STUDIES ON THE METABOLISM AND TOXICITY OF N-METHYL CONTAINING COMPOUNDS

by

Helen Margaret Whitby

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To my parents

The University of Aston in Birmingham

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Submitted for the degree of Doctor of Philosophy, 1983.

The influence of several factors on the oxidative metabolism of hexamethylmelamine (HMM), procarbazine, caffeine, aminopyrine (AP), N-methylformamide (NMF) and N,N-dimethylformamide was investigated in vivo. Mice were administered ^{14}C-methyl labelled compounds and the fate of the N-methyl moiety was monitored by $^{14}CO_2$ exhaled with the breath. The $^{14}CO_2$ exhalation profile for caffeine, but not HMM, was shown to be dependent on the dose. The influence on caffeine metabolism of inhibition or induction of cytochrome P450 dependent enzymes using SKF 525A or phenobarbitone, respectively, also depended on the dose of caffeine. To assess the influence of a tumour on N-demethylation, mice bearing tumours were administered ^{14}C-methyl labelled drugs. Only |^{14}C| AP administered to CBA/Ca mice with TLX5 tumours showed any altered $^{14}CO_2$ exhalation profile, probably due to cytochrome P450 inhibition. Characterisation of the $^{14}CO_2$ exhalation from |^{14}C-methyl| NMF suggested that zero order kinetics were involved in the rate determining step to $^{14}CO_2$, as exhalation proceeded at a constant low rate.

A study was made of possible mechanisms of hepatotoxicity of NMF. In isolated mouse hepatocytes, NMF, in contrast to other related formamides, produced changes in glutathione status and increased lipid peroxide production. Histopathology of livers and plasma liver enzyme measurements showed hepatotoxicity was dependent on the strain of mouse and dose dependent in BALB/c mice. The characteristic periacinar necrosis was not apparent until 12 hours after NMF. Survivors of high dose NMF showed signs of liver regeneration. Lipid peroxidation was observed only in livers showing severe necrosis and liver glutathione was unchanged even after multiple doses of NMF.

Assessment of calcium uptake by microsomes or mitochondria from livers of mice pretreated with NMF revealed a marked inhibition, one hour after the dose, only in mitochondria. This inhibition of mitochondrial calcium uptake was seen only with NMF.

The alteration in glutathione and lipid peroxidation in vitro may be relevant to the hepatotoxicity of NMF seen in vivo. However, the lack of effect on glutathione levels after multiple doses and the presence of lipid peroxidation only as a sequela of severe liver damage suggested that these may be secondary events. Inhibition of mitochondrial calcium uptake one hour after NMF suggested that this could be an early toxic effect important to the in vivo hepatotoxicity of NMF. The region of necrosis induced by NMF in vivo suggested that a metabolite of NMF may be responsible.

Keywords: Metabolic N-demethylation, $^{14}CO_2$ exhalation, N-alkylformamide hepatotoxicity, hepatocellular glutathione, intracellular calcium transport.

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ABBREVIATIONS

ALT	alanine aminotransferase
AP	1,2-dihydro-2,3-dimethyl-4-(N,N-dimethylamino)-1-phenylpyrazol-5-one; aminopyrine.
AST	aspartate aminotransferase
DMF	N,N-dimethylformamide.
F	formamide
GSH	reduced glutathione
GSSG	oxidised glutathione
HMF	N-hydroxymethylformamide
HMM	2,4,6-Tris(dimethylamino)-1,3,5-triazine; hexamethylmelamine.
i.p.	intraperitoneal
i.v.	intravenous
NADP(H)	nicotinamide adenine dinucleotide phosphate (reduced form)
NMF	N-methylformamide
PB	phenobarbitone, sodium salt
PCZ	4-(2-methylhydrazinyl)-methyl-N-(prop-2-yl) benzamide; procarbazine
SDH	Sorbitol dehydrogenase
s.c.	subcutaneous
SKF525A	2-diethylaminoethyl 2,2-diphenyl valerate; proadifen
TARM	Thiobarbituric acid reactive material
2VP	2-vinylpyridine

PREFACE

This thesis describes work which was planned to elucidate details of, firstly, the metabolism of certain N-methyl containing compounds and secondly, the mechanism by which one of these drugs causes toxicity. Consequently the thesis is divided into two parts. Part one is concerned with studies of the oxidative metabolism of some N-methyl containing compounds, including hexamethylmelamine, procarbazine, caffeine and N-methylformamide. Although very different in their spectrum of pharmacological activity and chemical structure, the compounds investigated have several similar features. The anti-neoplastic compounds among them appear to require metabolic activation and the presence of the N-methyl moiety is considered essential for their activity (see 1.I). In an attempt to compare some characteristics of the metabolism of these compounds, the effect of different factors, such as dose dependency or cytochrome P-450 inducers and inhibitors, on the fate of the N-methyl group was investigated. Carbon dioxide is the final product of the metabolic oxidation of the N-methyl group via formaldehyde. The method of investigation used here was the determination of the amount of $^{14}CO_2$ exhaled by animals dosed with ^{14}C-methyl labelled compounds.

Part two is concerned with the metabolism and toxicity of N-methylformamide (NMF) both *in vivo* and *in vitro* by a variety of methods. Chemically, NMF is the most simple of the N-methyl compounds dealt with in this thesis. *In vitro* studies of the biochemical mechanisms associated with the metabolism and especially with the hepatotoxicity of NMF were made by observing changes in glutathione status, lipid peroxide production and morphology of isolated mouse hepatocytes incubated with drug. In the *in vivo* studies, changes in

glutathione status, lipid peroxide production and calcium homeostasis were observed in livers of mice treated with NMF. In addition, characterisation of the hepatotoxicity was performed by measuring the release of certain liver enzymes into the plasma and by studying the histopathology of livers from mice treated with NMF.

PART ONE

Studies on the metabolism of N-^{14}C-methyl containing compounds *in vivo* by analysis of $^{14}CO_2$ exhalation.

SECTION I

INTRODUCTION

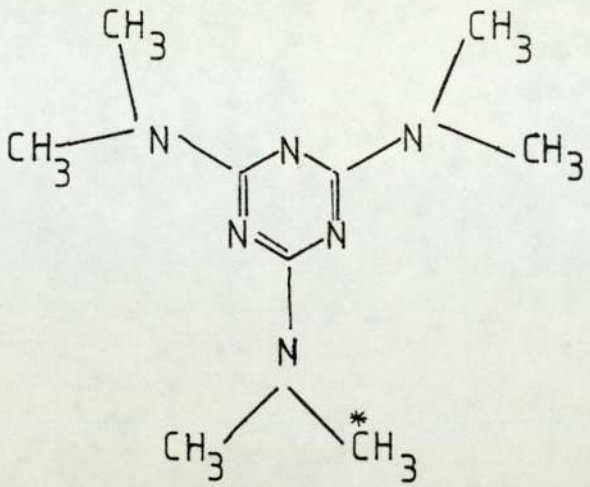
1.I Properties and metabolism of the N-methyl containing compounds investigated in this study

This thesis is concerned with the metabolism of some N-methyl group containing agents and the relationship with some pharmacological or toxicological properties of these agents. In the following, some of the pharmacological and toxicological properties of the drugs, hexamethylmelamine (HMM), procarbazine (PCZ) and N-methylformamide (NMF) (Figure 1) which are antitumour agents, and caffeine (Figure 1) are briefly outlined.

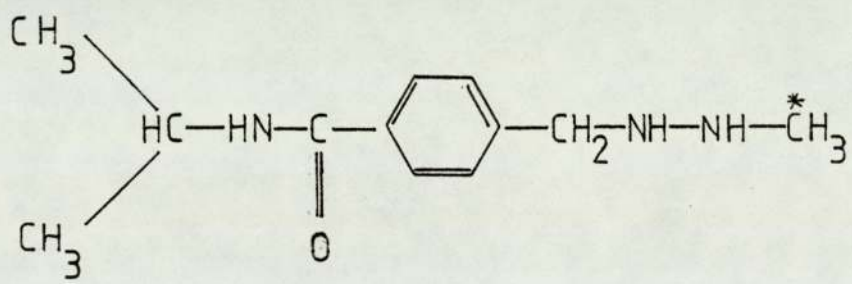
HMM has been shown to have activity against several murine tumours including the ADJ/PC6A plasma cytoma (Mitchley et al., 1975) and the M5076/A reticulum cell sarcoma (Brindley et al., 1982a). HMM has been used singly or in combination with other cytotoxic agents in a number of human malignancies, especially those of the lung (Takita and Didolkar, 1974) and the ovary (Wharton et al., 1979; Bonomi et al., 1979; Vogl et al., 1979). The major dose limiting toxicities of HMM are gastrointestinal, neurological and haemopoeitic (Hahn and Black 1980).

Several reports have indicated that metabolic activation of HMM to cytotoxic intermediate species is required for antitumour activity (Rutty and Abel, 1980; Rutty and Connors, 1977). It has also been reported that replacement of the N-methyl group by NH or N-ethyl moieties in the melamine molecule resulted in a corresponding reduction in antitumour activity and also host toxicity (Rutty and Connors, 1977; Lake et al., 1975), thus emphasising the requirement for the N-methyl group for both activity and toxicity. Oxidative demethylation of HMM, as shown in Figure 2, is therefore a major pathway of metabolism and activation. The demethylation of HMM (I)

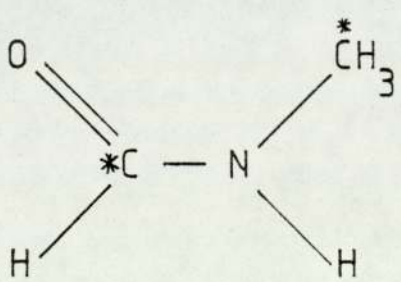
A.



HEXAMETHYLMELAMINE (HMM)



PROCARBAZINE (PCZ)



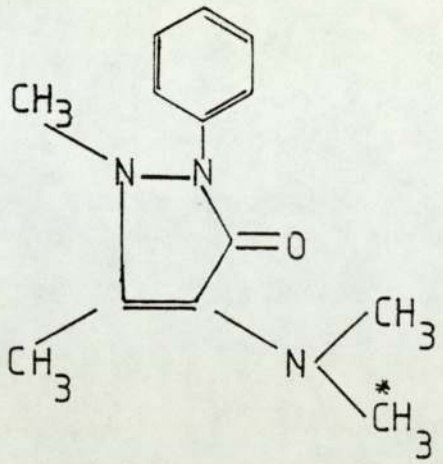
N-METHYLFORMAMIDE (NMF)

(labelled at either methyl or formyl positions)

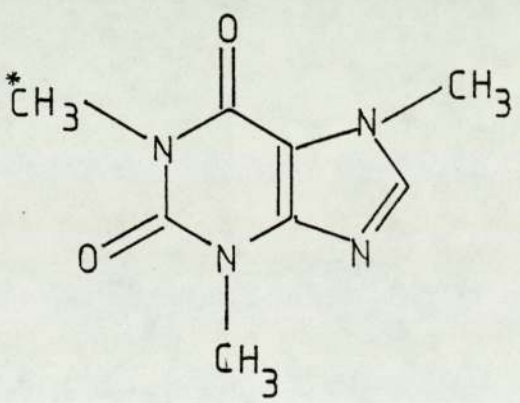
Figure 1 : Chemical structures of N-methyl containing compounds investigated with A antitumour activity and B (overleaf) no antitumour activity.

(asterix denotes position of ^{14}C label for $^{14}CO_2$ exhalation studies)

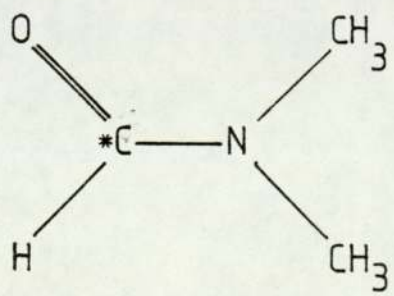
B.



AMINOPYRINE (AP)



CAFFEINE



N,N-DIMETHYLFORMAMIDE (DMF)

is considered to proceed via C-hydroxylation to N-hydroxymethylpentamethylmelamine (HMPMMM, II) a major *in vitro* metabolite (Gescher *et al.*, 1980). This decomposes to pentamethylmelamine (PMM, III) with liberation of formaldehyde (IV) (De Milo and Borkovec, 1968). HMPMM, unlike HMM, has been shown to be directly cytotoxic when incubated with murine or human tumour cells (Rutty and Abel, 1980; D'Incalci *et al.*, 1980). It appears that formation of more stable methylols such as HMPMM constitutes an activation whereas the cleavage of the N-C bond in the N-methylols to N-desmethyl derivatives appears to be a detoxification process.

PCZ has been shown to be an effective antitumour agent against several tumours both in the laboratory and in the clinic (Bollag, 1963 ; Reed, 1975) and is established as a component of combination regimens for the treatment of Hodgkins disease and other neoplasias (De Vita *et al.*, 1975; Spivack, 1974). Toxicity includes delayed bone marrow and CNS depression, nausea and vomiting. PCZ is also a potent teratogen, mutagen and carcinogen (Chaube and Murphy, 1969; Sieber *et al.*, 1978).

As with HMM, the requirement of the N-methyl group for cytotoxic activity has been reported for PCZ. Bollag and Grunberg (1963) showed that the most marked antitumour activity was found with methyl-substituted hydrazines. The biochemical reactivity of the methyl group of PCZ was confirmed in experiments using ^{14}C-methyl and ^{14}C-ring labelled azoprocarbazine, the major metabolite of PCZ, in which preferential covalent binding of the methyl portion to microsomal protein was observed when compared with the ring labelled portion of the molecule (Wiebkin and Prough, 1980).

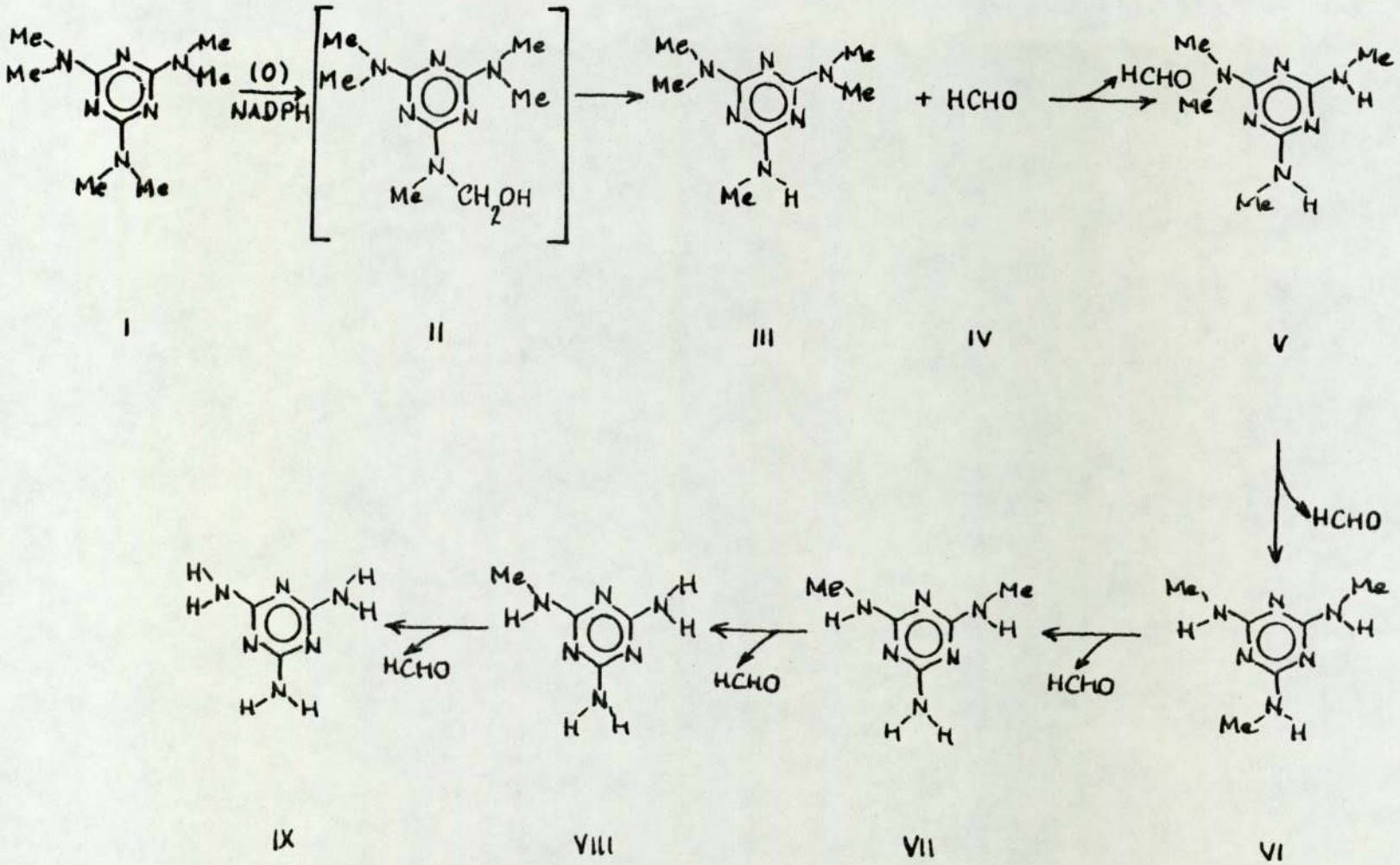


Figure 2 : Suggested pathway for the oxidative N-demethylation of HMM : HMM(I) is metabolised to HMPMM(II) which decomposes to PMM (III) and formaldehyde (IV). Further demethylation yields lower melamines. Each oxidised step is believed to proceed via an N-hydroxylated intermediate with production of HCHO.

The mechanism of action of PCZ as an antitumour agent or as a carcinogen is unclear and many complex metabolic pathways have been proposed, that result in a number of toxic species. Several experiments have shown the involvement of cytochrome P-450 dependent processes in these metabolic steps (Wiebkin and Prough, 1980; Dunn et al., 1979; Dewald et al., 1968). PCZ (I) rapidly undergoes oxidation to azoprocarbazine, 4-(2-methylazo)-methyl-N-(prop-2-yl)-benzamide (II, Figure 3). This reaction can occur chemically or enzymatically in vivo (Bagglioni et al., 1969; Weinkam and Shiba, 1978). The azo compound has been shown to be the major stable metabolite produced by rat liver microsomes incubated with PCZ (Dunn et al., 1979) and retains some cytosolic activity (Bollag et al., 1964). In vitro and in vivo investigations have shown that N-oxidation of the azo compound leads to two azoxyisomers (IIIa and IIIb), which may be hydroxylated to two hydroxyazoxy isomers or hydrazones (IVa and IVb). Formation of an aldehyde, 4-formyl-N-(prop-2-yl)-benzamide (VII), from this sequence was suggested (Bagglioni et al., 1969) and confirmed by experiments by Dunn et al., (1979). The 4-carboxy-N-(prop-2-yl) benzamide (IX), the inactive, major urinary metabolite was found to be the final product of the pathway. Dost and Reed (1969) also reported that carbon dioxide and methane were expired products following administration of radiolabelled PCZ to rats. It was postulated that the hydrazone intermediates produced could undergo several reactions, including hydrolysis to methylhydrazine (VIII) or the formation of an alkylating species (V) and formaldehyde (VI). Both methylhydrazine and formaldehyde could be further metabolised to carbon dioxide (X). Weinkam and Shiba (1978) also suggested that a number of free radical products could be formed following

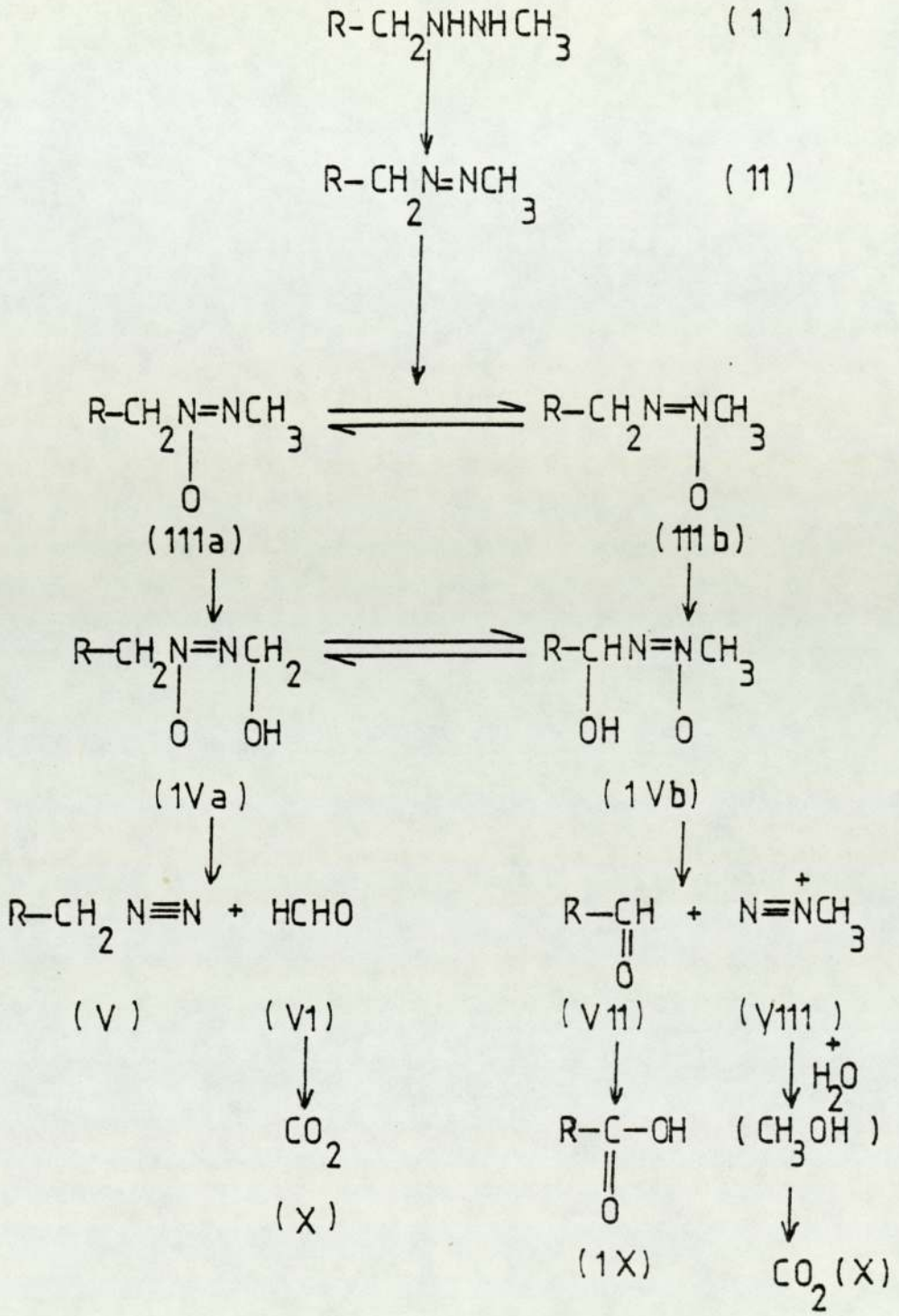


Figure 3 : Simplified proposed metabolic pathway of PCZ(I) in vivo, resulting in the formation of formaldehyde (VI) and CO_2 (X)

(R = $(CH_3)_2CHNHCOC_6H_4$)

C-hydroxylation of the azo compound (II). Methane, formaldehyde and subsequently carbon dioxide would be by-products of these reactions. Dunn et al (1979) observed that many facets of the metabolic sequence described here are similar to those described for the carcinogen 1,2-dimethylhydrazine (Fiala et al., 1975) and proposed that one or more of the reactive intermediates of PCZ metabolism could be responsible for the carcinogenicity and anti-tumour properties of the drug.

NMF is an antitumour agent that has demonstrated antineoplastic activity against both murine tumours and human tumour xenografts in mice (Clarke et al., 1953; Furst et al., 1955; Gescher et al., 1982; Dexter et al., 1982). It is now undergoing Phase 2 clinical trials.

NMF is hepatotoxic, as is the structurally related formamide, N,N-dimethylformamide (DMF, Figure 1). NMF appears to be more toxic in rats than DMF (Scailteur et al., 1981). In the first clinical trial of NMF as an antitumour agent, hepatotoxicity was the major side effect of treatment (Myers et al., 1956) and liver damage has been reported in rats and dogs (Lundberg et al., 1981; Newman et al., 1981). Unlike most anticancer drugs, NMF appears to have no myelosuppressive properties in laboratory animals (Newman et al., 1981; Langdon et al., submitted for publication; Dexter et al., 1982) or in man according to the results of recent Phase 1 clinical trials (McVie et al., 1983). This lack of bone marrow toxicity makes NMF an ideal candidate for combination regimens with myelosuppressive antitumour agents, if activity can be established for NMF in the recent Phase 2 investigations.

As with PCZ, the N-methyl group appears essential for optimum antitumour activity. Only NMF showed significant antitumour activity when compared with N-ethylformamide or formamide (F), (Gescher et al., 1982). DMF also showed marginal antitumour activity (Clarke et al., 1953).

