biochem. Z., 49, 333-369

Mitchley,B.C.V., Clarke,S.A., Connors,T.A. and Neville,A.M. (1975)
Hexamethylmelamine Induced Regression of Human Lung Tumours Growing in Immune Deprived Mice.
Cancer Res., 35, 1099-1101

Muindi,J.R.F., Rutty,C.J. and Harrap,K.R. (1981)
DNA Cross-linking Induced by Pentamethylmonomethylolmelamine In-Vitro.
Brit. J. Cancer, 44, 281.

Mukundrai, P.A. (1981)
Formulation, Sterilisation and Stability Studies on New Antitumour Drugs.
MSc. Project, University of Aston in Birmingham, U.K.

Myers, W.P.L., Karnofsky, D.A. and Burchenal, J.H. (1956)
The Hepatotoxic Action of N-methylformamide in Man.
Cancer, 9, 949-954

Nadkarni, M.V., Goldenthal, E.I. and Smith, P.K. (1954)
The Distribution of Radioactivity Following Administration of Triethyleminimo-s-triazine-C^{14} in Tumour Bearing and Control Mice.
Cancer Res., 14, 559-562

Nash, T. (1953)
The Colorimetric Estimation of Formaldehyde by Means of the Hantzsch Reaction.
Biochem. J., 55, 516-421

Nebert, D.W. and Gielen, J.E. (1972)
Genetic Regulation of Aryl Hydrocarbon Hydroxylase Induction in the Mouse.
Fed. Proc., 31, 1315-1325

Newman, K.N., Meeks, R.G. and Frick, S. (1981)
Phase I Report of Task I Preclinical Intravenous Toxicity Study of N-methylformamide (NSC-3051) in CDFI Mice and Beagle Dogs.
Southern Research Institute, Birmingham, Alabama.

Nony, C.R., Bowman, M.C., Holder, C.L., Young, J.F. and Oller, W.L. (1976)
Trace Analysis of 2,4,5-Trichlorophenoxyacetic Acid, its Glycineamide, and their Alkaline Hydrolyzable Conjugates in Mouse Blood, Urine and Faeces.
J. Pharm. Sci., 65, 1810-1816

Omura, G.A. (1981)
Hexamethylmelamine in Mustard Resistant Ovarian Adenocarcinoma.
Cancer Treat. Rep., 65, 530

Paget, W.M. and Hmmer, W.F. (1958)
The Infra-red Spectra of Some Derivatives of 1,3,5-triazene.
J. Am. Chem. Soc., 80, 803-808

Pederson, T.C. and Aust, S.C. (1970)
Aminopyrine Demethylase. Kinetic Evidence for Multiple Microsomal Activities.
Biochem. Pharmac., 19, 2221-2230

Philips, F.S. and Thiersch, J.B. (1950)
The Nitrogen Mustard-like Actions of 2,4,6-Tris (ethylenimino) - s - triazine and Other Bis(ethylenimines).
J. Pharmac. Exptl. Ther., 100, 398-407

Poland, A.P. and Nebert, D.W. (1973)
A Sensitive Radiometric Assay of Aminopyrine N-Demethylation.
J. Pharmac. Exp. Ther., 184, 269-277

Potter, H.P. (1973)
Dimethylformamide-Induced Abdominal Pain and Liver Injury.
Arch. Environ. Health, 27, 340-341

Ross, E.W., McMillan, D.R. and Ross, C.F. (1981)
Comparison of DNA Damage by Methylmelamines and Formaldehyde.
JNCI, 67, 217-221

Rutty, C.J. and Abel, G. (1980)
In-Vitro Cytotoxicity of the Methylmelamines.
Chem. Biol. Interac., 29, 235-246

Rutty, C.J. and Connors, T.A. (1977)
In-Vitro Studies with Hexamethylmelamine
Biochem. Pharmacol., 26, 2385-2391

Rutty, C.J., Connors, T.A., Nguyen-Hoang, N., Do-Cao-Thang and Hoellinger, H. (1978)
In-Vivo Studies with Hexamethylmelamine.
Eur. J. Cancer, 14, 713-720

Saegusa, T., Kobayshi, S., Hirota, K. and Ito, Y. (1969)
Synthetic reactions by complex catalysts. XIII - Carbonilation of amines by group IB and IIB metal compound catalysts.
Bull. Chem. Soc., Japan, 42, pp 2610-2614