The mode of antineoplastic activity of NMF is unknown. NMF and F are relatively stable compounds when compared with some other antitumour compounds and the possibility of a direct chemical reaction with cellular components seems unlikely. However an NMF metabolite may be a reactive species. Several theories have been proposed for the mechanism of action of NMF. It was suggested that NMF is able to interfere with metabolism of C^1 units by inhibition of dihydrofolate reductase but the administration of either folic acid or citrovorum factor did not lead to decreased tumour growth inhibition or host toxicity by NMF (Clarke et al., 1953). Interference with the synthesis of nucleic acid bases by NMF has been reported (Clarke et al., 1953; Skipper et al., 1955). No cross resistance was observed between NMF and PCZ when tested against the TLX5 lymphoma, indicating that the mode of action of the two drugs is different (Gescher et al., 1982).

The metabolism of NMF is not well understood. The majority of metabolism studies on formamides have been concerned with the industrial solvent DMF, in efforts to elucidate the mechanisms of hepatotoxicity of this compound. DMF is rapidly demethylated in vivo (Massmann, 1956) and, according to one report, in vitro (Barnes and Ranta, 1972). Kimmerle and Eben (1975) reported that DMF was metabolised to NMF and F in rats and dogs in vivo, although Gescher

et al., (1982) have since cast doubt on these findings because of lack of specificity. It is possible that what was identified as NMF by gas chromatography was probably N-hydroxymethyl,N-methylformamide, the immediate product of C-hydroxylation of DMF. This carbinolamine is stable under physiological conditions, but decomposes readily on heating on the gas chromatography column (Brindley et al., in press). However it has been proposed that the hepatotoxic property of DMF could be attributed to its metabolite, NMF (Scailteur et al., 1981). In recent studies, oxidative N-demethylation of NMF to F and formaldehyde in vitro was not observed (Gescher et al., 1982). However, in vivo, NMF underwent extensive metabolism. Using ^{14}C-methyl labelled NMF (400mg/kg i.p.), 74% of the injected radioactivity was excreted in the urine in 24 hours but only 26% of this was shown to be unchanged NMF (Brindley et al., 1982b). Less than 2% of the injected dose was recovered as F in the urine. Urine samples gave a positive test for formaldehyde after alkaline hydrolysis, which suggested the presence of formaldehyde precursors, such as N-hydroxymethylformamide (HMF,V, Figure 4). Only authentic HMF released HCHO on alkaline hydrolysis (Brindley et al., 1982b). The presence of HMF in the urine of NMF treated mice has recently been confirmed by thin layer chromatographic and gas chromatographic methods (Kestell et al., 1983, in preparation). HMF lacks the tumour inhibitory properties of NMF against the TLX5 lymphoma or M5076 reticulum cell sarcoma in mice but is cytotoxic in vitro against the TLX5 lymphoma. The latter result can be accounted for by the very slow degradation of HMF to formaldehyde (Cooksey et al., 1983, in press). The presence of F and HCHO in the urine after NMF administration to mice (Brindley et al., 1982b) and the presence of F and NMF in the urine after DMF administration to rats (Kimmerle and Eben,

1975; Barnes and Ranta, 1972) have suggested that both DMF and NMF are demethylated *in vivo*, probably as described in Figure 4.

As outlined above, the N-methyl group appears to be essential for the antitumour activity of HMM, PCZ and NMF and metabolic activation or detoxification is also considered a common feature of the three agents. The *in vivo* antitumour activity of several drugs, for which the presence of a N-methyl moiety is essential for activity, has been tentatively ascribed to the formation of N-hydroxymethyl intermediates (Hickman, 1978; Gescher *et al.*, 1979; Ross *et al.*, 1982) which degrade to form formaldehyde, a known cytotoxic agent (Kini and Cooper, 1962). All three agents described here are thought to produce hydroxymethyl compounds at some stage of their metabolism presumably with subsequent formation of formaldehyde.

Caffeine (1,3,7-trimethylpurin-2,6-dione, Figure 1) is an example of an N-methyl containing compound with no antitumour activity that undergoes metabolism to a complex mixture of dimethyl, monomethyl and hydroxy products in many species (Burg, 1975). Several of the metabolites, such as theobromine (3,7-dimethylpurin-2,6-dione) and theophylline (1,3-dimethylpurin-2,6-dione) are pharmacologically active. Metabolites formed by processes other than N-demethylation have been identified and the existence of unknown polar biotransformation products has been reported in the rat (Arnaud, 1976). The quantitative importance of demethylation in total caffeine metabolism is therefore unclear.

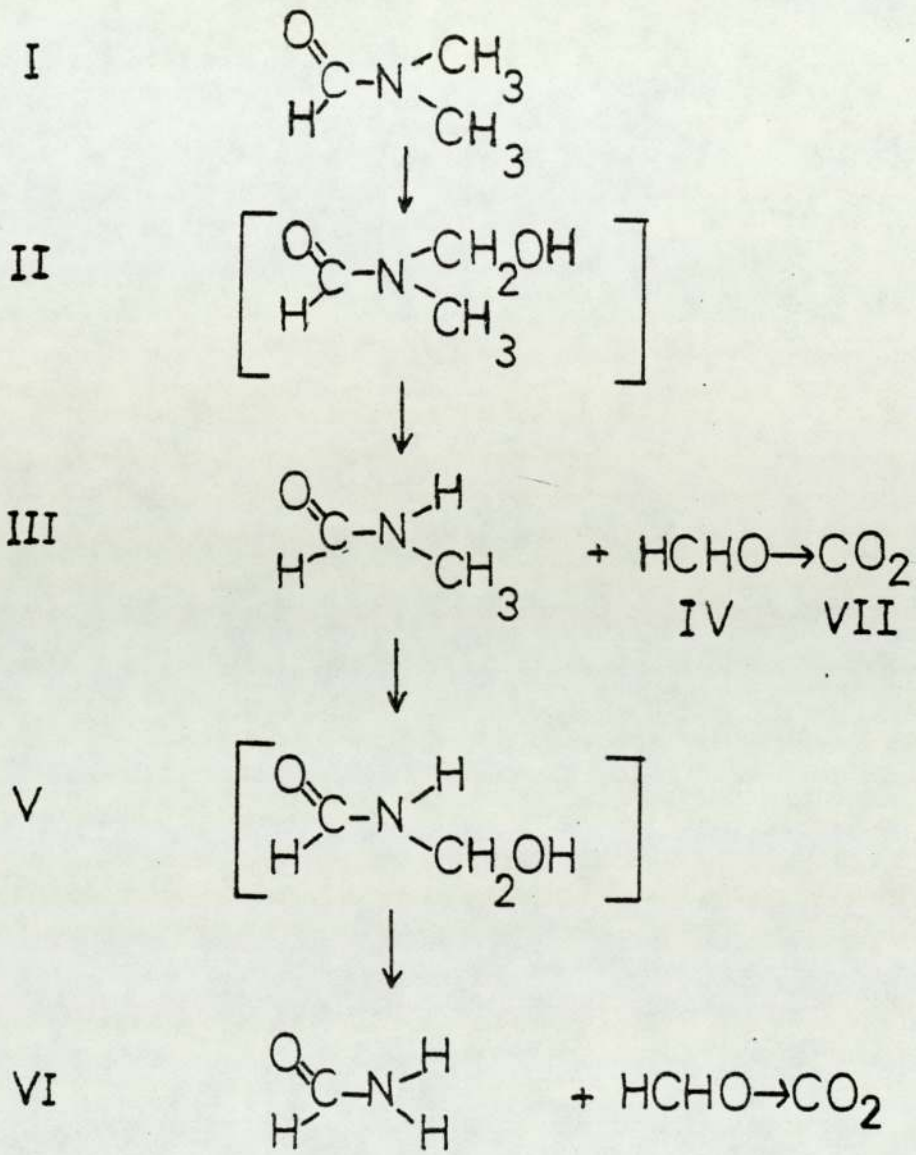


Figure 4 : Suggested pathway for metabolism of DMF(I) to NMF (III) and F(VI), via hydroxylated intermediates : N-hydroxymethyl NMF (II) and N-hydroxymethylformamide (V). Each demethylation step results in the formation of formaldehyde (IV) and CO_2 (VII).

1.II Background and applications of the $^{14}CO_2$ breath test

The suggestion has been made that the metabolic hydroxylation of an N-methyl moiety in HMM or PCZ leads to the formation of an antitumour species (1.I). If this is so, one has to explain why a drug such as caffeine, which also undergoes oxidative N-demethylation, is not biotransformed to a reactive or antineoplastic species. There are probably qualitative or quantitative differences in the oxidative N-demethylation of different N-methyl containing drugs. N-methyl compounds that undergo oxidative metabolism, catalysed by cytochrome P-450 dependent mixed function oxygenases, usually form formaldehyde, which is further oxidised to formate and finally to carbon dioxide (Figure 5). Monitoring the end product of this metabolic pathway, exhaled CO_2 with the breath, has been shown to reflect *in vivo* drug metabolising capacity (Vesell, 1980) and has been considered to probably characterise the N-demethylations of different compounds.

One compound that undergoes extensive N-demethylation, yielding CO_2 as an end product, is aminopyrine (AP), an obsolete analgesic (Figure 1). The measurement of $^{14}CO_2$ appearing in the breath after administration of ^{14}C-methyl labelled AP has been used for the clinical assessment of liver function and has been accepted as a valuable tool for detection of changes in the metabolism of drugs (Hepner and Vesell, 1975; Henry *et al.*, 1979; Vesell, 1980). The test has also been used for the estimation of the rate of oxidation of xenobiotic metabolism *in vivo* in animals (Lauterburg and Bircher, 1976; Roots *et al.*, 1980). Quantitative analysis of $^{14}CO_2$ exhaled following administration of other ^{14}C-methyl labelled N-methyl compounds to mice has subsequently been studied (Gescher and Raymont, 1981).

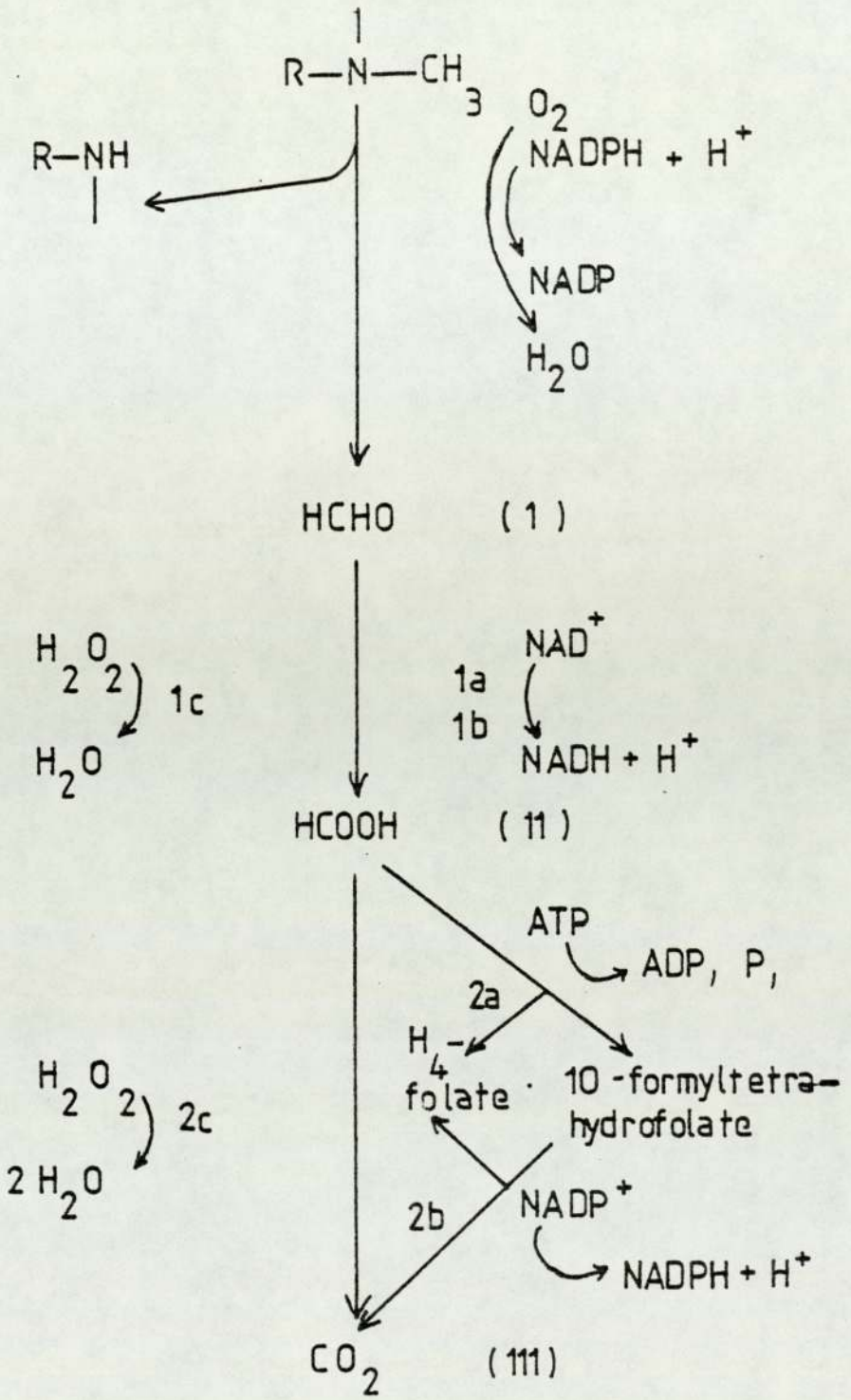


Figure 5 : Simplified reaction sequence of drug N-demethylation with the production of formaldehyde (I), formate (II) and carbon dioxide (III). (Waydhas et al, 1978).

(Enzymes : 1a= formaldehyde dehydrogenase (GSH dependent); 1b=aldehyde dehydrogenase; 1c= catalase; 2a= 10-formyltetrahydrofolate synthetase; 2b= 10-formyltetrahydrofolate dehydrogenase; 2c= catalase)

The analysis of $^{14}CO_2$ exhaled in this way has provided a relatively simple and rapid method for investigation of factors affecting drug metabolism. Examples of such factors are the influence of dose on drug metabolism and the effect of the presence of malignancies and of inhibitors or inducers of mixed function oxygenase enzymes. These factors have been studied in this work and are outlined in the following discussion.

The kinetics of drug disposition may be dependent on the dose administered. If this occurs, saturation of one or more of the processes involved in the physiological disposition of the drug is suggested. Saturation at any step in the disposition of a drug for which a metabolite appears important for either activity or detoxification, such as HMM or caffeine, may be important for the therapeutic or toxic effect of the agent. Monitoring of $^{14}CO_2$ exhaled with the breath could be useful in the investigation of the dose dependency of oxidative metabolism of a radiolabelled agent. Such an investigation has been reported for the N-demethylation of N-[^{14}C]-methyl labelled cocaine and AP (Stewart et al., 1978).

The influence of inhibitors and inducers of mixed function oxygenase enzymes on the characteristics of $^{14}CO_2$ exhalation following the administration of ^{14}C-labelled AP (Lockwood and Houston, 1980; Gescher and Raymont, 1981) and of ^{14}C-labelled HMM (Gescher and Raymont, 1981) have been studied. The extent to which the exhalation of $^{14}CO_2$ from these compounds is dependent on cytochrome P-450 dependent monoxygenase reactions has been evaluated. In the case of those compounds for which a metabolic pathway is essential for activity (or detoxification), characterisation of the enzyme systems involved is crucial.

There have been many reports of differences in drug metabolising capacity between animals with and without neoplastic growth. A variety of reasons for this have been suggested including a reduction or inhibition of cytochrome P-450 content or activity (Rosso et al, 1971; Brown et al, 1971; Litterst et al, 1977) and in drug metabolising enzyme activity (Litterst et al. 1977) in tumour bearing animals. In the latter report, N-demethylase activity was reduced in guinea pigs dosed with ethylmorphine. It has been postulated that tumours may excrete a product that is capable of inhibiting microsomal enzyme activity (Boulos et al, 1972; Tanaka et al, 1972). A decreased rate of pentobarbital metabolism in normal rats following cross over experiments, involving transfusion of blood from tumour animals, has been reported as evidence for the existence of such a substance (Bartošek et al, 1972). The administration of enzyme inducers such as PB has been shown to reverse the changes in drug metabolising capacity induced by the presence of tumours (Rosso et al, 1971). Such a reversal tended to support the theory that inhibition of cytochrome P-450 was important in the changes induced by tumour growth. It has been postulated that liver microsomal enzyme impairment may be different in different tumours and thus the effect on mixed function oxygenase enzymes would also differ. This differing influence on the metabolising enzymes by different tumours may help to explain the difference in therapeutic success of a particular agent against several tumours. Monitoring of $^{14}CO_2$ exhaled with the breath following administration of ^{14}C-methyl labelled drugs could be used to investigate the effect of tumour presence on the oxidative metabolism of N-methyl containing antitumour agents.

Although measurement of $^{14}CO_2$ exhaled with the breath may be a useful indication of N-demethylation of the ^{14}C-labelled parent compound, it is important to recognise the limitations of the method, as noted by Willson et al. (1979), in a discussion of the use of AP in the in vivo evaluation of hepatic function. The method measures the final product of a series of reactions and one can only speculate on the intermediate processes involved and their relative importance to the overall metabolic pathway. It is assumed that the rate at which $^{14}CO_2$ is exhaled with the breath is determined by the rate-limiting step in a series of first order reactions. That N-demethylation is this rate-limiting step is uncertain. The activity of several enzyme systems is measured and the hybrid nature of the technique makes conclusions limited.

Caution is especially required when comparing the rate of $^{14}CO_2$ exhaled after administration of different drugs. Differences in absorption, solubility, protein binding and drug actions on other physiological processes, such as hepatic blood flow or respiration, may also affect the final exhalation of $^{14}CO_2$ with the breath.

1.III Aims and scope of the present investigation

The common structural feature of HMM, PCZ, NMF, caffeine and AP is the N-methyl moiety and each of these agents is believed to be metabolised to a greater or lesser extent by N-demethylation (1.II). In some drugs - but not in all - this metabolic pathway leads to the formation of antineoplastic species (1.I). In the case of NMF, a metabolic pathway may well be associated with the major side effect of the drug, hepatotoxicity. A better understanding of the metabolism of these compounds, and in particular the fate of the important N-methyl moiety, may uncover qualitative or quantitative differences which may be related to the differences in their pharmacological or toxicological properties. In the first part of this thesis, work is described which is focussed on the generation of the ultimate metabolite of the N-methyl group, CO_2.

It is well known that N-demethylation results in the formation of formaldehyde and that this is further metabolised to CO_2 which is mainly exhaled in the breath (see Figure 5). By quantitative analysis of the $^{14}CO_2$ exhaled with the breath following administration of ^{14}C-methyl labelled compounds, an indirect estimation of the metabolic fate of the C1 unit is possible (1.II).

One of the aims of this investigation was to use the method of $^{14}CO_2$ analysis to study several aspects affecting the metabolism of HMM, PCZ, caffeine and AP in the mouse. A better knowledge of factors influencing the metabolism of the antitumour agents, HMM and PCZ, was considered to be especially important as the cytotoxic species of these compounds is believed to be formed on metabolism. Such knowledge may ultimately help to improve the

therapeutic usefulness of these compounds. For comparative purposes, the influence of such factors on the metabolism of AP and caffeine was also assessed.

The factors investigated were the influence of dose on drug disposition as reflected by CO_2 exhalation, the involvement of cytochrome P-450 dependent mixed function oxygenases in the metabolism of these drugs and the effect of the presence of malignancy on oxidative metabolism.

In the case of the dose dependency study, ^{14}C-HMM and ^{14}C-caffeine were investigated, as examples of one agent with and one without antitumour activity. The pharmacological efficacy of both compounds conceivably could be dramatically altered if a metabolic process e.g. one involved in N-demethylation, became saturated (1.II). Several reports have indicated previously that dose dependency may be important for the disposition and metabolism kinetics of HMM and caffeine (Burg and Werner, 1972; Broggini et al., 1981), but the $^{14}CO_2$ breath test, to our knowledge, has not been used for such an investigation.

The majority of oxidative metabolic reactions involve cytochrome P-450 dependent mixed function oxygenases and the activity of these enzymes may be altered by administration of inducers or inhibitors of cytochrome P-450. The extent to which cytochrome P-450 dependent enzymes were involved in the oxidative N-demethylation of ^{14}C-PCZ and ^{14}C-caffeine was assessed in this study by pretreatment of animals with either the inhibitor SKF525A or the inducer PB. Changes in the rate of cytochrome P-450 dependent steps in the metabolism of PCZ have an effect on the formation of antineoplastic metabolites

(Shiba et al., 1979). However the $^{14}CO_2$ breath analysis has not been used to assess the involvement of cytochrome P-450 in PCZ metabolism. The role of cytochrome P-450 in the metabolism of caffeine has been studied following PB treatment (Arnaud and Welsch, 1980), but, to our knowledge, inhibition experiments have not been performed with ^{14}C-caffeine and the $^{14}CO_2$ breath test.

The third factor influencing metabolism, that was assessed in this study was the presence of malignancy in the animal. Many investigations have shown that metabolism is indeed affected by neoplastic growth (1.II). For HMM and PCZ, two agents for which metabolic activation is essential for antineoplastic activity, alteration of metabolism by a tumour may prove crucial for efficacy. As for the other factors discussed here, the use of the $^{14}CO_2$ breath analysis has not been reported previously for such studies. ^{14}C-HMM or ^{14}C-PCZ were administered to animals bearing tumours which are known to be sensitive to these drugs and the $^{14}CO_2$ exhalation profile was compared with that from animals without tumours. ^{14}C-AP was used for comparison also.

In addition to the effects of several factors on the $^{14}CO_2$ profile and hence on the oxidative metabolism of HMM, PCZ, AP and caffeine, preliminary investigations into the fate of the N-methyl group of the alkylformamides were performed. The metabolism of DMF and NMF appears to be complex (1.I) and in further efforts to elucidate the fate of drug derived C1 units, the $^{14}CO_2$ exhaled following administration of ^{14}C-methyl labelled NMF, ^{14}C-formyl labelled NMF and ^{14}C-formyl labelled DMF was quantitatively analysed.

SECTION 2

ANIMALS AND MATERIALS

2.I Radiochemicals and scintillants

^{14}C-methyl-AP and ^{14}C-formate (sodium salt) were purchased from Amersham International. 1-^{14}C-methyl-caffeine was purchased from NEN, Southampton, U.K. ^{14}C-methyl-PCZ and 2,4,6-^{14}C-methyl-HMM were generously provided by Dr. R. Engle, Chemical Research Station, N.C.I., Bethesda, USA. ^{14}C-methyl-NMF, ^{14}C-formyl-NMF and ^{14}C-formyl-DMF were synthesised by Dr. M. Threadgill as described by Threadgill and Gate (1983).

The scintillant 2,5 bis(5-tert-butyl benzoxazol-2yl) thiophen (BBOT) was obtained from Fisons Limited. Toluene (Scintillation grade) was obtained from BDH Chemicals Limited. A solution containing 4g BBOT per litre toluene was used as the scintillant mixture.

2.II Miscellaneous chemicals and apparatus

Caffeine citrate, aminopyrine and sodium formate were purchased from BDH Chemicals Limited. Procarbazine hydrochloride was a gift from Roche Products Limited. HMM was synthesised in our laboratories by Dr. S.P.Langdon according to the methods of Thurston et al. (1951) and Paget and Hammer (1958). NMF was purchased from Aldrich Limited and DMF from BDH Chemicals Limited. Both were purified further by redistillation. Phenobarbitone sodium (PB) was purchased from BDH Chemicals Limited and SKF525A was a gift from SKF Pharmaceuticals.

The glass metabowls and accessories were purchased from Jencons Limited, Hemel Hempstead. All other reagents and chemicals used were of Analar grade and were purchased from BDH Chemicals Limited or Aldrich Limited.

2.III Animals, tumour maintenance and injections

Male and female BALB/c, male CBA/Ca and female BDF_1 mice were obtained from Bantin and Kingman Limited, Hull, U.K. Animals were 10-20 weeks old (20-25g) and were maintained on Heygates 41B Breeding diet. Food and water were allowed ad libitum during all experiments.

In those experiments involving animals bearing tumours, female BALB/c mice were inoculated with the plasma cytoma PC6A i.p., male CBA/Ca mice were inoculated with the TLX5 lymphoma i.p. and female BDF_1 mice were inoculated with the reticulum cell sarcoma M5076 by trochar injection. Details of tumour passage and maintenance are given in Gescher et al (1982). In all experiments, radiolabelled compounds were injected on the day when the tumour was routinely passaged into another mouse, so that the tumour was definitely established and the animals would survive for several more days. Exhalation experiments were performed on PC6A and TLX5 bearing animals on the seventh day and on M5076 animals on the twentyfirst day after tumour inoculation.

All injections of drugs containing label were prepared by mixing radiolabelled material with unlabelled compounds in solvent. Injection volume was 0.1ml. The amount of radioactivity injected with unlabelled compound was 1μCi for ^{14}C-HMM, ^{14}C-caffeine, ^{14}C-methyl-NMF, ^{14}C-formyl-NMF and ^{14}C-formyl-DMF, ^{14}C-formate and 0.6μCi for ^{14}C-AP and ^{14}C-PCZ. In the case of the formamide experiments, the specific activity of the solution in the three injections used was 5.5 - 5.8μCi per mmole per mouse. All drugs, with the exception of HMM, were dissolved in 0.9% w/v sterile saline. HMM was suspended in arachis oil.

In induction experiments, animals were injected with PB, dissolved in saline, i.p. at a dose of 50mg/kg for 4 consecutive days prior to injection of radiolabelled compound (Conney et al, 1960). In inhibition experiments, animals were injected with SKF525A, dissolved in saline, i.p. at a dose of 60mg/kg one hour prior to injection of radiolabelled compound. Control animals were injected with sterile saline for both experiments.