Segal, I.H. (1975)
in: Enzyme Kinetics, Wiley, New York

Scailter, V., Buchet, J.P. and Lauwrys,R. (1981)
The Relationship Between Dimethylformamide Metabolism and Toxicity.
in: Organ Directed Toxicity (ed., Brown, S. and Davies, D.).
Pergamon Press, pp 160-174

Silvarajah, K., Lasker, J.M., Eling, T.E., and Abou-Doria, M.B. (1982)
Metabolism of N-alkyl Compounds During the Biosynthesis of Prostaglandin:
N-dealkylation During Prostaglandin Biosynthesis.
Mol. Pharmac., 21, 133-141

Simmonds, R.J. and Stevens, M.F.G. (1982)
Triazenes and Related Products. Part 25. Methods for Attachment of Sugar Residues to Cytotoxic 1,3,5-Triazines.
J. Chem. Soc. Perkins Trans., 1, 1821-1825

Sipes, I., Slocumb, M. and Holtzman, G. (1978)
Stimulation of Microsomal Dimethylnitrosamine-demethylase by Pretreatment of Mice with Acetone.
Chem.-Biol. Interact., 21, 155-166

Smith, I.E., Muindi, J.F.R., Newell, D.R., Merai, K., Rutty, C.J., Wilman, D.E.V. and Taylor, R.E. (1980)
Pentamethylmelamine (PMM): Phase I and Pharmacokinetic Studies.
Proc. Am. Assoc. Cancer Res., 21, 136

Skipper, H.E., Schabel, F.M., Binns, V., Thompson, J.R. and Wheeler, G.P. (1955)
Studies on the Mechanism of Action and Anticancer Activity of N-methylformamide.
Cancer Res., 2, 425-492

Smith, P.K., Nadkarni, M.V., Trams, E.G. and Davison, C. (1958)
Distribution and Fate of Alkylating Agents.
Ann. N.Y. Acad. Sci., 68, 834-850

Smith, R.L. and Williams, R.T. (1961)
The Metabolism of Aryl Thioureas. III. (a) The Toxicity of Hydrogen Sulphide in Relation to that of Thiourea. (b) The Protection of Rats Against the Toxic Effects of Phenylthiourea, with 1-Methyl-1-Phenylthiourea.
J. Med. Chem., 4, 137-146

Stolinsky, D.C. and Bateman, J.R. (1973)
Further Experience with Hexamethylmelamine (NSC-13875) in the Treatment of Carcinoma of the Cervix.
Cancer Chemother. Rep., 57, 497-499

Stotz, E. (1943)
A Colorimetric Determination of Acetaldehyde in Blood.
J. Biol. Chem., 148, 585-591

Supniewski, J.V. (1926)
The Toxicological Properties of Certain Thiocarbamine Compounds.
J. Pharmac. Exp. Ther., 28, 317-325

Takita, H. and Didolkar, M.S. (1974)
Effect of Hexamethylmelamine (NSC-13875) on Small Cell Carcinoma of the Lung (Phase II Study).
Cancer Chemother. Rep., 58, 371-374

Testa, B. and Jenner, P. (1976)
In: Drug Metabolism, Chemical and Biochemical Aspects
M. Dekker, New York, p. 82

Thurston,J.T., Dudley,J.R., Kaiser,D.W., Hecherbleinkner,I., Schaefer,F.C. and Holm-Hanser,D. (1951)
Cyanuric Acid Derivatives. I-Aminochloro-s-triazines.
J. Am. Chem. Soc., 73, 2981-2983

Ungar,H., Sullman,S.F. and Zuckerman,A.J. (1976)
Acute and Protracted Changes in the Liver of Syrian Hamsters Induced by a Single Dose of Aflotoxin B1.Observations on Pathological Effects of the Solvent (Dimethylformamide).
Br. J. Exp. Path., 57, 157-164

Venditti,J.M. (1975)
Relevence of Transplantable Animal Tumour Systems to the Selection of New Agents for Clinical Trial.
In:Pharmacological Basis of Cancer Chemotherapy. Baltimore, Williams and Wilkens (1975): pp 245-270

Venkatesan,N., Arcos,J.C. and Argus,M.F. (1971)
Amino Acid Induction and Carbohydrate Repression of Dimethylnitrosamine-demethylase in Rat Liver.
Cancer Res., 30, 2556-2562