SECTION 3

METHODS

3.I Method of analysis of $^{14}CO_2$ exhaled in vivo

The method originally described by Lauterburg and Bircher (1976) and modified by Gescher and Raymont (1981) was used for analysis of the $^{14}CO_2$ exhaled. After drug administration, animals were placed in individual glass metabowls, which were securely sealed to prevent any air leakage. Air was drawn through the metabowl at a rate of 200-400ml per minute, after removal of atmospheric CO_2 by passing through 1M NaOH. Exhaled CO_2 was collected continuously, after passage through concentrated sulphuric acid, in 15ml of a trapping mixture containing methanol:ethanolamine (4:1) in a series of glass vessels. Over 99% of the $^{14}CO_2$ exhaled during experiments was trapped in the first of these vessels. Samples (2ml) of the trapping fluid were taken at 10 minute intervals for the first hour, at 20 minute intervals for the next four hours and at 30 minute intervals for the next two hours. $^{14}CO_2$ exhaled was monitored for approximately seven hours after radiolabelled drug administration. Methanol (3ml) and scintillant (Section 2.II) (15ml) were added to samples. Radioactivity was determined by liquid scintillation counting using a Packard Tricarb 2660 scintillation counter. Counting efficiency was estimated to be approximately 80% using the single channel ratio method.

The $^{14}CO_2$ exhalation profiles for each drug were characterised by several parameters. The percentage of administered radioactivity exhaled per minute as $^{14}CO_2$ was plotted semi-logarithmically against time after injection. The slopes of the linear phases of the curves obtained were estimated by linear regression. For aminopyrine and formate, the method of residuals (Gibaldi and Perrier, 1975) was used to strip the curve into a number

of exponential components and to obtain slopes for the two phases of the exhalation profile. Half life values were calculated from the slopes. The total percentage of administered radioactivity exhaled was calculated for the total collection period. The time of peak exhalation and the rate of $^{14}CO_2$ exhalation at the peak time were also evaluated. (Appendix).

Statistical analysis of the results was performed using either the Student's 't' test or Wilcoxon's Sum of Ranks test as described by Langley (1979).

3.II Investigation of dose dependency of oxidative metabolism of ^{14}C-HMM and ^{14}C-caffeine

Male CBA/Ca mice were injected i.p. with either ^{14}C-HMM at 1,5,40 or 80mg/kg or with ^{14}C-caffeine at 0.2, 2, 20 or 40mg/kg.

3.III Investigation of the effect on oxidative metabolism of ^{14}C-PCZ and ^{14}C-caffeine of pretreatment with SKF525A or PB

Male CBA/Ca mice were injected i.p. with either ^{14}C-PCZ 100mg/kg, ^{14}C-caffeine 40mg/kg or ^{14}C-caffeine 2mg/kg, after pretreatment with either SKF525A, PB or saline as described in 2.III.

3.IV The effect of the route of injection on $^{14}CO_2$ exhalation following administration of ^{14}C-HMM, ^{14}C-AP or ^{14}C-PCZ

Male CBA/Ca mice were injected either i.p. or s.c. with either ^{14}C-AP 38mg/kg, ^{14}C-HMM 90mg/kg or ^{14}C-PCZ 100mg/kg. Female BALB/c mice were injected either i.p. or s.c. with either ^{14}C-AP 38mg/kg or ^{14}C-HMM 90mg/kg.

3.V Investigation of the effect of tumour presence on the oxidative metabolism of ^{14}C-HMM, ^{14}C-PCZ, ^{14}C-AP and ^{14}C-formate.

Female BALB/c mice inoculated with PC6A tumour (2.III) were injected i.p. with either ^{14}C-HMM 90mg/kg or ^{14}C-AP 38mg/kg. Male BDF_1 mice inoculated with M5076 (2.III) were injected i.p. with either ^{14}C-HMM 90mg/kg or ^{14}C-AP 38mg/kg. Male CBA/Ca mice inoculated with TLX5 tumour (2.III) were injected s.c. with either ^{14}C-PCZ 100mg/kg, ^{14}C-AP 38mg/kg or ^{14}C-formate 2mg/kg. In all experiments, animals without tumours were used as controls.

3.VI Investigation of $^{14}CO_2$ exhalation following administration of ^{14}C-labelled N-methylformamides

Male CBA/Ca mice were injected i.v. into the tail vein with either ^{14}C-methyl-NMF, ^{14}C-formyl-NMF or ^{14}C-formyl-DMF, at 6.8 mmoles/kg. The i.v. route was used to correlate with clinical studies and with other metabolic studies. NMF and DMF are volatile and preliminary experiments were performed to check whether exhaled radioactivity contained any unmetabolised DMF or NMF. Gas chromatographic analysis (Brindley et al, 1982b) revealed no measurable amounts of either compound. The majority of exhaled radioactivity was considered to be in the form of $^{14}CO_2$.

SECTION 4

RESULTS

4.I Investigation of dose dependency of oxidative metabolism of ^{14}C-HMM and ^{14}C-caffeine

4.I.i. Introduction

Dose dependence of the kinetics of drug disposition for a drug, that is believed to be activated or detoxified via a bio-transformed product, may be relevant for effective usage (1.II). In this study, the dependence of the fate of the methyl groups of HMM or caffeine on the dose of drug administered was investigated. Dose dependent kinetics have been implicated in the disposition of caffeine previously but no study utilising the $^{14}CO_2$ exhalation method has been reported. Following oral administration of different doses of caffeine to mice, differences in tissue half lives and areas under the plasma concentration of drug vs time curve (AUC) have been reported in two studies (Burg and Werner, 1972; Latini et al., personal communication).

When pentamethylmelamine (PMM), one of the demethylated metabolites of HMM, was administered i.v. at a dose of 10mg or 50mg/kg to rats, the rate of PMM elimination, as measured by its plasma clearance, appeared to be influenced by the dose (Columbo et al., in press). This conclusion was similar to that obtained from a study of plasma levels of PMM following oral administration of two doses of PMM to mice. Peak levels and AUC increased with dose and $T_{\frac{1}{2}}$ remained unchanged. However, levels of PMM in the plasma after 100mg/kg were much higher than twice the value obtained for 50mg/kg, implicating dose dependent kinetics (Broggini et al., 1981). In the same study, administration of HMM at two doses resulted in an increase in AUC and peak plasma levels of HMM in proportion to the dose, together with $T_{\frac{1}{2}}$ values similar to each other. This suggested a dose dependent

metabolism for PMM and not for HMM. Monitoring demethylation of ^{14}C-HMM by $^{14}CO_2$ breath exhalation was considered a useful method to confirm these findings.

4.I.ii Results

The parameters obtained for the different doses of HMM and caffeine are shown in Table 1. For HMM, little difference was evident in either the parameters or in the shape of the $^{14}CO_2$ exhalation rate profile (Figure 6) with changing dose. In the experiments using caffeine, data for the 20mg and 40mg/kg doses were similar. Statistical tests revealed no significant difference between the two doses. Data for the 2mg and 0.2mg/kg doses were also similar to each other but were markedly different from the higher doses. Figure 7 shows the difference in $^{14}CO_2$ exhalation profile between the 2mg and the 40mg/kg doses. Statistical analysis, however, revealed only a significant difference between the half life values of 2mg/kg compared to the higher doses of caffeine ($p < 0.05$).

4.I.iii Discussion

The similarity of the $T_{\frac{1}{2}}$, % dose exhaled, peak rates and exhalation profiles with increasing doses of ^{14}C-HMM suggested that the kinetics of the metabolic steps leading from the HMM methyl moiety to CO_2 did not appear to be saturated. There was no evidence of dose dependence of the step which determined the rate of $^{14}CO_2$ exhalation. These results tend to confirm the inference based on plasma level data for HMM after oral dosing (Broggini et al., 1981), where AUC's and plasma peak levels increased in proportion to the

dose, with a corresponding lack of change of $T_{\frac{1}{2}}$. It is important to realise that $^{14}CO_2$ measured in this study had been generated by demethylation of not only HMM and PMM but from other methylmelamines present as metabolites of HMM (Figure 2). This complexity made extrapolation to possible dose dependent kinetics at therapeutic doses inadvisable.

The results obtained from the caffeine experiments suggested dose dependency and the possibility that saturable processes were involved in the disposition of caffeine. The similarity in $^{14}CO_2$ exhalation rate profiles between the two higher doses was difficult to explain if the assumption was made that metabolic processes were near to saturation at these doses. At the two lower doses, the disposition of caffeine was not governed by saturation kinetics. The lack of difference in the % exhaled for all doses, which is equivalent to the AUC in conventional pharmacokinetic studies, was unexpected when one considered the marked difference in the shape of the profiles obtained. Theoretically, for a process in which metabolic steps which are saturated govern the overall disposition, a decrease in the percentage of administered dose exhaled as a metabolite with increasing dose would be predicted.

The technique used here may be insensitive to small metabolic differences between doses due to the heterogeneous nature of the overall metabolism that ultimately leads to the exhalation of $^{14}CO_2$. In addition, the possibility that N-demethylation is only a minor pathway of caffeine metabolism in the mouse may invalidate the test. With different doses of drug, the fraction of the total metabolism attributable to N-demethylation may not have varied but

other metabolic processes may have shown dose dependency. However, these results were comparable with those of Burg and Stein (1972). The half life values were similar to those obtained by Burg and Werner (1972), when measuring caffeine in tissues and plasma after administration of 5mg or 25mg/kg orally to mice. A $T_{\frac{1}{2}}$ value of 102 minutes for caffeine after administration of 25mg/kg was comparable with 94 minutes for the $^{14}CO_2$ rate profile after administration of a 20mg/kg dose reported here.

This apparent similarity between plasma caffeine $T_{\frac{1}{2}}$ value with that of the $^{14}CO_2$ exhalation rate after ^{14}C-caffeine in mice suggested that the $^{14}CO_2$ exhalation reflected the plasma clearance of parent drug. The metabolism of caffeine to theobromine (Figure 8) is believed to be a minor metabolic pathway in the mouse. The monitoring of the $^{14}CO_2$ originating from the ^{14}C in the C^1 position and thus the conversion to theobromine here appeared to reflect the plasma elimination of parent drug. This was similar to the conclusion of Willson and Hart (1981), who reported that $^{14}CO_2$ breath elimination rates reflected plasma radioactivity clearance after i.v. administration of ^{14}C-caffeine.

DRUG	Dose Administered (mg/kg)	Time of peak exhalation (min.)	% dose exhaled as $^{14}CO_2$	Peak rate (nmoles $^{14}CO_2$ per min.)	$T_{\frac{1}{2}}$ (min.)
HMM	1(1)	30.0	28.0	30.6	100.4
	5(1)	50.0	20.0	33.1	81.5
	40(2)	52.5	20.5	49.7	76.3
	80(2)	55.0± 7.1	18.9± 2.6	69.9± 21.4	98.4± 2.9
CAFFEINE	0.2(2)	22.5	15.3	0.7	50.8
	2 (6)	33.3± 13.3	20.0± 4.7	1.0± 0.5	62.1± 8.6
	20(5)	124.0± 52.7	14.4± 2.4	2.1± 0.5	75.6± 4.3
	40(9)	116.9± 62.8	10.9± 4.0	2.6± 1.4	94.4± 10.1

Table 1 : $^{14}CO_2$ breath analysis produced from varying doses of ^{14}C-HMM or ^{14}C-caffeine i.p. to male CBA/Ca mice (numbers in brackets refer to number of animals used; values for parameters are means ± s.d.)

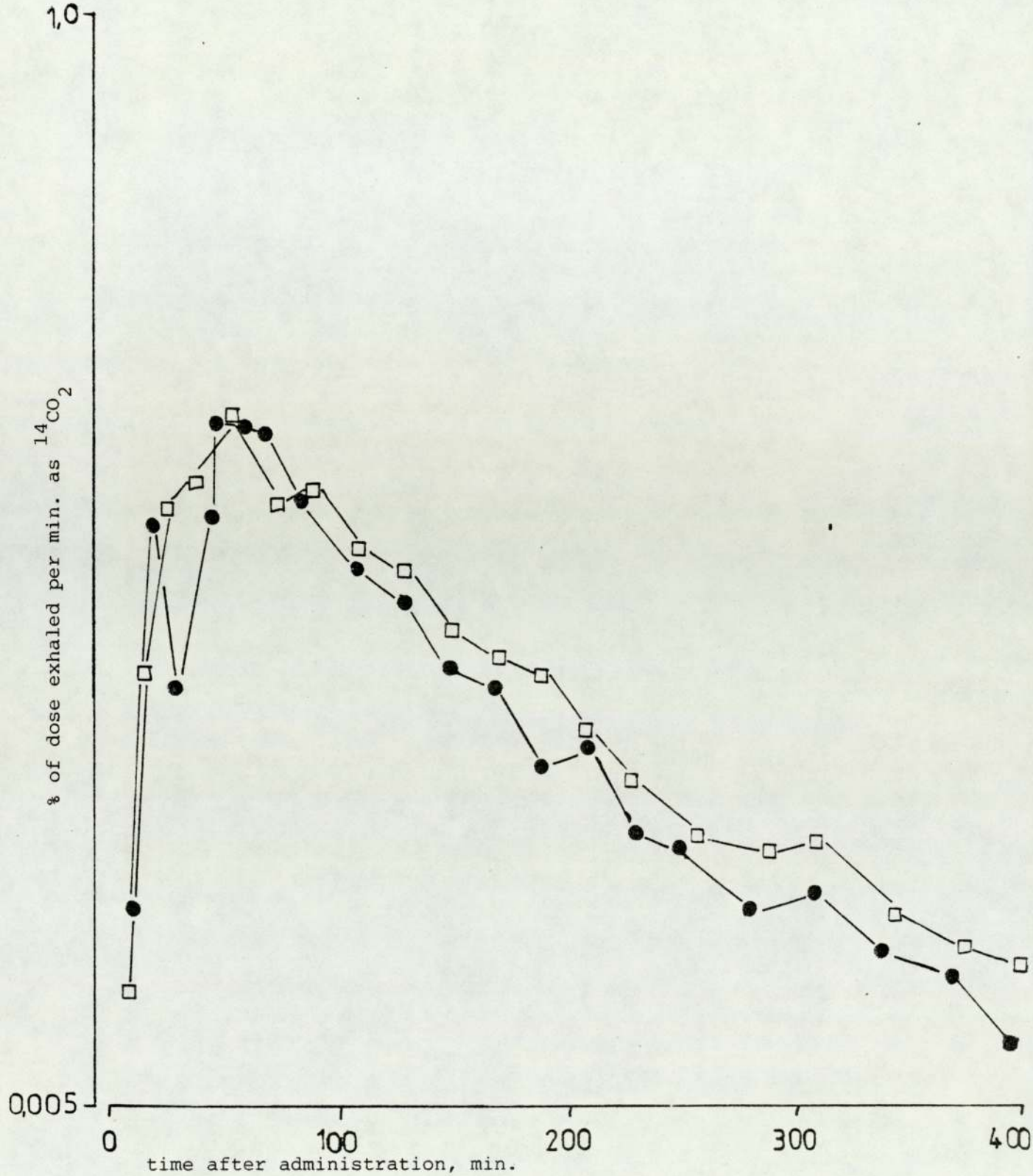
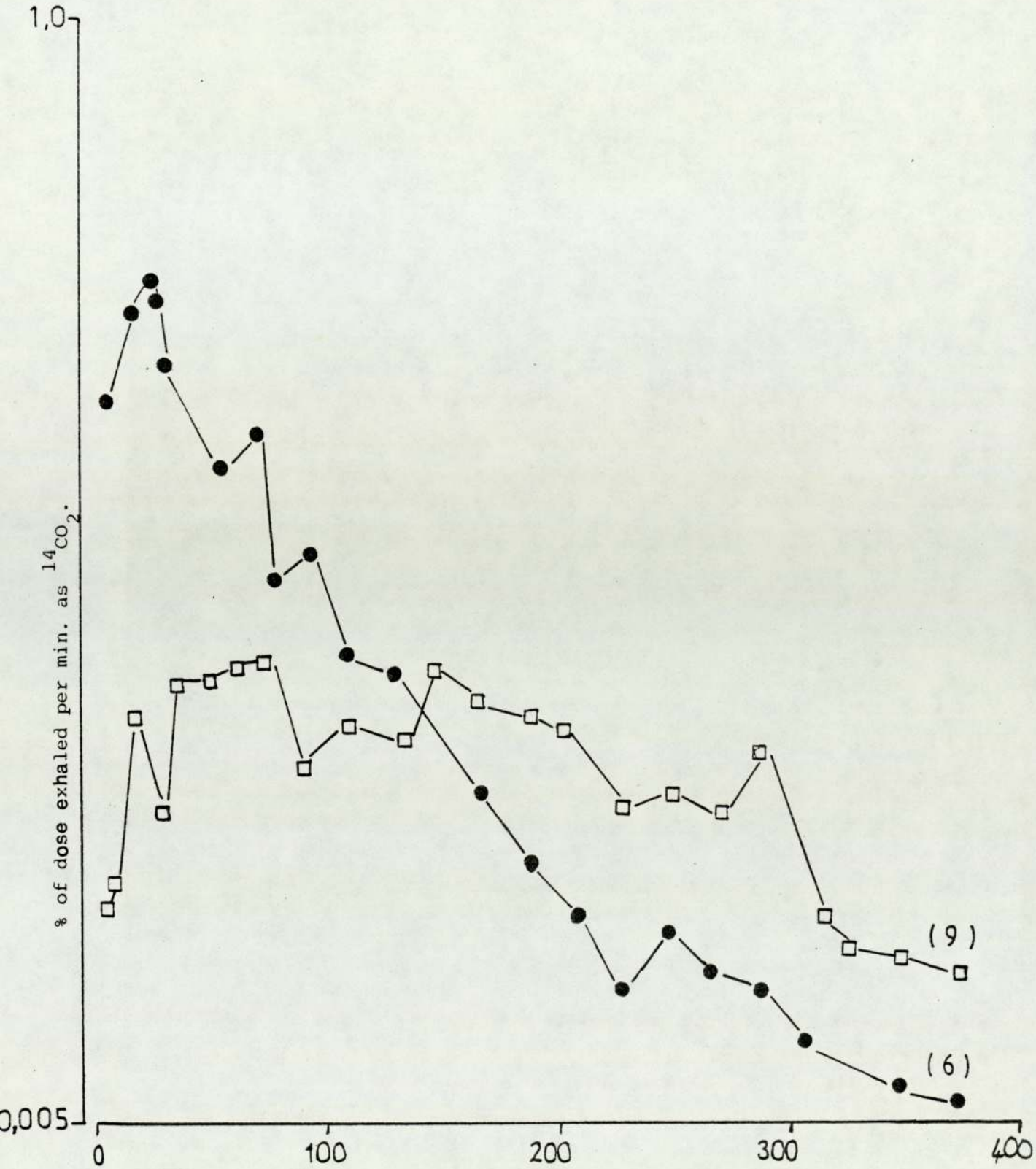


Figure 6 : $^{14}CO_2$ breath analysis after i.p. administration of ^{14}C-HMM to CBA/Ca mice at two doses, 80mg/kg (□) and 5mg/kg (●). (mean of 2 experiments).



time after administration, min.

Figure 7 : $^{14}CO_2$ breath analysis after i.p. administration of ^{14}C-caffeine to CBA/Ca mice at two doses, 40mg/kg(□) and 2mg/kg (●).

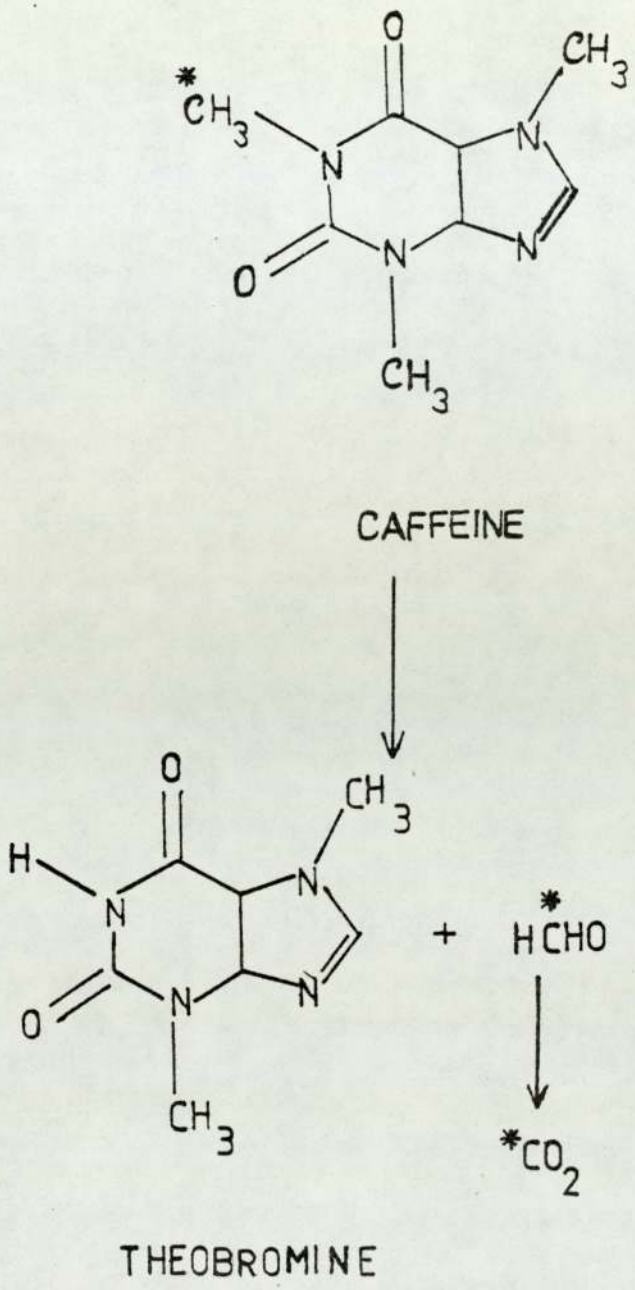


Figure 8 : The metabolic conversion of caffeine to theobromine (asterix denotes position of ^{14}C in ^{14}C-caffeine)

4.II Investigation of the effect on oxidative metabolism of ^{14}C-PCZ or ^{14}C-caffeine of pretreatment with SKF525A or PB

4.II.i Introduction

$^{14}CO_2$ exhalation analysis has been used previously to investigate the influence of metabolic inducers or inhibitors on oxidative metabolism (see 1.II). In order to characterise the metabolism of PCZ and caffeine and to investigate the applicability of the $^{14}CO_2$ breath test, animals were pretreated with either PB, an inducer of hepatic mixed function oxidases, or SKF525A, an inhibitor. As for the dose dependence study (4.I), high and low doses of caffeine were used for comparison. Mixed function oxidases have been implicated in at least three of the oxidation steps of PCZ metabolism (Dunn et al., 1979). Characterisation of the microsomal enzymes involved in PCZ metabolism has shown that N-demethylase activity was inducible by PB pretreatment (Prough et al., 1970) and Dewald et al (1968) noted a definite effect on the "steady state rate of $^{14}CO_2$ production" in rats following SKF525A or PB pretreatment. Shiba et al (1979) reported an increased antitumour activity of PCZ in mice bearing L1210 tumour following PB pretreatment. An acceleration of active metabolite production was implicated in the latter study.

In vitro and in vivo experiments on caffeine demethylation in rats following PB pretreatment have demonstrated a small increase in metabolism or plasma clearance of parent drug when compared with control animals (Arnaud and Welsch, 1980; Welsch, 1977). Together with other workers investigating caffeine these authors reported greater induction observed in vivo following pretreatment with 3-methylcholanthrene (3MC) or other polycyclic aromatic hydrocarbons,

known to be cytochrome P-448 inducing agents. Such evidence has suggested the use of quantification of caffeine demethylation as a test for cytochrome P-448 related hepatic function (Arnaud et al., 1980) in the same way as AP is used to study processes catalysed by cytochrome P-450. In all reports, caffeine or demethylated primary metabolites were monitored. In contrast to the many induction experiments reported, there appears to be little reference to inhibition experiments with caffeine.

4.II.ii Results

Breath analysis parameters for both induction and inhibition experiments are given in Table 2. For PCZ, pretreatment of mice with PB did not result in a change when compared with controls. Pretreatment with SKF525A produced a reduction in peak exhalation time and the % of dose exhaled, with other parameters remaining unchanged. Statistical comparison of values (Wilcoxon's Sum of Ranks Test) produced a significant difference ($p < 0.05$) only for the % of dose exhaled. Prior treatment of animals with either PB or SKF525A had an effect on the half life of the terminal phase of the exhalation curve for caffeine 40mg/kg, but not on any other parameter. PB markedly increased and SKF525A pretreatment markedly decreased this half-life value ($p < 0.05$). In contrast, SKF525A treatment of mice prior to caffeine 2mg/kg produced no significant change in half life values.

4.II.iii. Discussion

The absence of any effect on $^{14}CO_2$ exhalation of PCZ following PB treatment and the minor changes seen after SKF525A

pretreatment of animals are surprising compared with evidence reported elsewhere and the known cytochrome P-450 dependence of several metabolic steps (Dewald et al., 1968; Dunn et al., 1979). The relative insensitivity of the demethylation of PCZ to PB is similar to that noted for AP (Gescher et al., 1981). A similar explanation may apply to PCZ as to AP: if the cytochrome P-450 dependent stages in PCZ metabolism are not rate determining for the production of $^{14}CO_2$ in the breath, any subtle changes in the velocity of these steps will not be apparent by this method. The small change obtained after SKF525A is again similar to that seen with AP. By reducing the rate of PCZ N-demethylation, SKF may render this metabolic step rate determining for the $^{14}CO_2$ production. The metabolism of PCZ is complex and it may be that the relative contribution of cytochrome P-450 enzyme systems may be low. Hence inducer or inhibitor effects may not become apparent by use of the potentially insensitive $^{14}CO_2$ breath test assay. Another consideration, proposed by Prough et al., (1970) was the existence of two types of N-demethylase in the metabolism of arylhydrazines in rats, only one of which being inducible by PB. A similar situation may occur with the mouse and may explain the lack of effect of PB treatment on the metabolism of PCZ to CO_2.

The alteration in the half life values for $^{14}CO_2$ exhalation following administration of ^{14}C-caffeine 40mg/kg and following both pretreatments indicates that a cytochrome P-450 dependent process must be implicated in the rate-determining step for $^{14}CO_2$ production. However the lack of alteration in any other parameters measured may suggest that the rate determining step is not wholly cytochrome P-450

dependent and that cytochrome P-448 dependent processes, as described previously, may play an equally important role. The latter hypothesis would accommodate the absence of any change in parameters following caffeine 2mg/kg administration to pretreated animals. It is conceivable that when saturation of the kinetic processes is achieved at higher dose levels, cytochrome P-450 dependent processes become more dominant than at lower dose levels. In the latter case, cytochrome P-448 dependent processes may predominate.

Drug administered and pretreatment	No. of mice	Time of peak exhalation (min.)	Rate at peak exhalation (nmoles $^{14}CO_2$/min.)	% dose exhaled as $^{14}CO_2$	$T_{\frac{1}{2}}$ (min.)
PCZ Control	5	42.0± 8.4	20.4± 7.3	21.6± 3.5	82.5± 7.8
PCZ after PB 50mg/kg	3	43.3± 2.9	26.9± 1.8	24.0± 3.4	70.5± 9.7
PCZ after SKF525A 60mg/kg	4	44.0± 7.4	11.1± 2.0	12.9± 2.7	81.2± 3.3
Caffeine 40mg/kg control for PB	3	90.0± 60.0	1.6± 0.8	8.8± 5.7	99.2± 12.8
Caffeine 40mg/kg after PB 50mg/kg	3	90.0± 52.9	1.7± 0.2	7.1± 3.3	55.2± 7.6
Caffeine 40mg/kg control for SKF	4	165.5± 44.1	2.2± 0.5	10.3± 1.6	94.8± 8.8
Caffeine 40mg/kg after SKF 60mg/kg	4	135.5± 85.3	1.7± 0.4	8.5± 0.7	251.2± 82.3
Caffeine 2mg/kg control for SKF	2	40.0	0.3	10.1	64.3
Caffeine 2mg/kg after SKF 60mg/kg	2	49.0	0.3	13.4	59.2

Table 2 : $^{14}CO_2$ breath analysis produced from ^{14}C-PCZ 100mg/kg, ^{14}C-caffeine 40mg/kg or 2mg/kg i.p. to male CBA/Ca mice following various pretreatments.

(values are means ± s.d.)

4.III The effect of the route of injection on $^{14}CO_2$ exhalation following administration of ^{14}C-HMM, ^{14}C-AP or ^{14}C-PCZ

4.III.i Introduction

In most experiments, compounds were administered i.p. In experiments with mice bearing PC6A or TLX5 tumours, tumours were inoculated i.p. and tumours developed in the ascites. Therefore i.p. drug injection into the tumour cell suspension could yield misleading results. Therefore, in tumour bearing animals, drugs were injected s.c. A comparison of $^{14}CO_2$ exhalation profiles due to the route of injection was possible in BALB/c and CBA/Ca mice following injection of ^{14}C-HMM, ^{14}C-AP or ^{14}C-PCZ.

4.III.ii Results

In both BALB/c female and CBA/Ca male mice, injection of ^{14}C-AP or ^{14}C-PCZ either s.c. or i.p. produced $^{14}CO_2$ exhalation profiles similar in shape and AUC for both routes of administration. Table 3 shows results for s.c. or i.p. injection of ^{14}C-HMM to BALB/c or CBA/Ca mice. Figure 9 shows the $^{14}CO_2$ exhalation profile following s.c. or i.p. administration of HMM to BALB/c mice. In BALB/c mice it appeared that s.c. administration of HMM led to an increase in the time of peak exhalation and the half-life of the exhalation rate profile and a reduction of the rate of exhalation at peak time and the percentage of the dose exhaled when compared with the i.p. route. Results in CBA/Ca mice were similar, with the exception of the time of peak exhalation for which there is little difference between different routes. Statistical comparison of data obtained with BALB/c mice revealed a significant difference for all values between the two routes of injection ($p < 0.01$).

4.III.iii Discussion

The marked difference in $^{14}CO_2$ exhalation following i.p. against s.c. administration of ^{14}C-HMM may have a pharmaceutical explanation. The lipophilicity of the HMM injection preparation (in arachis oil) may explain the apparent retention in tissues following s.c. administration. It has been postulated that the slower exhalation of $^{14}CO_2$ after s.c. injection could indicate a slower rate of metabolism probably leading to a reduction in anti-tumour efficacy of HMM as shown by a decrease in survival time of tumour bearing animals treated with HMM. However, Langdon (1982, unpublished results) has shown that BDF_1 mice bearing the M5076 tumour survived for a similar number of days after injection of HMM, regardless of the route of injection. Therefore the difference in $^{14}CO_2$ breath exhalation between the two routes may essentially be an absorption phenomenon, with no apparent effect on antitumour activity.

	Time of peak exhalation (min.)	Rate at peak exhalation (nmoles $^{14}CO_2$ per min.)	% dose exhaled	$T_{\frac{1}{2}}$ (min.)
CBA/Ca mice i.p. (2)	55.0	79.3	18.0	98.4
CBA/Ca mice s.c. (3)	35.0± 10.0	18.6± 19.9	7.0± 1.5	265.8± 87.2
BALB/c mice i.p. (3)	43.3± 2.9	61.6± 12.7	13.6± 1.4	96.7± 23.8
BALB/c mice s.c. (6)	155.0± 20.0	14.9± 4.0	7.5± 2.8	276.3± 97.0

Table 3 : $^{14}CO_2$ breath analysis produced from ^{14}C-HMM 90mg/kg i.p. or s.c. to BALB/c mice or CBA/Ca mice.

(numbers in brackets are numbers of animals used; values are means ± s.d.)

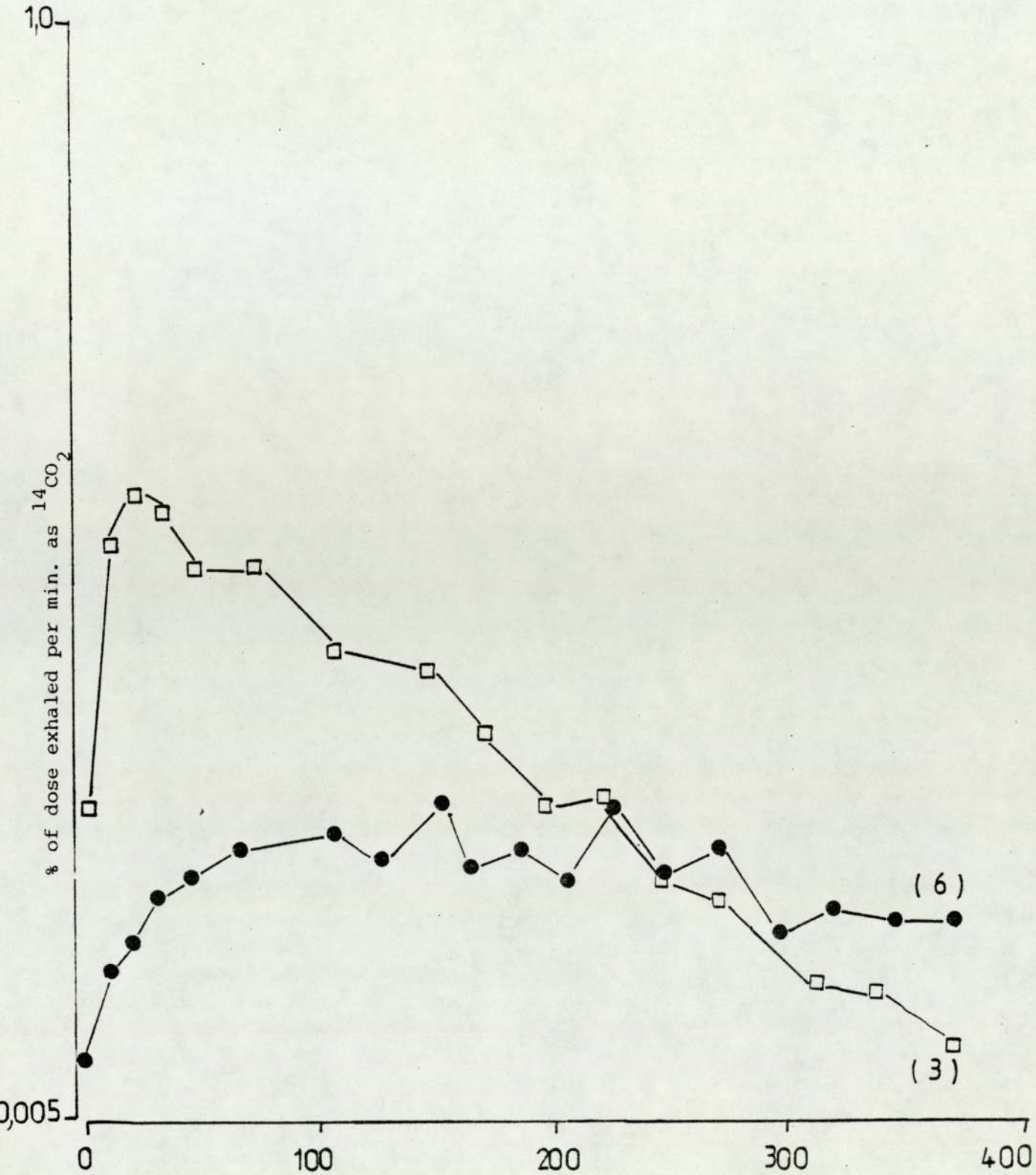


Figure 9 : $^{14}CO_2$ breath analysis after s.c.(●) or i.p.(□) administration of ^{14}C-HMM to BALB/C mice

4.IV Investigation of the effect of tumour presence on oxidative metabolism OF C-HMM, ^{14}C-PCZ, ^{14}C-AP and ^{14}C-formate

4.IV.i Introduction

There have been many reports of alterations in the disposition of antitumour compounds due to the presence of a tumour (I.II). It has been suggested that factors excreted by the tumour may affect liver microsomal enzyme activity and this may represent one of the factors explaining the different sensitivities of various tumours to a given antitumour agent. It is important to consider the effect of tumours on the metabolism of cytotoxic agents requiring metabolic activation. In this study, animals bearing tumours sensitive to HMM (PC6A and M5076) or PCZ (TLX5) were dosed with either ^{14}C-HMM or ^{14}C-PCZ and $^{14}CO_2$ exhalation profile compared with those obtained in animals without tumours. For all tumours, ^{14}C-AP was tested for comparison (3.V).

4.IV.ii Results

The presence of the PC6A tumour in BALB/c mice or the M5076 tumour in BDF_1 mice appeared to have no significant effect on the exhalation of $^{14}CO_2$ following administration of ^{14}C-HMM or ^{14}C-AP, when compared with controls. A similar lack of effect, following ^{14}C-PCZ administration was found in CBA/Ca mice with TLX5 tumour (Table 4). However in this tumour group, the biphasic $^{14}CO_2$ exhalation profile obtained with ^{14}C-AP in control animals was altered in TLX5 bearing animals to a curve with a monophasic decline, exhibiting a $T_{\frac{1}{2}}$ value of 58 minutes. Peak time, peak exhalation rate and the total % of dose exhaled for this $^{14}CO_2$

exhalation analysis were unchanged. In previous studies it was postulated that the steps in the metabolic pathway involving the oxidations of formaldehyde to formate and then to CO_2 may have been rate-limiting for the metabolic formation of CO_2 from aminopyrine, as ^{14}C-formate administered to CBA/Ca mice produced exhalation profiles similar to those of AP (Gescher and Raymont, 1981). ^{14}C-Formate was administered to CBA/Ca mice with TLX5 tumour to investigate if a change in exhalation profile occurred similar to that seen with ^{14}C-AP. The results are shown in Table 4. No change was noted for either the profile or the parameters obtained.

4.IV.iii Discussion

With the exception of AP in CBA/Ca mice bearing the TLX5 tumour, all drugs administered to tumour bearing animals produced exhalation rate profiles similar to those of control animals. Interpretation of these results is complex as a single dose of drug was administered at only one arbitrarily set time after tumour inoculation. It can be concluded that a tumour of a certain age or size does not influence the demethylation of a single dose of drug when monitored by the $^{14}CO_2$ exhaled. Extrapolation of these results to possible effects of tumours on the oxidative metabolism of drugs administered on a multi-day schedule should be made with caution.

The alteration in the $^{14}CO_2$ profile obtained from CBA/Ca mice bearing TLX5 tumour, who were dosed with ^{14}C-AP, indicates that the rate limiting step for $^{14}CO_2$ exhalation is indeed affected. The monophasic profile is not unlike that obtained from mice pretreated with SKF525A who were dosed with ^{14}C-AP (Gescher and Raymont, 1981).

Similarly, the lack of effect on ^{14}C-formate oxidation is like that reported for experiments with ^{14}C-formaldehyde in animals pretreated with SKF525A. Gescher and Raymont (1981) suggested that, in the mouse, the rate of metabolism of the N-methyl group of aminopyrine to CO_2 was determined by the rate of oxidation of formate or formaldehyde. Agents which decrease the rate of cytochrome P-450 dependent demethylation of aminopyrine, like SKF525A, could inhibit the demethylation to the extent of rendering that step in the metabolic pathway rate determining for the appearance of $^{14}CO_2$ in the breath, instead of the steps involving formate or formaldehyde. This could explain the effect of SKF525A on ^{14}C-AP oxidative metabolism and the lack of effect of SKF525A on the oxidation of ^{14}C-formate or ^{14}C-formaldehyde. One could tentatively propose that the change in oxidative metabolism of AP reported here in animals bearing TLX5 tumour could be due to an inhibition of cytochrome P-450. A reduction in the N-demethylation of AP has been reported in rats with the Walker 256 tumour, due to the inhibition of cytochrome P-450 (Kato et al., 1970).

It may have been useful to dose animals bearing tumours that were unresponsive to HMM or PCZ, with labelled HMM or PCZ. Any difference in $^{14}CO_2$ profiles obtained for different tumours might help to explain differences in the chemosensitivity of the tumours.

Strain/ tumour	Drug/ Route	Time of peak exhalation (min)	Rate at peak (nmoles $^{14}CO_2$/ min)	% dose exhaled	$T_{\frac{1}{2}}$ (min) of phases of exhalation
BALB/CONTROL	HMM/ S.C.	155.0± 20.0	14.9± 4.0	7.5± 2.8	276.3± 97.0
BALB/PC6A	HMM/ S.C.	147.0± 42.0	15.3± 5.9	4.9± 1.4	271.3± 66.0
BALB/CONTROL	AP/ S,C	15.0	58.7± 13.5	30.7± 3.5	=13.1±5.3 =84.4±13.9
BALB/PC6A	AP/ S.C.	15.0	57.0± 6.5	28.3± 3.4	=20.1±6.6 =83.5±6.1
BDF/CONTROL	HMM/ i.p	41.5	116.9	13.8	68.6
BDF/M5	HMM/ i.p	31.7± 5.8	57.6± 18.9	10.8± 1.8	112.1±50.5
BDF/CONTROL	AP/ i.p	15.0	96.5	43.6	= 11.8 = 91.3
BDF/M5	AP/ i.p	15.0	53.8	55.3	= 11.2 = 86.6
CBA/CONTROL	PCZ/ S.C.	35.0	9.4± 0.8	17.9± 5.5	138.3± 2.5
CBA/TLX5	PCZ/ S.C.	45.0± 8.2	6.1± 1.0	14.6± 2.6	132.0± 7.5
CBA/CONTROL	AP/ S.C.	20.0± 5.0	91.2± 17.8	54.7± 17.4	=16.1±1.8 =117.9±30.2
CBA/TLX5	AP/ S.C.	26.0± 2.2	52.0± 15.7	42.4± 6.9	57.6±3.4
CBA/CONTROL	FORMATE /S.C	22.5± 9.6	12.5± 3.7	57.5± 7.7	=12.2±0.9 =169.4±47.5
CBA/TLX5	FORMATE /S.C	25.0± 8.2	9.2± 2.2	57.1± 1.5	=14.7±1.7 =132.4±12.8

Table 4 : $^{14}CO_2$ breath analysis produced from ^{14}C-HMM 90mg/kg, ^{14}C-PCZ 100mg/kg, ^{14}C-AP 38mg/kg, or ^{14}C-formate 2mg/kg s.c. or i.p. to tumour bearing animals and controls.

(values are means ± s.d.)

4.V Investigation of $^{14}CO_2$ exhalation following administration of ^{14}C-labelled N-methylformamides

4.V.i Introduction

In order to investigate the oxidative metabolism and possibly the difference in antitumour activity and toxicity of the N-methylformamides described in 1.I., NMF, labelled in the methyl or formyl groups, and DMF, labelled in the formyl moiety were obtained. It has been postulated that DMF and NMF, for which the methyl group appears important for activity, undergo metabolism to formaldehyde and CO_2, in common with other compounds with N-methyl moieties, such as aminopyrine. Comparison of the $^{14}CO_2$ exhaled from mice dosed with the labelled methylformamides (3.VI) at equimolar doses and relating the exhalation data to plasma and urinary levels of radioactivity and levels of the parent compound could help to elucidate methylformamide metabolism and disposition.

4.V.ii Results

The $^{14}CO_2$ exhalation profiles for the three labelled compounds, ^{14}C-methyl-NMF, ^{14}C-formyl-NMF and ^{14}C-formyl DMF, are shown in Figure 10. Surprisingly, radioactive CO_2 is exhaled at a constant rate for all compounds, with no definable peak exhalation rate or declining sections of the profiles within 7 hours. Consequently, of the parameters defined in 3.I, only the percentage of the dose exhaled could be quantified. The mean (of 2 experiments) % dose exhaled was 3.1%, 14.5% and 10.9% for ^{14}C-methyl-NMF, ^{14}C-formyl-NMF and ^{14}C-formyl-DMF respectively. $^{14}CO_2$ was exhaled at a constant rate of 0.007, 0.038 and 0.028 nmoles per minute for ^{13}C-methyl-NMF, ^{14}C-formyl-NMF and ^{14}C-formyl-DMF respectively.

4.V.iii Discussion

The constant rate of $^{14}CO_2$ exhalation reported here for ^{14}C-methyl-NMF contrasts markedly with the profiles of other N-methyl containing compounds that undergo N-demethylation e.g.HMM, caffeine,AP. It suggests that the rate determining step in the overall metabolism to CO_2 is governed by zero order kinetics, probably due to saturation of this step. The $^{14}CO_2$ exhalation appears to be independent of plasma NMF concentrations. The total percentage of radiolabelled drug administered exhaled is remarkably low when compared with other N-methyl compounds.

In other metabolism experiments, injections of 400mg/kg ^{14}C-methyl-NMF produced plasma concentration-time curves for unchanged NMF very similar to that for total radioactivity in plasma, during the first 24 hours after drug administration (Brindley et al., 1982b). Measurement of radioactivity in urine of these animals showed that 73.6% (±6.8) of the radioactivity administered was recovered in the urine after 24 hours. Of this radioactivity, only 26.4% (±2.9) was unchanged NMF and less than 2% was identified as F or HMF, the assay method being unable to distinguish between these two species. The results suggested that only a small amount of metabolite was present in the plasma during 24 hours, but the large amount of metabolite recovered in the urine suggested that plasma clearance of metabolites was rapid. Alkaline hydrolysis of urine produced evidence of formaldehyde, suggesting that HMF may well be a urinary metabolite (see Figure 4). The analytical evidence for metabolic demethylation of NMF in vivo, together with that reported earlier (Kimmerle and Eben,1975; Barnes and Ranta, 1972) would

suggest that formaldehyde and hence CO_2 might be formed as part of the metabolic pathway. As discussed by Brindley et al., (1982b), the N-hydroxymethyl metabolite of NMF formed in vivo (HMF) is a remarkably stable compound so that further metabolism (i.e. cleavage of N-C bound to form HCHO) may not be a chemical process but may require catalysis by an enzyme that is ready saturated at the large doses of NMF employed. This could explain the slow zero order formation of CO_2 shown above, at a rate independent of plasma NMF concentration. Repetition of the experiment using lower doses of NMF may help to elucidate whether dose dependent and hence saturable metabolic processes are involved.

The greater amount of radioactivity exhaled as $^{14}CO_2$ following injection of formyl labelled NMF when compared with methyl labelled NMF provokes speculation of a pathway by which the formyl group could be metabolised to CO_2. One obvious possibility is the hydrolytic cleavage of the amide bond leading to formate, which undergoes rapid conversion to CO_2. The constant rate of $^{14}CO_2$ exhalation for both ^{14}C-formyl-DMF and ^{14}C-formyl-NMF and the similarity in the total percentage of the dose exhaled for both compounds again suggests that a saturable process is involved.

It is worth pointing out that the pathways by which carbon atoms in xenobiotic molecules are metabolised to CO_2 are most likely very complex, e.g. the ethyl moiety of phenacetin is metabolised to CO_2 which reflects the elimination of phenacetin from the animal body (Desmond et al., 1980). Undoubtedly the ethyl group has to undergo a series of biotransformations involving the Krebs cycle before CO_2 is formed. A N-methyl grpup, however, does not require

such a multitude of transformations as an ethyl moiety. That the $^{14}CO_2$ exhalation in the case of both aminopyrine (with a ^{14}C-methyl) and phenacetin (with a ^{14}C-ethyl) can be used to detect disturbances in liver function, exemplifies that the $^{14}CO_2$ breath analysis is based on a black box approach - the metabolic details involved are obscure.

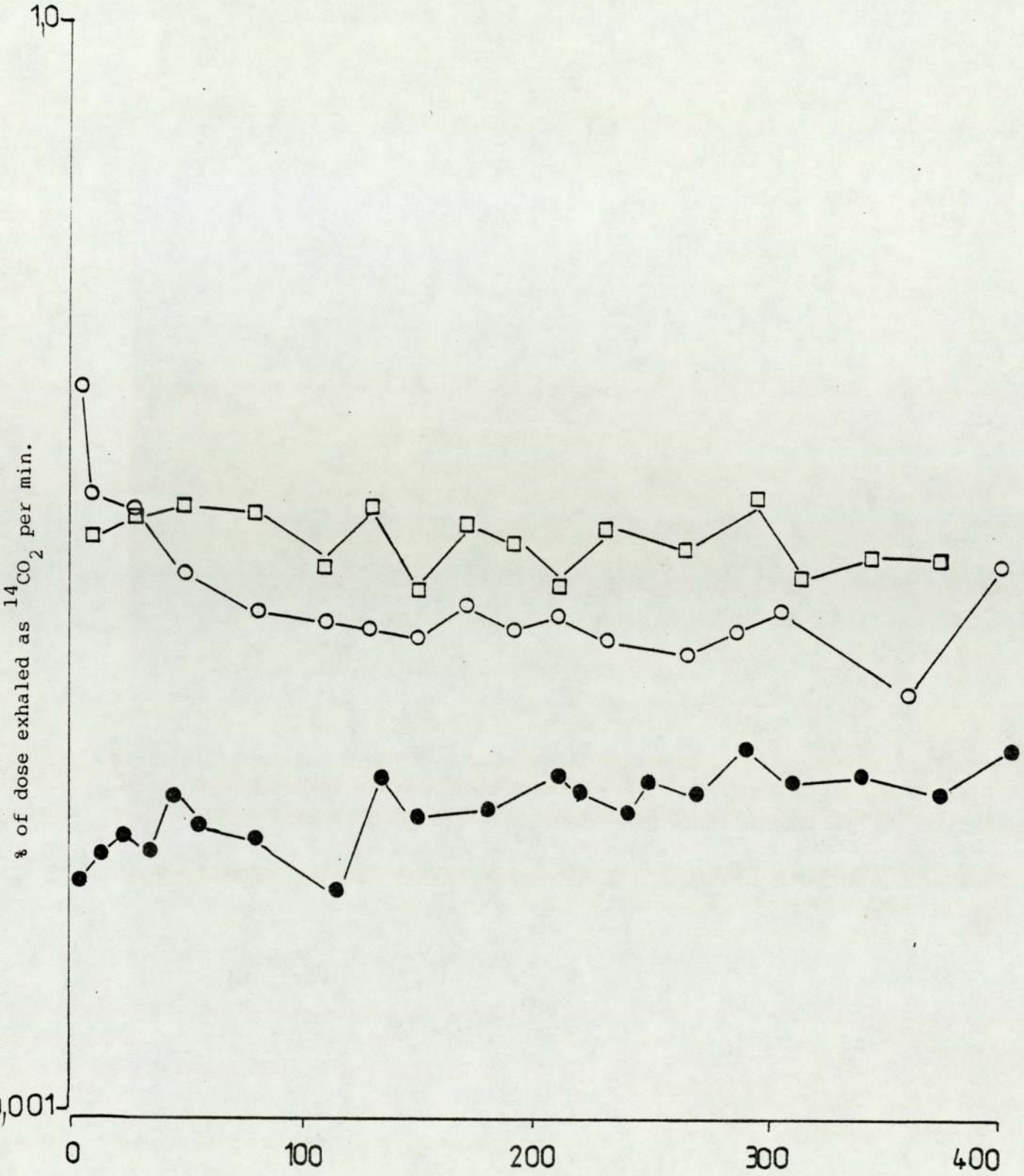


Figure 10 : $^{14}CO_2$ breath analysis after i.v. administration of ^{14}C-methyl-NMF (●), ^{14}C-formyl-NMF (□) and ^{14}C-formyl-DMF(○) 6.8mMol/kg to CBA/Ca mice.