Vogl,S.E., Berenzweig,M., Kaplan,B.H., Moukhtar,M. and Bulkin,W. (1979) (a)
The CHAD and HAD Regimens in Advanced Ovarian Cancer: Combination Chemotherapy Including Cyclophosphamide, Hexamethylmelamine, Adriamycin and cis-Dichlorodiammine-platinum (II).
Cancer Treat. Rep., 63, 311-317

Vogl,S.E., Greenwald,E., Kaplan,B.H., Mouktar,M. and Wollner,D. (1979) (b)
Ovarian Cancer. Effective Treatment After Alkylating Agent Failure.
JAMA, 241, 1908-1911

Wagner,J.G. (1973)
A Modern View of Pharmacokinetics.
J. Pharmacokin. Biopharm., 1, 363-401

Wagner,J.G. (1975)
in: Fundamentals of Clinical Pharmacokinetics.
Drug Intelligence Inc., Illinois. pp. 347

Wampler,G.L., Mellette,S.J., Kuperminc,M. and Regelson,W. (1972)
Hexamethylmelamine (NSC-13875) in the Treatment of Advanced Cancer.
Cancer Chemother. Rep., 56, 505-514

Weinkam,R.J. and Shiba,D.A. (1978)
Metabolic Activation of Procarbazine.
Life Sci., 22, 937-945

Wietzel,G., Schneider,F. and Fretzdorff,A.M. (1964)
Cytostatischer Wirkingsmechanismus der Methylhydrazine.
Experientia, 20, 38-39

Werringloer,J. (1978)
in: Methods in Enzymology, Vol. 52, part C (eds., Fleischer,S. and Packer,C.),
Academic Press, London, p.257

Wharton,J.T., Rutledge,F., Smith,J.P., Herson,J. and Hodge,M. (1979)
Hexamethylmelamine: an Evaluation of its Role in the Treatment of Ovarian Cancer.
Am. J. Obstet. Gynecol., 133, 833-844

Wilkinson,G.N. (1961)
Statistical Estimations in Enzyme Kinetics.
Biochem. J., 80, 324-332

Wilson,W.L. and de la Garza,J.G. (1965)
Phase I Study of Hexamethylmelamine (NSC-13875).
Cancer Chemother. Rep., 48, 49-52

Wilson,W.L., Schroeder,J.M., Besel,H.F., Mrazek,R. and Hummel,R.P. (1969)
Phase II Study of Hexamethylmelamine (NSC-13875).
Cancer, 23, 132-136

Winters,W.D., Spector,E., Wallach,D.P. and Studeman,F.E. (1955)
Metabolism of Thiopental-S^{35} and Thiopental-2-C^{14} by a Rat Liver Mince and Identification of Phenobarbital as a Major Metabolite.
J. Pharmac. Exp. Ther., 114, 343-357

Workman,P. (1980) (a)
Dose-dependence and Related Studies on the Pharmacokinetics of Misonidazole and Desmethylmisonidazole in Mice.
Cancer Chemother. Pharmac., 5, 27-37

Workman,P. (1980) (b)
Pharmacokinetics of Hypoxic Cell Radiosensitisers. A review.
Cancer Clinical Trials.

Worzalla,J.F., Johnson,B.M., Ramirez,G. and Bryan,G.T. (1973)
N-Demethylation of the Antieoplastic Agent Hexamethylmelamine by Rats and Man.
Cancer Res., 33, 2810-2815

Worzalla, J.F., Kaiman, B.D., Johnson, B.M., Johnson, R.O. and Bryan, G.T. (1974) (a)
Biotransformations of a Series of Methylmelamine Homologs of Hexamethylmelamine (HMM) In-Vivo
Am. Assoc. Cancer Res., 15, 15

Worzalla, J.F., Kaiman, B.D., Johnson, B.M., Ramirez, G. and Bryan, G.T. (1974) (b)
Metabolism of Hexamethylmelamine - Ring - ^{14}C in Rats and Man
Cancer Res., 34, 2669-2674

Worzalla, J.F., Lee, D.M., Johnson, J.O. and Bryan, G.T.(1972)
Effect of Microsomal Enzyme-inducing Chemicals on the Metabolism of Hexamethylmelamine (HMM, NSC-13875) in Rats and Hamsters.
Proc. Am. Assoc. Cancer Res., 13, 41

Zeffren, E. and Hall, P. (1975)
The Study of Enzyme Mechanisms.
Wiley, New York. pp. 63-64