(mean of 2 experiments)

PART TWO

Studies on the metabolism and mechanisms of toxicity of NMF and other N-methyl containing compounds *in vivo* and *in vitro*

SECTION 5

INTRODUCTION

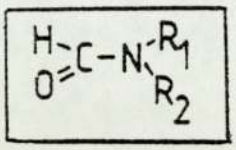
5.I Metabolism and toxicity of NMF and related formamides

The second part of this thesis is mainly concerned with studies of the mechanisms of hepatotoxicity of NMF in the mouse. During the course of the experimental work, certain features relating to the metabolism of NMF were investigated and other N-methyl containing compounds were also included.

The scope and mode of antitumour activity, together with some details of the metabolism and toxicity of NMF have been discussed in section 1.I. Other aspects of the metabolism and toxicity of NMF will now be discussed. Brindley et al. (1982b) found that the plasma disposition profile of radioactivity during the first 24 hours after administration of ^{14}C-methyl-NMF was virtually superimposable on that of unlabelled NMF. It was concluded that only a small amount of metabolites, if any, appeared in the plasma during this time. However, during the same time, only 26% of the radioactivity injected was found to be unchanged NMF in the urine, indicating that most of the urinary radioactivity was due to metabolites of NMF which appeared to be rapidly eliminated into the urine from plasma. NMF has been shown to be distributed throughout the body with the highest concentration, apart from the plasma, being in the liver (Barlow, 1982). When comparing the disposition of radioactivity in the plasma with that in the liver, the peak concentration was reached one hour after the dose for the plasma,but drug concentration in the liver did not reach a peak concentration until 8 hours after the dose. The rapid disappearance of metabolites from the plasma into the urine and the obvious importance of the liver in the disposition of radioactivity administered with NMF, could mean that the metabolites are conjugates e.g. of hydroxylated derivatives of NMF with either glucuronic acid, sulphate or glutathione.

HMF (Figure 11), as described in 1.I., has been shown to be present in the urine of NMF treated animals and raised levels of thio-ethers in the urine of such animals have also been found (Kestell et al. 1983, in preparation). Gescher et al. (1982) measured the non-protein thiol levels in livers of mice one hour after a single dose of NMF 400mg/kg. A depletion of 59.8% over control values was observed, that appeared to be specific for NMF, was dose dependent and was reversed by pretreatment of mice with SKF525A. It was then suggested that metabolic oxidation of NMF produced a reactive metabolite capable of reaction with soluble thiols. Mitchell et al. (1973a) postulated that a similar effect on hepatic glutathione by paracetamol was due to an electrophilic metabolite and subsequent covalent binding to hepatic macromolecules resulted in the necrotic lesions of paracetamol hepatotoxicity.

The hepatotoxicity of NMF has been mentioned previously (1.I). Myers et al (1956) observed that NMF caused reversible liver damage in patients, as indicated by serum liver enzyme tests. The hepatotoxic potential of NMF has been confirmed in mice (Langdon et al, submitted for publication; Newman et al, 1981) dogs (Newman et al, 1981) and rats (Scailteur et al, 1981; Lundberg et al, 1981). In all these studies, plasma levels of liver enzymes were determined and in some cases these were correlated with evidence of liver necrosis (Lundberg et al, 1981). It appears that no studies on the possible biochemical mechanisms of NMF hepatotoxicity have been reported. The hepatotoxicity of DMF (1.I) has been attributed to NMF (Kimmerle and Eben, 1975) and Lundberg et al (1981) have suggested that NMF itself is a metabolic precursor of another toxicant which is responsible for the hepatotoxicity



	R_1	R_2
N-methylformamide (NMF)	CH_3	H
N,N-dimethylformamide (DMF)	CH_3	CH_3
N-hydroxymethylformamide (HMF)	CH_2OH	H
formamide (F)	H	H

Figure 11 : Formulae of formamides in this study

associated with DMF. Scailteur et al (1981) observed that the metabolism of DMF to NMF and from NMF to F (Figure 11) could be assumed to be activation and inactivation reactions respectively, on the basis of the individual toxicities of each of these compounds to rats. Chanh et al (1971) in experiments with mice also reported on the increased hepatotoxicity of NMF when compared with DMF. However,Lundberg et al (1981) and Dexter et al (1982) found that DMF and NMF were equally hepatotoxic in rats and mice. However, DMF has only marginal activity against murine tumours, when compared with NMF (Cooksey et al, in press). Recent work by Langdon et al (submitted for publication) showed that administration of NMF to mice on a chronic schedule led to optimum antitumour activity with reduced hepatotoxicity, as assessed by plasma liver enzyme levels. On the basis of these observations it may be speculated that the antitumour activity and hepatotoxicity of NMF result from separate events. It may be that a metabolic intermediate with no antitumour activity may be responsible for the hepatotoxic damage.

NMF appears to cause no myelosuppression (1.I) and in recent Phase 1 clinical trials, the major toxicity appeared to be severe nausea and vomiting. Hepatotoxicity, as determined by serum liver enzymes, appeared to be unpredictable, transient and reversible at the low doses of NMF used. (Newlands, personal communication).

Another reported toxic manifestation associated with DMF and NMF is presumably the consequence of the inhibition of aldehyde dehydrogenase activity. Symptoms of alcohol intolerance as an adverse response to DMF exposure in industrial workers have been described by Reinl et al (1965) and Lyle et al (1979). Dermal flushing with severe

headaches had been observed following exposure to DMF and subsequent intake of alcohol. Such an effect was similar to that obtained with disulfuram, which interferes with the metabolic pathway of ethanol by impairing the oxidation of acetaldehyde, due to inhibition of aldehyde dehydrogenase (Dietrick et al, 1971). Hanasono et al (1977) investigated the adverse effect of DMF on alcohol metabolism in rats. It was found that after equimolar doses of DMF or NMF, acetaldehyde levels were raised above control levels when ethanol was administered. However, the maximum effect was only reached when DMF or NMF were given orally either 18 hours or 3 hours respectively before alcohol, indicating that NMF was a more efficient inhibitor of acetaldehyde metabolism than DMF. As the effects of NMF and DMF are so similar to those of disulfuram it is believed that aldehyde dehydrogenase inhibition by the formamides is the cause of the alcohol intolerance.

5.II The role of glutathione in the metabolism and detoxification of xenobiotics.

Glutathione or ɣ-glutamyl-cysteinyl-glycine (Figure 12) is a tripeptide which plays an important role in many biochemical and physiological functions of cells. In this discussion, the function of glutathione in the metabolism and toxicity of xenobiotics will be emphasised. Glutathione accounts for approximately 90% of the intracellular content of non-protein thiols (Reed and Beatty, 1980). Free reduced glutathione (GSH) exists in the cell in dynamic equilibrium with the oxidised form (GSSG). GSSG can be formed from GSH by enzymic action of glutathione peroxidase and the reverse reaction is catalysed by glutathione reductase. Glutathione is found mainly intracellularly and normally more than 95% of this is reduced glutathione. Extracellular levels of glutathione are usually very low (Anderson and Meister, 1980).

Glutathione occurs in all tissues, but in nearly all species investigated the highest concentrations are found in the liver. The concentration of GSH in mouse liver has been reported to range from 3-8mM depending on the age of the animal (Kosower and Kosower, 1978). Translocation of glutathione across cell membranes appears to be an important part of glutathione disposition. The efflux of intracellular GSH and GSSG to extracellular spaces has been studied in perfused liver (Bartoli and Sies, 1978) and in isolated hepatocytes (Jones et al, 1978; Eklöw et al, 1981).

There have been many reviews of the biosynthesis, degradation and function of glutathione in the cell (Kosower and Kosower, 1978; Reed and Beatty, 1980;Orrhenius and Jones, 1978). Examples of the three major types of reaction in which glutathione is involved in metabolism

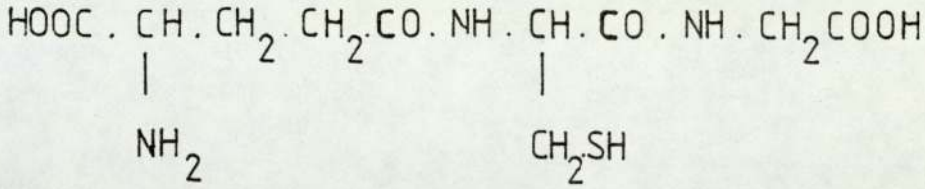


Figure 12A : GLUTATHIONE

(ɣ -glutamyl-cysteinyl-glycine)

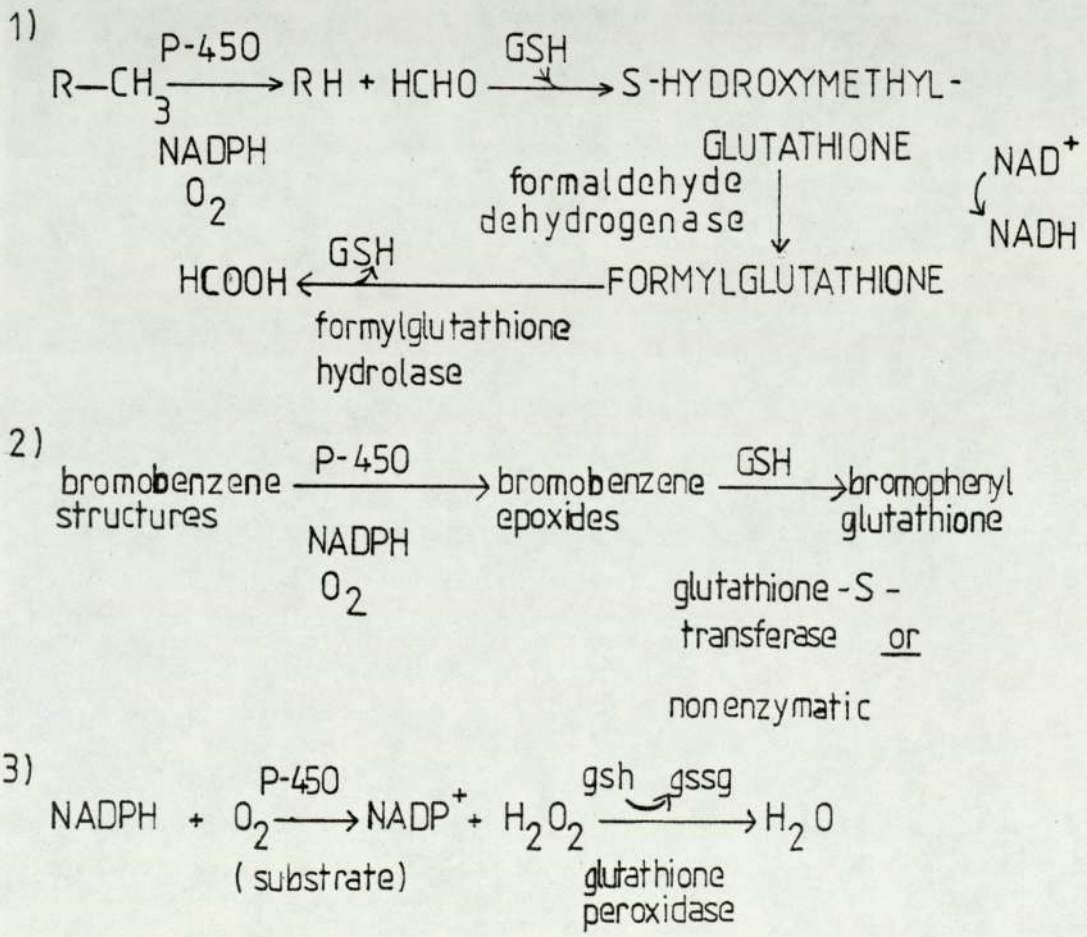


Figure 12B: Functions of GSH in detoxification,

1) as a cofactor in aldehyde oxidation;

2) as a nucleophile in drug conjugation;

3) as a reductant in peroxide reduction.

(Orrhenius and Jones, 1978)

and detoxification are summarised in Figure 12b. (Orrhenius and Jones, 1978). GSH can act as a cofactor (1), as a nucleophile (2), or as a reductant (3). All three of these reactions are concerned with further metabolism or detoxification of substances generated by cytochrome P-450 monoxygenase enzymes in the liver.

The example of the role of GSH as a cofactor (1) is in the type of reaction described in part one of this thesis; N-demethylation, with subsequent formaldehyde oxidation, the metabolic process which forms the central theme of this thesis. The metabolic pathway from $N\text{-}CH_3$ to CO_2 is summarised in Figure 11. The oxidation of formaldehyde to formic acid appears to involve two enzyme catalysed reactions for which GSH is required as a cofactor (Strittmatter and Ball, 1955). Formaldehyde reacts non-enzymatically with GSH to form S-hydroxymethyl glutathione (Uotila et al, 1974) which is the substrate for formaldehyde dehydrogenase. The resulting formylglutathione is hydrolysed enzymatically to yield formic acid and GSH. The enzyme in the latter reaction is formyl glutathione hydrolase. GSH is also a cofactor for the enzyme glyoxalase, which catalyses the conversion of methylglyoxal into lactic acid (Wills, 1981).

The remaining two examples of types of reaction involving glutathione shown in Figure 12b are concerned with metabolic detoxification. GSH functions as a nucleophile (2) towards a wide variety of electrophilic compounds. The reaction leads to GSH conjugates which are either excreted as such or are metabolised further to mercapturic acids which are excreted either in the urine or the bile (Kosower and Kosower, 1978). The reaction between electrophiles and nucleophiles may be catalysed by enzymes known as GSH-S-transferases. Several of these enzymes have

been shown to have low substrate specificity and have been isolated from rat liver (Jakoby, 1978). Alternatively, these conjugation reactions may occur spontaneously under physiological conditions without enzyme catalysis. The conjugation reaction with xenobiotics or their metabolites is probably the best understood biological role of GSH. Electrophiles known to react with GSH include organophosphorous compounds, quinones and epoxides, such as are formed from bromobenzene (Figure 12b, 2). Endogenous compounds, such as steroid hormones, also form conjugates with GSH and this is believed to be of importance in the regulation of steroid levels (Chasseaud, 1979). These reactions not only facilitate the excretion of foreign compounds but also serve to deactivate electrophilic compounds before they can covalently bind to tissue nucleophiles and elicit toxic damage. Mitchell et al (1974) observed that in the case of paracetamol there was a direct relationship between hepatic glutathione depletion, GSH conjugate formation, covalent binding and liver cell necrosis. Many chemicals are known to deplete GSH in liver after a single administration and it is generally believed that the maximum depletion by a sublethal dose of a reactive toxin cannot decrease levels below 20-25% of normal GSH levels (Reed and Beatty, 1980). Rapid resynthesis of GSH usually follows. In most cases, the metabolic routes of toxic compounds which can be ultimately detoxified by GSH conjugation are the formation of epoxides, N-hydroxy intermediates, insipient carbonium ions or free radicals. The availability of GSH is a critical factor and toxicity depends on the balance between the rate of reactive metabolite production and GSH synthesis (Prescott, 1982).

In the discussion of the role of GSH conjugation in the detoxification of compounds, it is important to realise that for some compounds, conjugation with GSH can result in an activated product. In particular, it appears that conjugation of bifunctional halogenated compounds such as 1,2-dibromoethane, leads to formation of alkylating species that are more reactive than the parent compound (Nachtomi, 1970).

The third function of glutathione, depicted in Figure 12b(3) is as a reductant. In this case, the oxidation-reduction properties of glutathione are used to reduce peroxides, which might otherwise be toxic to the cell (5.III). Free radicals formed on metabolic activation of GSH dependent hepatotoxins may subsequently produce a multitude of hydroperoxides and other oxidising substances (5.III). GSH is oxidised to GSSG with conversion of the hydroperoxides to water or alcohols under the catalytic action of the enzyme, glutathione peroxidase. The oxidised form of glutathione is readily reduced back to GSH by glutathione reductase.

It is therefore clear that glutathione plays a vital role in the protection of cells against metabolic oxidation products of many xenobiotics and any decrease in tissue concentration of glutathione or in the rate of glutathione biosynthesis could lead to toxic changes.

The majority of studies on the role of glutathione have concentrated on liver cells and xenobiotics that are toxic to the liver, but glutathione has also been shown to be important to tumour cells and to play a part in the metabolism and activity of antitumour drugs. Glutathione levels were reduced in the tumours of mice following BCNU

treatment in tumour bearing mice (McConnell et al, 1979) and experiments with cyclophosphamide showed that the drug causes a dose dependent decrease in hepatic glutathione (Hipken et al 1979). Glutathione transferase activity was important in the conversion of azothioprine to 6-mercaptopurine (Kaplowitz and Kuhlenkamp, 1978). The glutathione content of tumour cells has also been investigated, especially in studies of resistance of a tumour cell line to a cytotoxic agent. Several studies have demonstrated that tumour cells sensitive to certain alkylating agents generally have a lower cellular content of non-protein thiols, than do cells which have acquired resistance (Connors, 1966; Poynter, 1970). Suzukake et al (1983) showed that tumour cells resistant to L-phenylalanine mustard (L-PAM) could be completely sensitised to L-PAM by decreasing the intracellular concentration of glutathione. Cells resistant to L-PAM were found to have a higher content of glutathione than cells sensitive to the drug. Therefore, glutathione can be said to play a crucial role in the cytotoxicity of some xenobiotics and thus its consideration is important in studying hepatotoxins and cancer chemotherapeutic agents.

5.III Biochemical changes associated with hepatotoxicity

In studies of the biochemical mechanisms involved in the hepatotoxicity caused by a number of compounds, several common features have emerged. Firstly, the role of the tripeptide, glutathione, has been found to be important in the defence of liver cells against damage induced by many hepatotoxins (5.II). Secondly, the generation of free radicals with subsequent peroxidation of cellular lipids has been implicated in liver damage and thirdly, alterations in cellular calcium homeostasis have been observed in liver following exposure to hepatotoxins. The importance of and the relationship between these three factors in the elucidation of mechanisms of hepatotoxicity will now be discussed.

The first of these features, the importance of glutathione, has been discussed previously (5.II). Xenobiotics or their metabolically activated intermediates may form water soluble and excretable conjugates with GSH and therefore deplete the glutathione reservoir. In addition glutathione is important as a cofactor for the selenium dependent glutathione peroxidase enzyme. Glutathione peroxidase acts in the defence system of the cell against hydrogen peroxide and hydroperoxides (Chance et al, 1978). It has been suggested that xenobiotic metabolism could lead to peroxidation of membrane lipids by a reduction in hepatic glutathione. Cellular damage following glutathione depletion could be explained by lipid peroxidation that destroys the integrity of the membrane and so the life of the cell before alkylation of protein occurs by a reactive metabolite (Anundi et al, 1979). A decreased level of glutathione, together with a decrease in activity of those protective enzymes against oxidative stress, superoxide dismutase, catalase and glutathione peroxidase, could cause elevated levels of

hydroperoxides. Younes and Siegers (1981) suggested that during normal liver metabolism, lipid peroxides are formed and glutathione protects against peroxide damage to the cell. When a certain minimum threshold concentration of glutathione is reached (20% of the initial value) the cellular defence mechanism against lipid peroxidation is diminished and cellular damage ensues.

The initial steps of lipid peroxidation involve the formation of an organic free radical by hydrogen abstraction with subsequent diene conjugation and the formation of peroxide radicals by trapping molecular oxygen. The stable products of these reactions are hydroperoxides (Chance et al, 1978). The hepatotoxicity of CCl_4 has been considered to be due to the production and propogation of free radicals which initiate the peroxidative breakdown of polyunsaturated fatty acids in the membranes, thus inducing toxicity (Slater, 1972). Such peroxidation of polyunsaturated fatty acids present in the membrane lipids has been considered to be a basic mechanism of toxicity for a variety of chemicals (Smith et al, 1983).

In the case of CCl_4 hepatotoxicity, lipid peroxidation has been shown to occur at an early stage and prior to cell death. However, in the case of bromobenzene toxicity, lipid peroxidation appears to be of secondary importance, as it becomes evident at a much later stage and probably only occurs after cell death. It is believed that lipid peroxidation is only a consequence of an earlier toxic effect induced by bromobenzene, such as glutathione depletion, and not a cause. (Smith et al, 1983).

The biochemical consequences of lipid peroxidation are varied. In addition to the loss of unsaturated fatty acids of lipid membranes,

a number of breakdown products of lipid peroxides are formed, including fatty aldehydes and ketones, malondialdehyde and short chain alkanes such as ethane and pentane (Bus and Gibson, 1979). The nature of these precursors and the mechanisms of breakdown of these peroxidised lipids is still unknown. There is increasing evidence that many products formed during lipid peroxidation are cytotoxic. Esterbauer et al (1982) isolated a complex mixture of products including aldehydes such as 4-hydroxyalkenals and malondialdehyde (MDA). 4-Hydroxypental was shown to inhibit protein synthesis, causing toxicity both in vitro and in vivo. MDA, the reaction product of lipid peroxidation that is usually determined in experiments, has been shown to crosslink with proteins and nucleic acids (Tappel, 1980) and is thought to be mutagenic and carcinogenic (Marnett and Tuttle, 1980; Shamberger et al, 1974). Enzyme inhibition and the release of lysosomal enzymes have also been associated with lipid peroxidation (Wills and Wilkinson, 1966).

Indication of lipid peroxidation is usually by measurement of one of the by products of the peroxidation reaction. An increase in conjugated lipid dienes (Recknagel and Ghoshal, 1966), in the occurrence of low level chemiluminescence (Cadenas et al, 1981) or in the exhalation of ethane or pentane (Wendel and Dumelin, 1981; Müller et al, 1981) are frequently the methods used. The most usual method is to measure malondialdehyde production as described in 7.IV. However, this method has some disadvantages, which should be considered when analysing results. MDA is rapidly metabolised by oxidation in mitochondria and it has been estimated that in the presence of oxidising enzymes, assays underestimate the true level of peroxidation

by approximately 50% (Högberg et al, 1975). It has been suggested that of all the methods available for the measurement of lipid peroxidation, chemiluminescence and ethane exhalation are the most sensitive for hepatocyte preparations and MDA may be insensitive for measuring low levels of lipid peroxidation (Smith et al, 1982).

The third feature common to the mechanism of toxicity of several hepatotoxins is a change in calcium homeostasis. The calcium ion exerts a profound influence on a wide number of cellular processes and alterations in the free calcium ion concentration in the cytosol are believed to be the most important factors affecting cellular function. Cytosolic Ca^{2+} is maintained at a concentration of 0.05 to 0.5μM (Rasmussen, 1981). The calcium concentration outside the cell has been estimated to be approximately 10,000 fold higher than in the cytosol (Rasmussen, 1981). Passive diffusion of calcium into the cell down the steep concentration gradient is prevented by the permeability barrier of the plasma cell membrane. In addition, the membrane acts as a site of active efflux of calcium from the cell against this gradient. Two energy dependent calcium sequestration processes have also been found in the cell that help to maintain the steady state level of ionised calcium in the cell cytosol. These are the calcium pumps in the endoplasmic reticulum (Moore et al, 1975; Farber et al, 1977) and in the mitochondrion (Lehninger et al, 1978). There is some controversy as to the relative importance of the two pumps in regulating cellular calcium. It may be that the levels of calcium ions in the mitochondria are simply a function of the cytoplasmic excess of calcium secondary to a change in influx (Farber, 1977).

Farber (1979) suggested that the best hypothesis to explain liver cell necrosis by hepatotoxins was the interference with the permeability of the plasma membrane in which calcium ion influx becomes greater than the rate of active efflux. The increase in intracellular calcium would be partially contained by the activity of the endoplasmic reticulum and mitochondrial pumps, but if influx was sustained, intracellular calcium would rise to toxic levels. The dependence of toxic cell death on cellular calcium concentration has been described (Schanne et al, 1979) and is currently the subject of a controversy (Jewell et al, 1982; Farber, 1979). Calcium has been shown to accumulate in necrotic tissue following administration of several hepatotoxins, but most reports have dealt with the effect of CCl_4 on cellular calcium. Early investigations with CCl_4 used cytochemical techniques (Reynold, 1963; Reynold, 1964) and the increased calcium in the cell was localised to the mitochondria, suggesting that the primary toxic lesion occurred in this organelle. However, the changes in cellular calcium content together with the morphological changes in the mitochondria did not occur until several hours after exposure to CCl_4. In an effort to discover an earlier site of toxicity in the cell, Moore et al (1975) reported that the ability of the hepatic endoplasmic reticulum to sequester calcium was severely inhibited 30 minutes after administration of CCl_4. Similar changes in the sequestration of calcium by the endoplasmic reticulum from isolated hepatocytes (Pencil et al, 1982) or by microsomes in vitro (Lowrey et al, 1981), following incubation with CCl_4, have also been reported.

It is believed that early failure to sequester calcium ions by the endoplasmic reticulum following CCl_4 exposure may be one of the initial events that lead to ultimate tissue damage (Recknagel, 1983). It was hypothesised that failure of the endoplasmic reticulum to sequester calcium may lead to an unacceptable rise in cytosolic free calcium levels. The influence of other toxins on calcium homeostasis in the liver has also been investigated. The quinone, menadione (2-methyl-1,4-napthoquinone) has been shown to alter total cellular calcium in isolated hepatocytes (Thor et al, 1982a). Bellomo et al (1982) reported that metabolism of t-butyl hydroperoxide by rat hepatocytes caused a release of calcium ions from both the mitochondrial and extramitochondrial compartments and calcium release from perfused liver has been observed by Sies et al (1981). In all these cases, plasma cell membrane changes have also been noted (see 5.IV), consistent with the theory that plasma cell membrane damage may be related to calcium changes (Farber, 1979).

It has been shown that in hepatocytes, the glutathione redox state has an important bearing on the extramitochondrial pool of Ca^{2+} and this pool is highly susceptible to oxidative stress (Bellomo et al, 1982; Thor et al, 1982a). Previous experiments have suggested that the microsomal calcium pump is sensitive to sulphydryl reagents (Moore et al, 1975). Jones et al (1983) have confirmed these results in experiments with t-butylhydroperoxide where it was shown that administration of intracellular thiols, especially GSH, prevented changes in ATP-dependent microsomal Ca^{2+} uptake, by protecting the calcium pump from oxidative damage.

The interrelationship between the three features discussed here, glutathione status, lipid peroxidation and calcium homeostasis in mechanisms of hepatotoxicity is further confirmed by the work of Lowrey et al (1981) and Waller et al (1983). Minimal lipid peroxidation induced by either bromotrichloromethane or carbon tetrachloride was correlated with severe inhibition of calcium uptake in the endoplasmic reticulum. At a given level of lipid peroxidation, calcium uptake by the endoplasmic reticulum was more severely depressed than activities of either glucose-6-phosphatase or aminopyrine demethylase (Lowrey et al, 1981). This was confirmed by Waller et al (1983) who noted that microsomal calcium sequestration was sensitive to both lipid peroxidation and covalent binding of CCl_4.

It therefore appears that for many intrinsic hepatotoxins, for which metabolism appears to be an essential requirement for toxicity, evidence of changes in glutathione, lipid peroxidation and calcium homeostasis is important in the understanding of the mechanisms of liver damage.

5.IV Histopathological and ultrastructural changes associated with hepatotoxicity

For a better understanding of the mechanisms by which drugs cause hepatotoxicity, the study of morphological changes in liver cell structure is useful. The analysis of the type and localisation of the damage in the liver may help to distinguish between different mechanisms. It is possible to classify substances that produce hepatic injury on the basis of clinical and experimental observations into two main categories. These are the intrinsic hepatotoxins, towards which most exposed individuals are susceptible and the idiosyncratic hepatotoxins, where toxic effects result from a special characteristic feature which make individuals more vulnerable to the hepatotoxin (Zimmerman, 1978). Although such a classification is somewhat oversimplified, intrinsic hepatotoxins have a number of characteristics (Klatskin, 1975). These substances all produce a high incidence of hepatic injury in exposed individuals. The hepatotoxic lesion may also be reproduced in experimental animals and the development and degree of hepatic injury are dependent on the dose of the agent. For idiosyncratic hepatotoxins, all these criteria do not apply.

Because of the ease of reproduction of lesions in animals by intrinsic hepatotoxins, characterisation of the morphological changes induced has been relatively simple. Analysis of such changes has facilitated understanding of mechanisms of injury. Examples of well-known intrinsic hepatotoxins are carbon tetrachloride (CCl_4), aflatoxins, amanitine and dimethylnitrosamine (DMN), which all show the characteristics described above (Zimmerman, 1978).

First analysis is usually by light microscopy and in the case of acute hepatic injury, toxic agents may lead to either degeneration or necrosis of the hepatocytes (cytotoxic damage) or to altered bile flow (cholestatic damage) or to a combination of both. Intrinsic toxins mainly cause cytotoxic damage and idiosyncratic toxins tend to produce predominantly cholestatic damage. In this thesis, hepatotoxicity following acute exposure to NMF was investigated. Some of the morphological characteristics of acute liver injury induced by intrinsic hepatotoxins, together with the role of active metabolites in this process will be discussed below.

Cytotoxic damage to liver cells leads to degeneration, necrosis or steatosis. In general, the necrosis produced by intrinsic hepatotoxins is zonal and centrizonal necrosis is characteristically produced by CCl_4, bromobenzene, DMN, amanitine and paracetamol (Rouiller, 1964; Zimmerman, 1978). The site of necrosis in the liver can give clues as to the mechanism by which many instrinsic toxins cause injuries. It is believed that the most important factor in the special vulnerability of the liver to injury by chemicals is its role in the metabolism of foreign compounds and it has become clear that most hepatotoxins and hepatocarcinogens must be activated to toxic or carcinogenic metabolites to promote liver damage (Zimmerman, 1978). It has been suggested that the area of the liver containing the enzyme system responsible for the conversion of a toxin to its active metabolite is most likely to be the area that incurs damage (Mitchell et al, 1973b; Mitchell et al, 1976). Bromobenzene, paracetamol and CCl_4 are all converted to metabolites by hepatocytes in the centrilobular (or periacinar) region and produce mainly centrizonal (or periacinal) necrosis (Reid et al, 1971; Jollow et al, 1973;

Recknagel and Glende, 1973). Degeneration of liver cells may occur prior to the necrotic development and also in non-necrotic areas adjacent to the necrotic areas. Degenerative cell changes can also be produced by lower doses of necrogenic toxins. In degenerated tissue, cells are often enlarged or "ballooned". Fatty changes (steatosis) in liver cells may also be produced by hepatotoxins although to varying degrees. CCl_4 produces mild steatosis with dominant necrosis (Rouiller, 1964) whereas amanitine produces predominantly steatosis (Fiume, 1972).

In addition to studying the morphology of liver cells by light microscopy, investigations of the ultrastructural changes occurring to liver cells by using electron microscopy can give further information on early signs of damage and can also help to elucidate mechanisms of injury. One early ultrastructural change has been shown to occur in the plasma membrane of the hepatocyte, following exposure to hepatotoxins. Bromobenzene (Thor et al, 1982b), nitrosamines (Le Page et al, 1967) and the mushroom toxin phalloidin (Frimmer et al, 1974) have all been shown to produce swelling of the plasma membrane of hepatocytes with the formation of protrusions or blebs on the outer surface. Other compounds which elicit oxidative changes in the cell such as menadione and t-butylhydroperoxide also produce this ultrastructural damage (Thor et al, 1982a; Bellomo et al, 1982). Farber (1979) hypothesised that liver cell necrosis produced by several agents was primarily due to damage to the plasma cell membrane, as indicated by the changes outlined above. It was suggested that interference with the permeability of the plasma membrane led to changes in cellular calcium homeostasis. Thor et al (1982a; 1982b) and Bellomo et al (1982) have correlated changes in the plasma cell membrane with alterations in cellular calcium levels. As discussed in 5.III, calcium ions have been implicated in liver cell necrosis.

Histopathological damage to any tissue is frequently reflected by the release of endogenous substances into the plasma, from the injured tissue. Measurement of these substances may often be a sensitive index of cellular damage if the toxic stimulus exhibits a degree of organ specificity. Low levels of these substances - often enzymes - are normally found in the plasma or erythrocytes. The measurement of serum enzyme activities for clinical diagnosis of liver function is widely employed.

Of the many enzymes that may be released by the liver following cellular damage, the measurement of sorbitol dehydrogenase (SDH, EC 1.1.1.14) has been recommended as its release is specifically associated with damage to this organ. SDH is normally present in the plasma in very low levels. Significant quantities are found only the liver cells with lower quantities in kidney and prostate (Kachmar and Moss, 1976). Measurement of serum SDH levels has been shown to be a more sensitive index of hepatotoxic damage induced by CCl_4, thioacetimide, DMN and diethanolamine than several other serum enzymes (Korsud et al, 1972; Korsud et al, 1973). SDH has also been employed in studies of the hepatotoxicity of DMF (Scailteur et al, 1981; Lundberg et al, 1981) and NMF (Newman et al, 1981). The appearance of any measurable amount of SDH in serum invariably indicates parenchymal liver damage and in acute cases of hepatic damage, levels may increase dramatically. The use of the enzyme is, however, restricted to acute damage as in chronic hepatitis or cirrhosis, enzyme activity rapidly returns to normal after an initial rise (Kachmar and Moss, 1976).

Other enzymes that are frequently used to assess liver damage are the transferases, alanine aminotransferase (ALT, glutamate pyruvate transaminase, EC 2.6.1.2) and aspartate aminotransferase

(AST, glutamate oxaloacetic transaminase, EC 2.6.1.1). These enzymes are widely distributed in animal tissues and can be found in the heart, liver and muscle, indicating that organ specificity is not as high as in the case of SDH. AST is present in almost equal amounts in both the liver and the heart whereas ALT is present almost exclusively in the liver, with only small quantities in the heart and skeletal muscle (Woodman, 1981). Although observing that the theory seemed to be rather oversimplified, Woodman suggested, with caution, that early loss of cytoplasmic enzymes may well reflect alterations in plasma membrane permeability caused by the hepatotoxin. The use of both rather than one aminotransferase may enhance the usefulness of this type of measurement as an index of liver damage. An increase in activity of both enzymes is likely to be a solid indication of damage whereas an increase in AST levels alone points only to cardiac damage. In contrast to SDH, aminotransferase levels are elevated in cases of cirrhotic livers (Kachmar and Moss, 1976). Korsrud et al (1972) reported that although not as sensitive as SDH for measurement of mild forms of CCl_4-induced liver damage, measurements of serum aminotransferase levels did correlate well with the severity of grave liver damage.

In view of the work reviewed above it seems to be important not to rely on a single serum enzyme measurement for assessment of liver function because of the presence of these enzymes in other tissues. The assay of several plasma liver enzymes appears to provide a more reliable reflection of hepatotoxic damage. It must also be emphasised that measurement of serum enzyme activities does not substitute for hisopathological examination of tissues as liver damage is often detected by microscopy at lower doses than can be estimated by enzyme measurement alone (Korsrud et al, 1972).

5.V Aims and scope of the present investigation

The antitumour properties of the agent, NMF, together with details of the current state of knowledge regarding its metabolism and toxicity have been described in 1.I and 5.I. Despite the fact that NMF was first used as an antitumour agent more than 25 years ago (Myers et al, 1956), very little is understood concerning the mechanisms of action, metabolism or toxicity of the compound. In the second part of this thesis, an attempt was made to elucidate further some of these mechanisms, in particular those relating to the major toxic manifestation of NMF in the mouse: hepatotoxicity. A better understanding of such mechanisms could be beneficial for the clinical use of NMF. Aspects of the metabolism of NMF were also studied and in some investigations, comparison with other N-methyl containing compounds was made. It is worth noting that in the recent clinical re-evaluation of NMF, symptoms of hepatotoxicity were clearly observed even though this toxicity does not seem to be the major side-effect associated with this drug. The hepatotoxicity study in this part of the thesis may be divided into two major sections. Firstly an investigation of possible biochemical mechanisms of NMF induced liver damage was made. For many hepatotoxins, one or more of the three biochemical effects described in 5.II and 5.III, glutathione depletion, lipid peroxide production and alteration in intracellular calcium homeostasis, have been implicated in the mechanisms of liver damage. A depletion of hepatic glutathione has been observed previously following NMF treatment (Gescher *et al*, 1982) and this was further investigated following multiple doses of the drug for possible cumulative glutathione depletion. In addition, *in vitro* studies used an experimental model which has been used rarely in this type of study, the isolated mouse hepatocyte preparation. The majority of investigations

on hepatocytes have used the rat as a donor and preliminary studies on cell viability and the effect of substances with well described effects on rat hepatocytes were performed in this work, to validate the mouse hepatocyte preparation. To complement the *in vivo* experiments concerning glutathione and NMF, *in vitro* studies following incubation with physiological concentrations of NMF and the related formamides, DMF, HMF, F and D-NMF were performed with mouse hepatocytes.

Lipid peroxide production was also studied in similar *in vitro* incubations of isolated mouse hepatocytes with NMF, DMF, HMF, F and D-NMF and in microsomal or mitochondrial fractions with NMF. In addition, production of lipid peroxides *in vivo* was estimated after administration of varying doses of NMF to mice. To evaluate a possible intracellular site of lipid peroxidation, estimates were made in cytosol, mitochondria and microsomes. In both experiments on glutathione status and those on lipid peroxide production the hypothesis was tested that chemically reactive metabolites of NMF formed in the liver caused necrosis either by directly reacting with bionucleophiles or by initiating lipid peroxidation.

The theory of changes in intracellular calcium homeostasis as an early indicator of hepatotoxic damage was discussed in 5.III. It was considered that evaluation of any alteration in the uptake of calcium into the microsomal or mitochondrial pumps within a short time of NMF or any related formamide administration to mice would be important for the understanding of the mechanism of NMF hepatotoxicity. An *in vitro* investigation using microsomes and NMF was also made.

It was believed that studies of these three biochemical features, both *in vitro* and *in vivo* would provide valuable information on the biochemical mechanisms of NMF hepatotoxicity. As discussed in

5.III, the interrelationship between glutathione depletion, lipid peroxide production and changes in cellular calcium content have been tentatively suggested by several authors as the basis of the liver damage induced by a number of hepatotoxins.

The second section of this study involves an investigation to characterise the nature of the liver damage in the mouse, induced by NMF, both in vitro and invivo by use of the criteria discussed in 5.IV. The in vitro studies involved isolated mouse hepatocytes and observation of any ultrastructural changes to the cell membrane following incubation with NMF in a similar manner to that described by Bellomo et al (1982) for rat hepatocytes. In the in vivo studies to characterise the liver damage in the mouse following NMF, the dose dependency and time of onset of the damage were investigated, by histopathological examination of livers. From these investigations, together with examination of livers from surviving mice several days after NMF treatment, it was considered that clues as to the mechanism of damage following acute NMF treatment would be apparent. A comparison of damage induced by NMF in different strains of mice was also possible. In most of the in vivo studies, a parallel evaluation of toxicity was possible in terms of release of liver enzymes into plasma. SDH, ALT, and AST levels in plasma have been used previously as an index of liver damage in many studies (5.IV).

In addition to the study of glutathione status in liver cells following NMF incubation, a similar study in three types of tumour cells was made. Glutathione content has been shown to vary between tumour cells (5.II). If a similar difference was observed between the TLX5, PC6A or M5076 tumour cells in this study, this might help to explain the different sensitivities of the tumour cells to drugs. It was also considered that a depletion of glutathione content in the

cells following incubation with drugs to which the cells were sensitive might give an indication of the mechanisms of cytotoxicity of those drugs.

SECTION 6

ANIMALS AND MATERIALS

6.I Animals

Male BALB/c mice (19-25g) were used in all experiments except those described in 7.IV and 7.X.i. where CBA/Ca and BDF_1 mice were also used. Details of tumour maintenance have been described by Gescher *et al* (1982). Treatment with NMF or saline was initiated on day 3 or day 1 after inoculation for the TLX5 and M5076 tumours respectively. Details of purchase and diet are given in 2.III.

6.II Chemicals

NMF was purchased from Aldrich Limited and DMF and F from BDH Chemicals Limited. All were purified further by distillation. HMM was synthesised according to the method of Grady and Stott (1965) and N-(trideuteromethyl)-formamide (deuterated NMF, DNMF) as described by Threadgill and Gate (1983) by E.N.Gate. Reduced and oxidised glutathione (GSH and GSSG) for standards, were purchased from Sigma U.K.Limited. Malonaldehyde-bis-dimethylacetal (MDA), used as a standard for the lipid peroxide assay and 2-vinylpyridine (2VP), for GSSG determination, were purchased from Aldrich Limited, U.K. Collagenase was obtained from Boehringer Limited, U.K. and horse serum from Gibco Europe. For the calcium sequestration studies, ^{45} calcium (12.5 mCi per mg calcium), in the form of calcium chloride was obtained from Amersham International, NE260 scintillant was purchased from New England Nuclear and filters (0.45μ) were obtained from Millipore U.K., Limited. All other substrates, cofactors and reagents for buffer solutions were purchased either from Sigma U.K. Limited or BDH Chemicals Limited.

6.III Buffers, reagents and cofactor solutions

(i) Glutathione assays

Phosphate 125mM and EDTA 6.3mM buffer, pH 7.5

11.12g $Na_2HPO4 \cdot 2H_2O$

1.17g Na_2 EDTA

were dissolved in distilled water up to 500ml and adjusted to pH 7.5.

Na_3 PO_4 solution

Na_3 PO_4 12.5g was weighed into a beaker (25ml), a little distilled water added and using heat and stirring, the solid gradually dissolved. When dissolved, the volume was made up to 25ml. Constant stirring was required for the solid to remain in solution.

DTNB solution

5'5-dithiobis-(2-nitrobenzoic acid), DTNB or Ellman's Reagent, (23.8mg) was dissolved in phosphate-EDTA buffer, pH 7.5 (10ml). The solution was stored in the freezer in the dark until use.

NADPH solution

NADPH 6.3mg was dissolved just before the experiment in phosphate-EDTA buffer, pH 7.5 (25ml).

Glutathione reductase

Glutathione reductase (Type III, from yeast) was diluted with phosphate-EDTA buffer, pH 7.5 such that the final solution contained 50 I.U. per ml.

(ii) Hepatocyte preparation buffers (Renton et al, 1978)

Hepes buffer, pH 7.4

NaCl	8.3g
KCl	0.5g
Hepes	2.4g
NaOH 1M	5.5ml

The chemicals above were dissolved in distilled water, the volume made up to 1L and the pH adjusted to 7.4. The solution was gassed before use with 95% O_2, 5% CO_2.

Hepes-EGTA buffer, pH 7.4

EGTA, 49mg was dissolved in Hepes buffer, pH 7.4, volume adjusted to 100ml and the pH adjusted to pH 7.4. The solution was gassed before use with 95% O_2, 5% CO_2.

Krebs-Henseleit buffer, pH 7.4 (Krebs buffer)

A.	NaCl	6.87g	
	KCl	0.40g	
	$MgSO4.7H_2O$	0.14g	These were dissolved in distilled water.
	$CaCl_2$	0.28g	
	NaH_2PO4	0.14g	
	Glucose	2.00g	
B.	$NaHCO_3$	2.1g	was dissolved in distilled water.

Solution B was added to solution A, the volume adjusted to 1L with distilled water and the pH adjusted to 7.4.

Hepatocyte incubation mixture, pH 7.4.

Krebs buffer above containing 0.1% w/v bovine serum albumin and 1% horse serum was gassed prior to use with 95% O_2, 5% CO_2.

Collagenase solution

Collagenase 25mg was dissolved in Hepes buffer, pH 7.4, 25ml. This was sufficient for one mouse liver perfusion, yielding approximately 40-50 x 10^6 hepatocytes. Before use, 0.5ml of a $CaCl_2$ solution (170mg/ml) was added and the solution gassed with 95% O_2, 5% CO_2.

(iii) TARM assay

TRIS buffer, pH 8.0

TRIS (0.605g) was dissolved in distilled water, the volume made up to 500ml and the pH adjusted to 8.0.

NaH_2PO_4 solution

NaH_2PO4 (78g) was dissolved in distilled water, (50ml). The pH was adjusted to 3.5.

Thiobarbituric acid solution

Thiobarbituric acid (0.250g) was dissolved in distilled water and the volume adjusted to 50ml.

(iv) Solutions for the preparation of liver fractions

0.25M sucrose

Sucrose (8.56g) was dissolved in distilled water and the volume adjusted to 100ml.

Potassium chloride 0.15M

Potassium chloride (5.60g) was dissolved in distilled water and the volume adjusted to 500ml.

NADPH generating system (Slater and Sawyer, 1971)

KCl 0.311g (excluded when TRIS/MALEATE buffer used)

Glucose-6-phosphate 0.0706g

$NADP^+$ 0.0094g

Glucose-6-phosphate dehydrogenase 13 i.u.

These reagents above were dissolved either in TRIS buffer, pH 8.0 or TRIS/MALEATE buffer, pH 7.4 (for calcium studies) and the volume adjusted to 50ml.

(v) $^{45}Ca^{2+}$ uptake studies solutions (Lowrey et al.,1981; Moore et al., 1975)

Sucrose 0.35M

Sucrose (11.98g) was dissolved in distilled water and the volume adjusted to 100ml.

Sucrose 0.88M

Sucrose (30.12g) was dissolved in distilled water and the volume adjusted to 100ml.

KCl-EDTA buffer, pH 7.4

KCl 5.74g

Na_2EDTA 0.558g

The reagents above were dissolved in distilled water and the volume adjusted to 500ml. The pH was then adjusted to 7.4.

TRIS-MALEATE buffer, pH 7.4

TRIS 0.606g

Maleic acid 0.580g

KCl 0.746g

The reagents above were dissolved in distilled water and the volume adjusted to 100ml. The pH was adjusted to 7.4.

Magnesium ATP solution, 5mM

Magnesium ATP (60mg) was dissolved in TRIS-MALEATE buffer, pH 7.4, 0.2ml. For each incubate (2.5ml) 0.02ml was added.

Imidazole-histidine buffer, pH 6.8

Imidazole	0.204g
Histidine	0.466g
KCl	0.746g

The reagents above were dissolved in distilled water and the volume adjusted to 100ml. The pH was adjusted to 6.8.

Imidazole-histidine calcium buffer (calcium buffer), pH 6.8

NaN_3	0.0163g (excluded from mitochondrial study)
$MgCl_2.6H_2O$	0.0509g
MgATP	0.1268g
NH_3 oxalate	0.0356g
Imidazole	0.1021g
Histidine	0.2328g
KCl	0.3728g

The reagents above were dissolved in distilled water and the volume adjusted to 50ml. The pH was then adjusted to 6.8

(vi) Cell Lysis Medium (Boyle, 1968)

NH_4Cl	14.94g
TRIS	4.12g

The reagents above were dissolved in distilled water and the volume adjusted to 2L. The pH was adjusted to 7.2.

(vii) Phosphate buffered glutaraldehyde solution 2%, pH 7.3, for cell fixation

A 25% glutaraldehyde solution in water was mixed with Millonig's buffer (Millonig et al., 1969) to form a 2% glutaraldehyde solution as follows:

Glutaraldehyde 25% w/v	12.0ml
$NaH_2PO4.\ 2H_2O$ 2.26% w/v	96.0ml
NaOH (2.52% w/v) approx.	19.0ml
Distilled water to	150ml

The pH was adjusted to 7.3.

SECTION 7

METHODS

7.I. Method of analysis for glutathione (reduced and oxidised)

A variety of assay methods have been reported for the determination of glutathione in tissues, including colorimetry (Beutler, 1963), fluorimetry (Hissin and Hilf, 1976), ion exchange cclumns (Tabor and Tabor, 1977), enzyme assays (Bernt and Bergmeyer, 1974; Tietze, 1969; Griffith, 1980) and high performance liquid chromatography (Reed et al, 1980; Jones et al, 1979). Of these, enzymatic methods were considered to be the most suitable in terms of specificity, stability of both reduced and oxidised glutathione during the assay, cost and availability of apparatus. The criteria for choice between suitable enzyme assays were enzyme specificity, comparative lack of inhibition by sample components, accuracy of both GSH and GSSG determination and a simple tissue extraction procedure.

Autoxidation of GSH to GSSG can be minimised by use of ice cold acidic deproteinising agents, followed by rapid assay. The majority of methods involved make use of glutathione reductase although glyoxalase was recommended by Bernt and Bergmeyer (1974). However glyoxalase is relatively unspecific for glutathione and is readily inhibited by S-methyl glutathione, (Vince et al, 1971), potentially a component of samples used in this study. GSSG levels are usually assumed to be the difference between GSH and total thiols determined, a relatively inaccurate procedure (McConnell et al, 1979). Sulphydryl group blocking agents such as N-ethyl maleimide (NEM) have been used to increase the specificity of enzymatic methods for GSSG (Tietze, 1969). However, rigorous removal of NEM is necessary as it is a potent glutathione reductase inhibitor (Guarnieri et al, 1979)

The method used by Tietze (1969) was modified by Griffiths (1980) by use of 2-vinylpyridine (2VP), instead of NEM, to block sulphydryl groups on the GSH molecule and so allow separate GSSG measurement (Figure 13a). On pretreatment of the sample with 2VP, and subsequent reaction of GSSG with glutathione reductase, GSH is liberated. The sulphydryl group on GSH reacts with DTNB and forms 2-nitro-5-thiobenzoic acid, which absorbs at 412nm (Figure 13b). Measurement of the rate of formation of this chromophore provides the basis for the assay.

Griffiths (1980) used a solution (10% w/v) of 5-sulphosalicylic acid to deproteinise tissues, followed by neutralisation with triethanolamine for optimum glutathione reductase activity and maximum electrophilic reactivity of 2VP. Owens and Belcher (1965) noted a marked inhibition of glutathione reductase in tissues extracted with sulphosalicylic acid and initial experiments carried out as part of this work, using standard glutathione solutions in 10% w/v sulphosalicylic acid, neutralised with triethanolamine, confirmed these findings. Deproteinisation with 10% (w/v) metaphosphoric acid, followed by neutralisation with saturated trisodium phosphate has been recommended (Oshino and Chance, 1976) and on trial this proved to be suitable. Therefore this procedure was used in all determinations of glutathione described.

Using a standard solution of glutathione, the crucial role of pH in this enzyme assay was elucidated. At pH values below 7.0 or above 7.5, no enzyme reaction was detectable. The pH of the final system was maintained at pH 7.0 - 7.5.

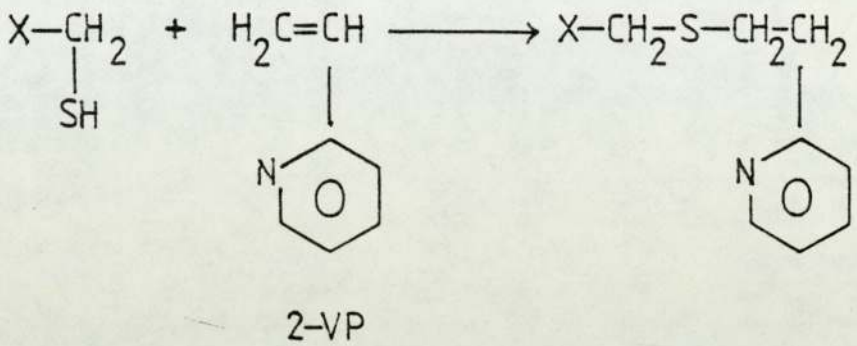


Figure 13A : Scheme showing the reaction of 2VP with SH group

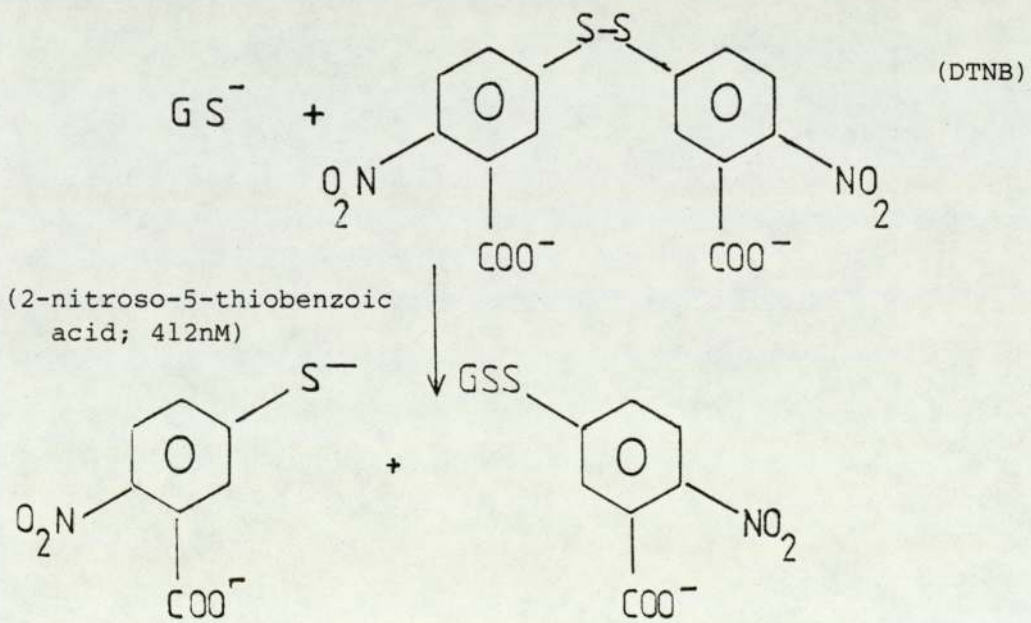


Figure 13B : Scheme showing the reaction of DTNB with SH group
(Tietze, 1969)

Experiments with known concentrations of GSH with and without 2VP addition revealed that the maximum SH blocking effect of 2VP did not occur before 20 minutes incubation at 30°C. With GSH concentrations ranging from 10-15μM, 85-95% of the sulphydryl groups reacted with 2VP. With similar concentrations of GSSG, a constant reduction of 10% of initial concentrations was noted when solutions were incubated with 2VP at 30°C for 20 minutes, which indicates that 2VP inhibits glutathione reductase to a minor extent.

Following preliminary investigations as described above, the following modification of the enzyme method was used:

Following addition of 10% (w/v) metaphosphoric acid to the sample and centrifugation, the supernatant (0.3ml) was pippetted into each of two plastic microcentrifuge tubes. To both, 0.12ml of a Na_3PO_4 solution (6.II) was added. To one tube, phosphate-EDTA buffer, 0.006ml, was added and to the other, neat 2VP, 0.006ml. Both tubes were then sealed and mixed thoroughly. After incubation for 20 minutes at 30°C in a water bath, tubes were centrifuged on a Beckmann microfuge for 2 minutes. The following reaction mixture was pippetted into a microcuvette (capacity 1.0ml, Hellmann Limited): the supernatant (0.142ml), DTNB solution (0.1ml), NADPH solution (0.70ml) and glutathione reductase suspension (0.01ml). After thorough mixing, the cuvette was placed in a temperature controlled cell holder at 30°C in a Cecil U.V. 5095 Spectrophotometer. A cuvette containing phosphate-EDTA buffer (0.71ml), DTNB (0.1ml), 10% (w/v) metaphosphoric acid (0.1ml) and Na_3PO_4 solution (0.04ml) was used as a reference. The change in absorbance at 412nm, with time, was recorded using the Cecil CE500 Scan Recorder. The initial rate of enzyme reaction,

calculated from the slope, was equivalent to the quantity of glutathione present. Glutathione levels determined from mixtures with or without 2VP were due to GSSG alone or total glutathione (GSH + GSSG) content respectively.

Standard solutions of GSH or GSSG were prepared in 10% (w/v) metaphosphoric acid and assays for glutathione were performed in order to produce calibration values. As expected, one mole of GSSG was found to produce 2 moles of GSH. Table 5 shows the calibration values for the initial rate of enzyme reactions.

Concentration (μM)	10	20	30	40	50
Rate of reaction (units/min)	0.072	0.171	0.269	0.395	0.520

Correlation coefficient = 0.995

Slope = 0.0105

Table 5 : Calibration of glutathione concentration (μM) against initial rate of enzyme reaction (units/minute)

Concentration (nmoles)	2.5	5.0	10.0	15.0	20.0	25.0
Absorbance at 532nm	0.136	0.291	0.572	0.829	1.176	1.449

Correlation coefficient = 0.999

Slope = 0.058

Table 6 : Calibration of malondialdehyde (nmole) against absorbance at 532nm

7.II The effect of incubation with N-methyl containing compounds on glutathione status in isolated mouse hepatocytes.

7.II.i Preparation of isolated mouse hepatocytes

Mouse hepatocytes were prepared according to the method of Renton et al (1978): Male BALB/c mice were anaesthetised with sodium pentobarbitone (200mg/kg) in saline, i.p. The inferior vena cava was canulated in a retrograde fashion via an incision in the right atria. The hepatic portal vein was cut to allow drainage of perfusion fluid. The liver was perfused with Hepes-EGTA solution for 1 minute, Hepes solution for 2 minutes, followed by collagenase solution for 5 minutes and finally by Hepes solution for a further 5 minutes. The liver was gently excised and hepatocytes were released from the capsule. After filtration through a nylon mesh, to remove debris and undigested connective tissue, hepatocytes were sedimented by standing on ice, washed and finally suspended in Krebs hepatocyte incubation mixture (6.III). Initial cell viability, measured by Trypan blue exclusion, was estimated at over 80%. Comparison of this method to assess viability with that by determining lactate dehydrogenase leakage (Leathwood and Plummer, 1969) gave similar results. The average number of hepatocytes per ml of incubation medium was 8-10 $\times 10^6$. The average yield of hepatocytes was 40-50 $\times 10^6$ cells per mouse liver. To check the glutathione assay in hepatocyte suspensions, standard solutions of GSH (25μM) and GSSG (10μM) were added to a sample cell suspension. Recovery of both standard solutions was over 90%.

7.II.ii Incubation conditions

Hepatocytes (2 x 10^6 cells per ml) were incubated in 2.5ml Krebs hepatocyte incubation mixture (6.III) at 37°C, with continuous gassing with a mixture of 95% O_2, 5% CO_2. The suspensions were contained in 25ml rubber stoppered, silanised conical flasks and were gently shaken. Compounds under study were added either in saline (0.9% w/v) or acetone (in the case of HMM) in volumes of 0.1ml. At the end of the incubation, the contents of each flask was transferred to centrifuge tubes and separation of cells and supernatant was achieved by standing on ice. A solution of 20% (w/v) metaphosphoric acid (0.5ml) was added to an equal volume of the supernatant and the cell pellet was suspended in 10% (w/v) metaphosphoric acid (0.1ml). After centrifugation, supernatants of each fraction were used for the glutathione assay (7.I). Incubations were carried out for not longer than 80 minutes. Compounds were incubated at the following concentrations: menadione (vitamin K) 2mM, formaldehyde 0.2mM, 2.0mM, HMM 2mM, NMF, DMF, HMF, F and D-NMF at 7mM. Control incubations contained either saline or acetone (control for HMM).

7.III The effect of incubation with NMF or HMM on the glutathione status in isolated tumour cells

7.III.i Tumour cells

PC6A, TLX5 or M5076 ascites tumour cells were obtained from female BALB/c, male CBA/Ca or female BDF_1 mice respectively. BDF_1 mice had been inoculated 10 days and BALB/c and CBA/Ca mice 7 days before cell harvesting with tumour cells as described by Gescher et al (1982). In these experiments cells were aspirated from the peritoneum and added to sterile saline 0.9% w/v. The cell yield was estimated using a Coulter Counter ZM. Cell suspensions were centrifuged in a Labofuge 6000 at Speed 5 for 5 minutes. PC6A or TLX5 cell pellets were then washed in Krebs incubation medium (6.III) and finally suspended in the medium such that the final concentration of cells was approximately 1.2×10^7 cells per ml. The large quantity of blood present with M5076 cells was removed by addition of cell lysis medium (6.III), 1ml, after the initial centrifugation, followed by mixing and standing for one minute. The suspension was recentrifuged on the Labofuge at speed 3 for 5 minutes. This procedure successfully lysed erythrocytes in the tumour cell suspension, leaving haemoglobin in the supernatant and M5076 cells intact in the pellet. Washing and resuspending procedures in Krebs incubation mixture was as for PC6A and TLX5 cells, with a final concentration of cells per ml of $0.8\text{-}1.2 \times 10^7$. Viability of all cells was estimated at over 95% by the Trypan Blue exclusion test.

7.III.ii Incubation conditions

Aliquots (2.5ml) of Krebs incubation mixture (6.III) containing tumour cells (2.0 - 3.0 x 10^7 cells) were incubated in covered silanised 25ml conical flasks, after gassing for 1 minute with 90% O_2, 10% CO_2. Flasks were shaken gently at 37°C for a maximum of 80 minutes. HMM 2mM, dissolved in acetone (0.1ml) was added to either the PC6A or M5076 tumour cells incubations. NMF 7mM, dissolved in saline (0.1ml) was added to either the TLX5 or M5076 incubation mixtures. Acetone or saline were added to control incubations respectively. At the end of each incubation, the contents of each flask were processed as described for hepatocytes. (7.II.ii).

7.IV The hepatic glutathione content in different strains of mice following repeated administration of NMF

Male BALB/c mice, female CBA/Ca mice, female CBA/Ca mice bearing TLX5 tumours and female BDF_1 mice bearing M5076 tumours (6.I) were injected i.p. with NMF 400mg/kg, dissolved in saline, daily for five consecutive days. 24 Hours after the 5th dose, animals were killed by cervical dislocation. Livers were rapidly excised, blotted, weighed, minced and homogenised in 10% (w/v) metaphosphoric acid (40ml/g liver). After centrifugation, aliquots of the supernatants were assayed for glutathione content (7.I).

7.V Assay for evidence of lipid peroxidation (Thiobarbituric acid reactive material, TARM)

Aldehydic material which reacted with thiobarbituric acid (TARM) was assayed spectrophotometrically according to the method of Fong (1973), with the following modification:

Hepatocyte or liver fraction suspensions were deproteinised by addition of 70% w/v trichloroacetic acid (final concentration 7% w/v). Aliquots (1.0ml) of the supernatant were adjusted to pH 3.5 by addition of a concentrated solution of sodium dihydrogen phosphate (6.III), 0.5ml. This pH has been described as optimum for the thiobarbituric acid reaction (Ohkawa et al, 1979) and also prevented the hydrolysis of NMF to form yellow products which would have interfered with the assay. Thiobarbituric acid 0.5% (w/v) was added to the mixture (1.0ml) and after mixing, the mixture was heated at 60°C for 60 minutes. After cooling, the absorbance at 532nm was determined using the Cecil U.V. CE5095 Spectrophotometer. The reference solution contained 7% (w/v) trichloroacetic acid (1.0ml), NaH_2PO_4 solution (0.5ml) and thiobarbituric acid (1.0ml), mixed together and heated as for the test mixture. MDA solutions were prepared as standards (Table 6). TARM values were expressed as nmoles MDA equivalents per 10^6 cells or mg protein or g liver.

7.VI The study of TARM in isolated mouse hepatocytes on incubation with NMF and related formamides

Mouse hepatocytes were isolated and incubated as described in 7.II, for a maximum of 240 minutes. NMF, DMF, HMF, F or D-NMF, in saline, at a concentration of 7mM were added to incubations. Control additions were of 0.9% w/v saline. In some incubations, hepatocytes were preincubated for 30 minutes with either DEM, 0.340μM, in acetone to deplete cells of glutathione, or acetone, before addition of NMF 7mM or saline. In other incubations, hepatocyte suspensions containing NMF 7mM or saline were co-incubated with either 0.5mM or 1mM ascorbic acid for up to 4 hours. For other incubations, hepatocytes were isolated from male BALB/c mice that had been pretreated with either phenobarbitone or saline, in order to induce mixed function oxidase enzymes (2.III). TARM was determined as described in 7.V.

7.VII The study of TARM in liver fractions on incubation with NMF

7.VII.i. Preparation of liver fractions

Male BALB/c mice were anaesthetised with sodium pentobarbitone (200mg/kg i.p.) and livers were perfused with Tris buffer 10mM, pH 8.0, to eliminate haemoglobin from the tissue as this interferes with the TARM assay. Livers were excised, blotted, weighed, minced and homogenised in ice cold sucrose 0.25M (10% w.v). Homogenates were centrifuged at 700g for 10 minutes to remove nuclei and cell debris. The supernatants were centrifuged at 10,000g for 10 minutes at 2°C using the MSE Pegasus 65 Ultracentrifuge. After washing, the pellet formed was suspended in 0.15M KCl and was used for the mitochondrial determinations. The post mitochondrial supernatant was centrifuged at 100,000g for 90 minutes at 2°C and after washing, the pellet formed was suspended in 0.15M KCl and was used for microsomal determinations.

7.VII.ii Incubation conditions

Microsomal or mitochondrial fractions from several animals were pooled so that the final solution contained a microsomal or mitochondrial fraction equivalent to 1g original liver weight per ml. A NADPH-generating stock solution in Tris buffer (6.III), as described by Slater and Sawyer (1971), was mixed with part of the suspension of liver. Aliquots (2.5ml) of the mixture (containing liver fractions equivalent to approximately 0.3g liver) were added to 25ml open beakers and incubated for a maximum time of 60 minutes, at 37°C, with shaking. Control incubations without the NADPH-generating system or without the liver fraction suspension were also prepared. To each flask was added either NMF 7mM or saline in volumes of 0.020 ml. Microsomal or mitochondrial protein was determined according to Lowry et al, (1951). TARM was determined as described in 7.V.

7.VIII The effect of NMF and related formamides in vivo and in vitro on Mg^{2+}-ATP dependant Ca^{2+} uptake in liver mitochondria or microsomes

7.VIII.i. Conditions for Ca^{2+} uptake in microsomes and mitochondria

The method for measurement of Ca^{2+} uptake in microsomal suspensions was a modification of that of Moore etal (1975), adapted by Lowrey et al (1981). Livers were homogenised in ice cold KCl/EDTA buffer, pH 7.4 (10ml/g liver), using 6 strokes of a cooled Teflon glass Potter-Elvehjem homogeniser, speed 5. The homogenate was centrifuged at 3000g for 10 minutes in the MSE Pegasus 65 Ultra-centrifuge at 4°C. The supernatant was centrifuged at 80,000g for 30 minutes at 4°C. The microsomal pellet formed was suspended in ice cold imidazole-histidine buffer, pH 6.8 (1ml) or ice cold Tris-maleate buffer, pH 7.4 (3ml) for the in vivo or in vitro experiments respectively.

For the in vitro experiments, the microsomal suspension was divided into two portions. One portion (1.5ml) was mixed with Tris-maleate buffer, pH 7.4 (12.5ml) and the other portion (1.5ml) was mixed with Tris-maleate buffer containing NADPH generating cofactors (12.5ml), as described in 6.III. Aliquots of each mixture (2.5ml) were added to 25ml silanised conical flasks. To each was added magnesium ATP 5mM. At the end of the incubation time, microsomes were recovered by centrifugation at 80,000g for 30 minutes on the Pegasus Ultracentrifuge. The microsomal pellet was resuspended in ice-cold imidazole-histidine buffer, pH 6.8 (1ml).

For both the in vivo and in vitro experiments, the microsomal suspension was assayed for uptake as described by Moore et al (1975).

Protein was determined according to the method of Lowry et al (1951). Microsomal suspensions at a protein concentration of 0.1-0.15mg per ml (usually 0.1ml) were added to 0.9ml calcium buffer, pH 6.8, (6.III) and preincubated for five minutes at 37°C, with shaking. The reaction was initiated by addition of Ca^{2+} solution, 0.01 ml, containing 0.1μCi $^{45}Ca^{2+}$ and 20μM Ca^{2+}. At various times up to 75 minutes, the microsomal suspension was filtered under vacuum on to a Millipore filter (0.45μ). After rinsing with imidazole-histidine buffer, pH 6.8, the filters were air dried and immersed in NE260 Scintillant (5ml). Radioactivity was counted on a Packard Tricarb 2660 liquid scintillation counter. Calcium uptake was expressed as either counts per minute (cpm) per mg protein or as a percentage of the control value (cpm/mg protein) at that time point.

Mitochondrial fractions were prepared as described by Landon (1967). Livers were homogenised in ice cold sucrose 0.35M (4mls/g liver) using 6 strokes of a cooled Teflon-glass Potter-Elvehjem homogeniser at speed 5. The homogenate was centrifuged at 500g for 10 minutes at 4°C on the MSE HiSpin 21 Ultracentrifuge. The supernatant was centrifuged at 1935g for 10 minutes at 4°C. The sediment formed was resuspended in ice cold sucrose 0.88M (3ml) and centrifuged at 7700g for 10 minutes at 4°C. The mitochondrial pellet was gently suspended in either imidazole-histidine buffer, pH 6.8 (6ml) or Tris-maleate buffer, pH 7.4 (3ml) for in vivo and in vitro experiments respectively.

The remaining procedure for calcium uptake was identical to that for microsomal suspensions with the exception that azide was ommitted from the calcium buffer mixture and the incubation time for $^{45}Ca^{2+}$ uptake did not exceed 30 minutes.

7.VIII.ii Incubations and dosage conditions

For in vitro experiments, microsomal or mitochrondrial suspensions in Tris-maleate buffer were incubated with either NMF 7mM in saline or saline (0.020ml) for up to 60 minutes, at 37°C with shaking. After incubation, the procedure as described in 7.VIII.i. was followed. For the in vivo microsomal experiments, male BALB/c mice were injected i.p. with either NMF 400mg/kg in saline or carbon tetrachloride (CCl_4) in arachis oil (2.5ml/kg). Control animals were injected with either saline or arachis oil. Animals were killed by cervical dislocation at 0.5, 1, 2 hours or 1 hour after dosing for NMF or CCl_4 treated animals respectively. For in vivo mitochondrial experiments, male BALB/c mice were injected i.p. with either NMF, DMF, HMF or F 6.8mmol/kg in saline. Control animals received saline injections. Diethylmaleate (DEM) 0.7ml/kg in acetone-arachis oil (1:9) was injected i.p. into some mice to deplete hepatic glutathione. Control animals received acetone-arachis oil injections. Animals were killed by cervical dislocation 1 hour after treatment. Livers in all in vivo experiments were prepared as described in 7.VIII.i.

7.IX Ultrastructural changes in isolated mouse hepatocytes following incubation with hepatotoxins

7.IX.i Incubation conditions and pretreatment of animals.

Male BALB/c mice were pretreated with either PB or saline as described in 2.III. Hepatocytes were isolated from both treatment groups and incubated as described in 7.II.i and 7.II.ii. NMF 7mM, dissolved in saline, was added (0.1ml) either to hepatocytes from PB (1 hour or 4 hour incubations) or from saline pretreated animals (1, 2, 4 hour incubations). t-Butylhydroperoxide (tbh) 4mM, dissolved in saline, was added (0.1ml) either to hepatocytes from PB or saline pretreated mice (0.5 or 1 hour incubations). Saline (0.1ml) was added to control incubations.

7.IX.ii Scanning electron microscopy details.

After each incubation, cells were sedimented on ice and the supernatant discarded. Cells were fixed directly by the addition of a 2% solution of glutaraldehyde in Millonig's buffer, pH 7.3 (5ml) (6.III). The hepatocytes were washed twice with deionised water and dried at room temperature on grids and sputter coated with gold. A Jeol model JEM-100 CX II scanning electron microscope was used to visualise and photograph hepatocytes.

7.X. Characterisation of NMF hepatotoxicity in vivo

7.X.i Histopathological examination.

Details of strains of mice, tumours, dose of and duration of treatment with i.p. NMF, in saline, for this study are shown in Table 7. Tumours were passaged and maintained as described by Gescher et al (1982). Animals were weighed at the beginning and end of the study. For the study of the recovery of animals after NMF, animals were weighed daily. After death, the livers were excised, blotted, weighed and examined for gross anatomical changes. Livers to be studied histopathologically were immediately fixed in Bouin's Fluid (Drury and Wallington, 1980). Twenty four hours after fixing, livers were rinsed and stored in 70% v/v alcohol. Sections were embedded in paraffin wax and stained with haemalum and eosin for microscopical examination.

7.X.ii Assays for plasma liver enzymes.

Male BALB/c mice dosed i.p. with NMF, in saline, at 400mg, 200mg, 100mg/kg singly or with 200mg or 100mg/kg daily for 5 days were anaesthetised with pentobarbitone sodium (200mg/kg) i.p., 24 hours after the last dose. Male BALB/c mice, injected with a single dose of 400mg/kg NMF i.p., were anaesthetised at 4,6,8,10,12, 24 hours or 7 days post dose. Blood samples (1ml) were collected in syringes containing 0.05ml sodium heparin (15mg/ml), after cardiac puncture. Plasma was obtained by centrifugation in a Beckmann Microfuge. Plasma SDH levels were determined spectrophotometrically according to the method of Rose and Henderson (1975). Plasma ALT and AST levels were assayed according to Kachmar and Moss (1976).

Strain of mouse	Sex of mouse	Tumour	Dose NMF mg/kg	Duration of treatment	Time of death
CBA/Ca	F	-	400	Daily x 5	24 hours last dose
CBA/Ca	F	TLX5	400	Daily x 5	24 hours last dose
BDF_1	F	-	400	Daily x 5	24 hours last dose
BDF_1	F	M5076	400	Daily x 5	24 hours last dose
BALB/c	M	-	400	Daily x 5	24 hours last dose
BALB/c	M	-	200	Daily x 5	24 hours last dose
BALB/c	M	-	100	Daily x 5	24 hours last dose
BALB/c	M	-	400	Single	24 hours post dose
BALB/c	M	-	400	Single	Up to 24 hours
BALB/c	M	-	400	Single	Recovery post dose
BALB/c	M	-	200	Single	24 hours post dose
BALB/c	M	-	100	Single	24 hours post dose

Table 7 : Experimental details of histopathology study

(For all experiments, control animals were dosed with saline; F = female; M = male).

7.X.iii TARM in liver fractions.

TARM was determined in liver fractions of all animals described in 7.X.ii, with the exception of those used for the time points prior to 24 hours after the dose of NMF. In addition, TARM in liver fractions of male BALB/c mice was estimated 1 hour after a single dose of NMF 400mg/kg. Microsomal and mitochondrial liver fractions were prepared as described in 7.VII.i. In addition, the post microsomal fraction was used as the cytosolic fraction. TARM was determined in each fraction, suspended in 0.15M KCl, as described in 7.V.

SECTION 8

RESULTS

8.I The effect of incubation with N-methyl containing compounds on the glutathione status in isolated mouse hepatocytes.

8.I.i Introduction

Glutathione plays an important role in drug metabolism and toxicity (5.II). In general, compounds which form chemically reactive species following hepatic metabolism are capable of depleting livers of glutathione (Plummer et al, 1981). It has been shown previously that administration of a single injection of NMF to mice produced an acute depletion in liver non-protein thiols (Gescher et al, 1982). This effect was specific for NMF when compared with other formamides, was dose related and it was postulated that oxidative metabolism of NMF to produce a reactive metabolite was responsible for the effect. As glutathione depletion has been implicated in the hepatotoxicity of many compounds (5.II), this result was considered to be relevant to the liver damage induced by DMF and NMF.

To investigate if similar effects occurred in vitro, the glutathione status of isolated mouse hepatocytes exposed to either NMF, DMF, HMF or F at equimolar doses, was determined (7.II). D-NMF was also investigated in order to examine whether a kinetic isotope effect existed in this class of molecules. Deuterium substitution has been used previously to slow the rate of oxidative metabolism of many different substrates (Sipes et al, 1980; Dagani and Archer, 1976). It is believed that the C-deuterium bond is stronger than the C-H bond (Morrison and Boyd, 1966) and therefore metabolic breakage of the C-deuterium bond is slower than for the C-H bond. If metabolism of the C-H bond was responsible for any of the effects seen with NMF, an alteration would be anticipated on deuteration. The concentration

of formamides used in incubations (7mM) was the peak plasma concentration of NMF achieved after a single dose of NMF 400mg/kg to mice (Brindley et al, 1982b).

Formaldehyde, formed by oxidative demethylation and a known product of HMM metabolism and claimed to be a metabolite of NMF (I.II) reacts readily with glutathione, producing S-hydroxymethylglutathione in hepatocytes (Uotila et al, 1974). It has been shown that substrates of cytochrome P-450 that yield formaldehyde, such as benzphetamine and aminopyrine, deplete glutathione in hepatocytes whilst those that did not yield HCHO such as hexabarbital, had only a minor effect on glutathione levels (Jones et al, 1978). The influence of HMM on the glutathione status in hepatocytes was therefore investigated. Formaldehyde was also added to hepatocyte incubations as a positive control.

The isolated hepatocyte preparation usually contains drug-metabolising enzymes in the same proportion and range as occur in vivo and therefore results obtained from experiments using hepatocytes are more relevant to metabolism and toxicity of compounds in vivo, than results obtained with other in vitro systems such as isolated liver fractions. These preparations may often give a misrepresentation of total drug metabolism as certain enzymes are lacking (Fry and Bridges, 1979). By using isolated mouse hepatocytes, cellular changes in glutathione status and flux together with toxicological effects can be studied. However, unlike rat hepatocytes, mouse liver cells have not been used extensively in studies of this kind. Therefore, the influence of an agent known to disturb glutathione homeostasis in rat hepatocytes, menadione (vitamin K), was investigated in mouse liver cells. Eklöw et al (1981) reported that menadione depleted intracellular GSH and increased extracellular GSSG in rat hepatocytes.

In order to validate the comparability of effects observed in rat and mouse hepatocytes, a similar experiment was performed in this study with mouse liver cells (7.II).

8.I.ii Results

Glutathione levels in hepatocytes immediately after isolation, were found to vary considerably, unrelated to slight differences in cell viability, but apparently dependent on the batch of collagenase used. For the majority of experiments, expression of results as the percentage of the value at the initial zero time point was therefore chosen. In some cases where the depletion was so rapid that the initial value was obviously reduced from the control, results were expressed as the percentage of the value of the control hepatocyte incubation at the relevant time point. Intracellular glutathione concentration was measured as 19.41 ± 7.93 nmoles per 10^6 cells in 19 experiments.

Figure 14 shows the intra- and extracellular glutathione levels, from one experiment (representative of two), in which mouse hepatocytes were incubated with menadione 2mM for up to 20 minutes. A dramatic decrease in intracellular GSH with a corresponding increase in extracellular GSSG was seen in menadione incubations when compared with control incubations. Intracellular GSSG rose initially and then remained at a relatively steady level. Extracellular GSH declined rapidly to negligible amounts.

Formaldehyde 0.2mM produced no marked effect on glutathione levels when incubated with mouse hepatocytes. However, at 2mM, HCHO reduced total intracellular glutathione levels after incubation for 80 minutes to 49.9% of the levels in the control incubations for the

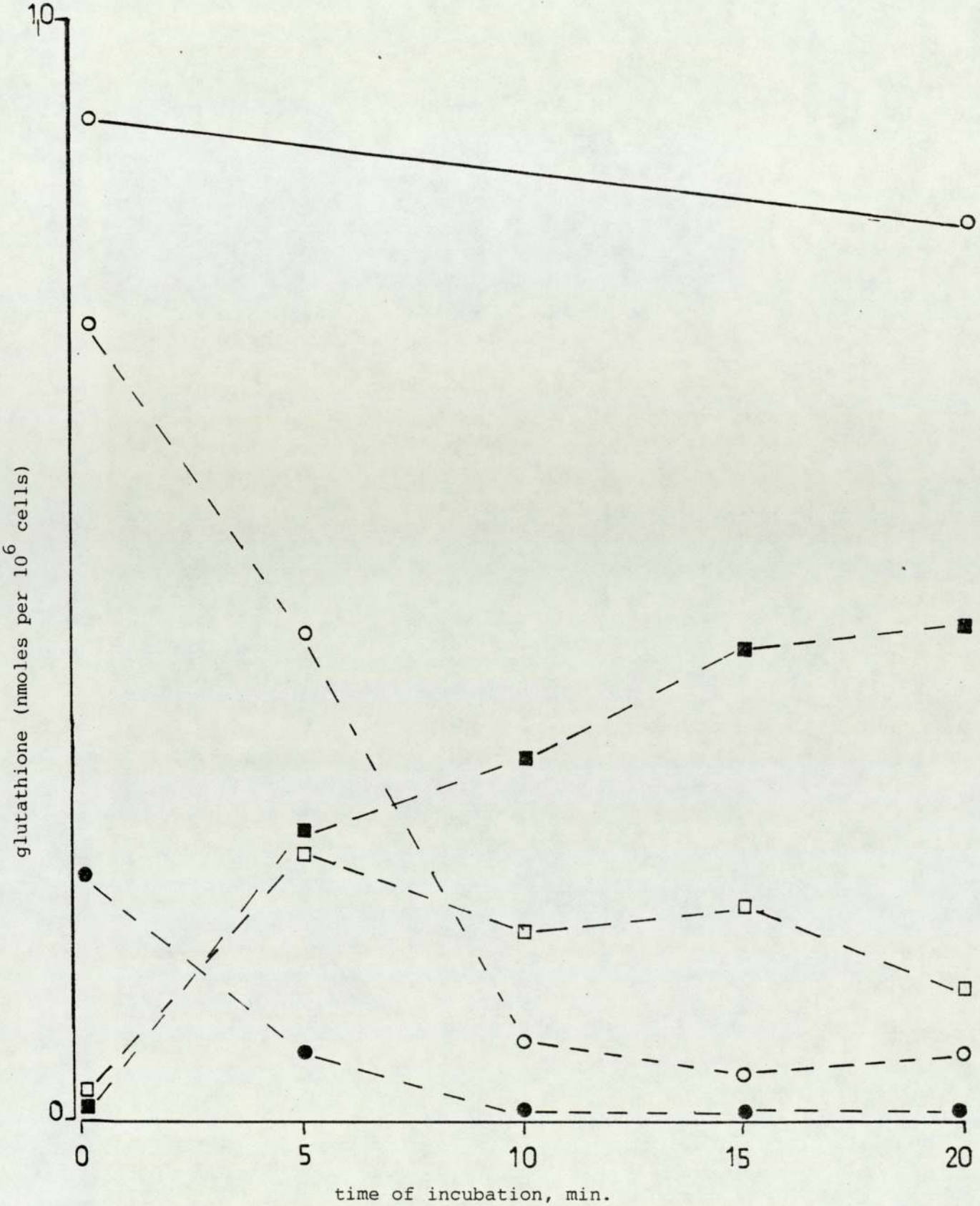


Figure 14 : Intracellular (○--○ gsh, □---□ gssg) and extracellular (●—● gsh, ■···■ gssg) glutathione levels in isolated mouse hepatocytes incubated with menadione 2mM and intracellular gsh (○—○) levels in saline incubations.

same time point. Results of experiments of incubation of hepatocytes with HMM 2mM are given in Table 8. The percentage change in glutathione levels for HMM or saline incubations varied a great deal between experiments. Using a paired 't' test, no significant difference was noted between HMM or saline incubations.

Figure 15 shows the marked decrease in total intracellular glutathione in hepatocytes incubated with NMF 7mM for up to 80 minutes, when compared with control incubations. At the 80 minute time point results were statistically different ($p < 0.001$). In a separate experiment, incubations of hepatocytes with NMF at concentrations from 7mM to 100mM did not lead to a marked decease in cell viability when compared with control incubations and when estimated by the Trypan blue exclusion method.

With the 7mM NMF incubations, the extracellular concentration of glutathione did not change significantly within the incubation period for either control or NMF incubations. Similarly for both control and NMF incubations, extracellular glutathione consisted almost exclusively of GSH at the beginning and almost entirely of GSSG at the end of the experiments. Initially intracellular GSSG was negligible but levels increased to 2.05 ± 1.42 nmoles per 10^6 cells (8.6% of the total intracellular GSH + GSSG, n = 5) after 80 minutes in control incubations and to 1.85 ± 0.24 nmoles per 10^6 cells (19.5% of total GSH + GSSG, n = 7) after 80 minutes incubation with NMF. Therefore, for NMF incubations, the reduction in total intracellular glutathione was mainly due to a decrease in GSH.

Figure 16 shows the total intracellular glutathione content (expressed as a percentage of the value initially) after 80 minutes

Total intracellular glutathione	
SALINE	HMM
103.49	100.00
43.47	39.16
97.46	57.70
52.52	23.49
66.35	45.28

Table 8 : Glutathione levels in isolated mouse hepatocytes after incubation with HMM 2mM for 80 minutes. (expressed as the percentage of glutathione value at the beginning of incubation; each HMM result is paired with the corresponding control for that experiment)

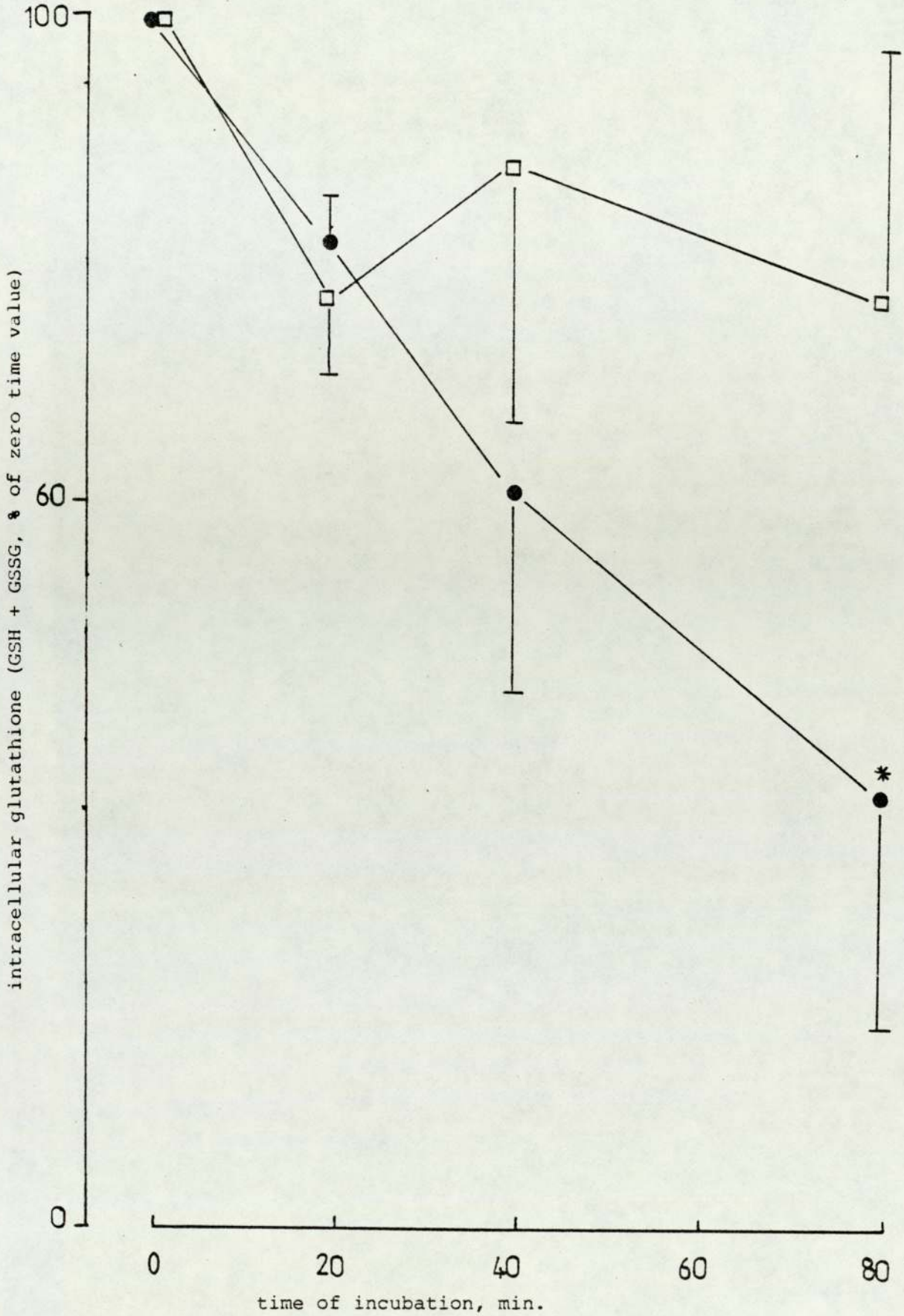


Figure 15 : Total intracellular glutathione content of isolated mouse hepatocytes incubated with NMF 7mM (●) or saline (□) for up to 80 minutes.
(asterix denotes a significant difference between values)

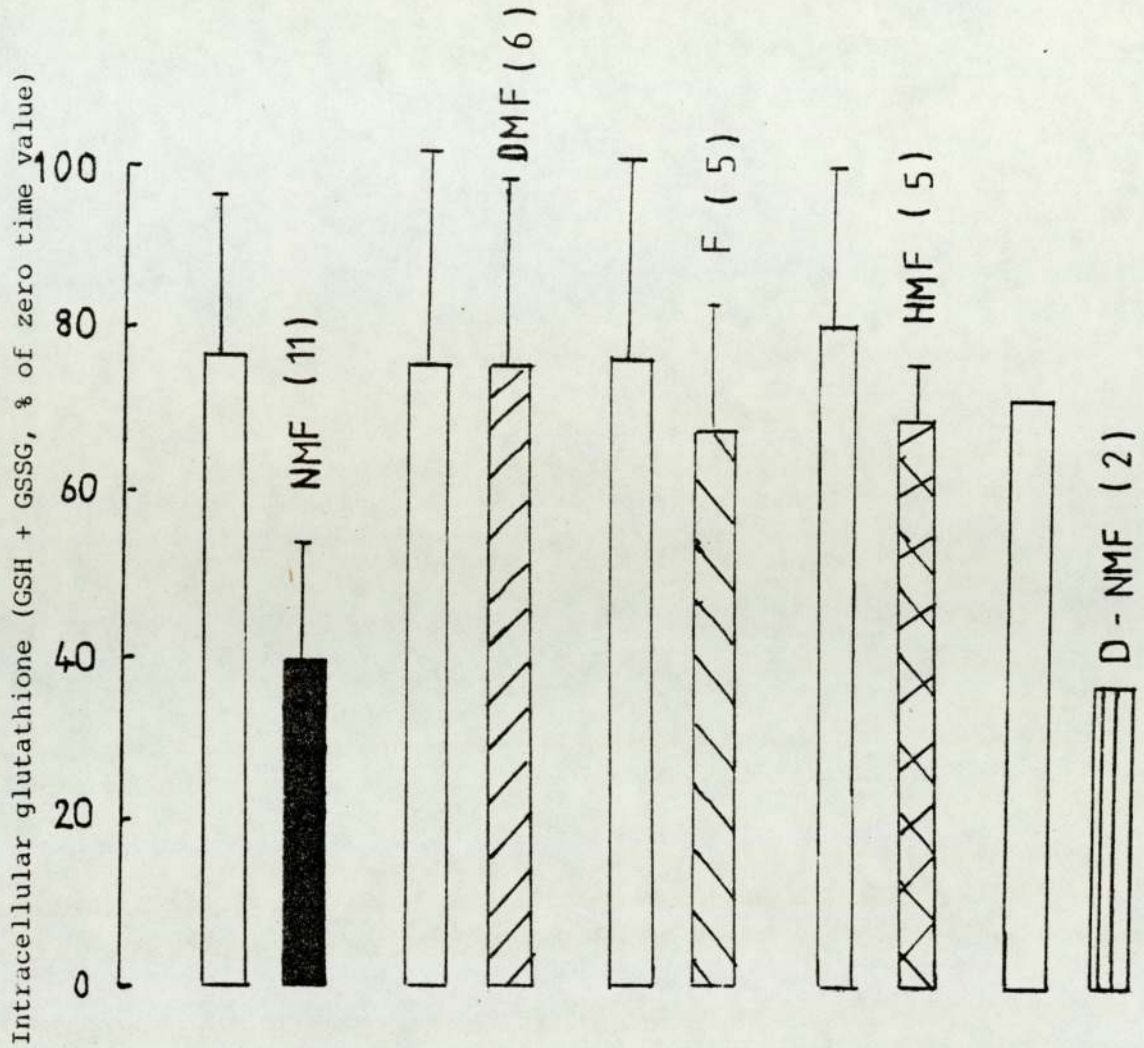


Figure 16 : Total intracellular glutathione in isolated mouse hepatocytes after 80 minutes incubation with either 7mM NMF, DMF, F, HMF, D-NMF or saline

incubation with either NMF, DMF, F, HMF, D-NMF or saline. It is apparent that only NMF produced a significant reduction in glutathione content of the hepatocytes when compared with the other formamides. Results from two experiments using D-NMF were almost identical to those obtained with NMF.

8.I.iii Discussion

Incubation of isolated mouse hepatocytes with either menadione 2mM or formaldehyde 2mM produced disturbances of GSH homeostasis which were similar to those reported previously for the rat. For menadione, the increase in extracellular GSSG and the decrease in intracellular GSH confirm the results of Eklöw et al (1981). Jones et al (1978) found that incubation of rat hepatocytes with HCHO 2mM for 1 hour resulted in a depletion of total glutathione in the cell to 44-52% of the control value at that time. This result is not unlike that found in this study for an 80 minute incubation with mouse hepatocytes.

The result of incubation of hepatocytes with HMM suggests that there is no evidence for a reaction of metabolites of HMM with glutathione within 80 minutes and therefore it may be assumed that any glutathione conjugate formation with HMM is limited. It has been shown previously that only traces of glutathione conjugates of metabolites of HMM are detectable in the bile of the rat, even though N-hydroxymethyl-PMM, the carbinolamine metabolite of HMM, reacts non-enzymatically with GSH (Gescher, unpublished). Hydroxymethyl metabolites of HMM (see Figure 2) are relatively stable metabolic intermediates and it may be that they degrade to formaldehyde at a very slow rate so that

it is immediately and efficiently oxidized further to formate. Alternatively, it could be argued that under the experimental conditions, carbinolamine metabolites of HMM are stable but possess low reactivity as electrophiles.

It is interesting that the depletion of glutathione caused by NMF in hepatocytes (Figure 15) occurred only after incubation for more than 1 hour. Glutathione levels in isolated hepatocytes are rapidly reduced by electrophilic agents such as diethylmaleate, which avidly undergo chemical and enzyme catalyzed reactions with glutathione. Compounds which are metabolised to electrophilic species, such as paracetamol, deplete hepatocytes of glutathione more gradually (Högberg and Kristoferson, 1978). The formation of an electrophilic metabolite is therefore suggested by the gradual depletion of glutathione in hepatocytes. It is conceivable that metabolites of NMF could deplete stores of glutathione in the liver by other mechanisms. Glutathione biosynthesis can be inhibited by xenobiotics (Griffiths, 1981). Drug-induced conversion of GSH to GSSG would also lead to depletion of GSH. Finally, agents like aspirin appear to deplete hepatic glutathione as a result of an increased leakage of GSH from hepatocytes (Kaplowitz et al, 1980). As GSSG levels are not increased in hepatocyte suspensions, it is unlikely that a redox process is involved in the NMF induced glutathione depletion. Furthermore, it is unlikely that NMF increases the efflux of GSH out of the hepatocytes as extracellular GSH levels did not differ significantly between NMF and control incubations.

Incubation of two possible metabolites of NMF, F and HMF, did not produce a similar depletion in glutathione (Figure 16). On the other hand, D-NMF gave an almost identical result for glutathione

depletion as NMF. These results suggest that an as yet unknown metabolite of specifically NMF is responsible for the delayed effect of NMF on hepatocyte levels of glutathione and this effect is not dependent on the rate at which C-H bonds in the NMF molecule are metabolically broken.

It is also unexpected that DMF, a well known hepatotoxin (Matthew et al, 1980; Unger et al, 1976) did not have an effect on glutathione levels in hepatocytes, but it may well be a much less potent toxin than NMF, a contention for which there is some evidence (Scailteur et al, 1981). Alternatively, DMF may cause hepatotoxicity via a mechanism different from the one operative for NMF.

8.II The effect of incubation with NMF or HMM on the glutathione status in isolated tumour cells.

8.II.i Introduction

The depletion of intracellular glutathione levels in hepatocytes following incubation with NMF (8.I) and the toxicological implications of this result suggest that a similar effect on glutathione levels in those tumour cells sensitive to NMF may be associated with the toxicity of the drug to cancer cells. It is also possible that the toxicity of NMF and HMM may be due in part to metabolism of the compounds by the tumour cells themselves. For these reasons, the effect of NMF or HMM incubation on the glutathione status in isolated tumour cells, sensitive to these agents, was investigated (7.III). Cells were incubated for up to 80 minutes as with hepatocytes. It was also possible to determine the base levels of glutathione in each tumour cell type. Similar information for other tumour cell lines has been found to be useful in studies of the resistance of tumours to a number of agents (Connors, 1966; Poynter, 1970).

8.II.ii Results

The total intracellular glutathione levels determined in each tumour cell type, with or without NMF 7mM or HMM 2mM are shown in Table 9, expressed as % of the value at the beginning of each incubation. For all experiments no GSSG was detectable. It is seen that incubation with either NMF or HMM produced no marked change in glutathione cells in any tumour cell type when compared with control incubations. The mean glutathione levels in untreated tumour cells were estimated to be 2.01 (± 0.44, n = 5), 0.56 (± 0.13, n = 6) and 1.90 (± 0.52, n = 6) nmoles glutathione per 10^6 cells respectively.

	Compounds in incubate			
Tumour cells	HMM	ACETONE	NMF	SALINE
PC6A	82.2± 6.3 (3)	83.7± 4.6 (3)	-	-
TLX5	-	-	59.6 (2)	65.1 (2)
M5076	46.2± 12.7 (4)	40.0± 9.9 (4)	36.5 (2)	30.9 (2)

Table 9 : Total intracellular glutathione levels in tumour cells PC6A, TLX5, M5076 following incubation for 80 minutes with or without HMM 2mM, NMF 7mM, acetone or saline.
(expressed as % of initial value; numbers in brackets denote number of experiments).

8.II.iii Discussion

The lack of effect of HMM on glutathione levels in both PC6A and M5076 cell incubations after 80 minutes suggests that formation of a glutathione conjugate of an HMM metabolite was unlikely or occurred to a very minor unmeasurable extent. It thus appears that glutathione status may not be relevant to the mechanism of action of HMM in these tumour cells. The lack of effect on glutathione levels in incubations of TLX5 or M5076 cells with NMF 7mM differed from the effect in mouse hepatocytes (8.I), for the same concentration of drug and time of incubation. This result has several implications. Firstly it may indicate that NMF does not undergo metabolism in tumour cells to the reactive metabolite that was suggested to be responsible for the glutathione depletion in hepatocytes. Secondly, it also suggests that the toxicity of NMF in TLX5 and M5076 cells is not due to the same mechanisms as the hepatotoxicity of the drug, if the latter toxicity is considered to be closely associated with hepatic glutathione depletion, as discussed in 5.II. It must also be pointed out that 80 minutes may have been too short an incubation time for an effect to become apparent in these experiments. Tumour cells are more able to survive long incubations with no loss of viability, than hepatocytes. It may be that the metabolising capacity of tumour cells is lower than that of hepatocytes and a longer contact time between cells and drugs may be required for adequate and toxic levels of reactive metabolites to be formed.

It is noticeable from Table 9 that levels of glutathione in control incubations of all three tumour cells were reduced by varying degrees after incubation for 80 minutes. PC6A cells appear to lose less glutathione than TLX5 and these less than M5076 cells. This

suggested that glutathione leakage from M5076 cells was greater than for the other cells, with no corresponding decrease in cell viability. This may have been due to the extra preparative step in the isolation of the M5076 cells for incubation (7.II), which may have predisposed the cells to a greater efflux of glutathione from the cell than under normal circumstances. However, one cannot exclude that there are differences in permeabilities in the cell membrane between the PC6A, TLX5 and M5076 cells.

Comparison of the glutathione content in each tumour cell type revealed similar levels for PC6A and M5076 cells but a much lower level for M5076 cells immediately after isolation. The lower level of glutathione in TLX5 cells, together with the knowledge that glutathione has an important role to play in the defence of the cell against oxidiative stress (5.II), could suggest that agents which produce free radical intermediates and initiate peroxidative reactions may be useful agents against the TLX5 cells. It is conceivable that the difference in glutathione content of PC6A and TLX5 tumour cells may be relevant to their different sensitivites to NMF.

8.III The hepatic glutathione content in different strains of mice following repeated administration of NMF

8.III.i Introduction

In a previous study, it was shown that administration of a single dose of NMF to mice caused an acute depletion of non-protein thiols in the liver (Gescher et al, 1982). This may have implications on both the metabolism and toxicity of NMF in mice (5.II, 5.III). The optimum antitumour regimen for NMF against a number of murine tumours involves repeated administration of the drug over several days (Gescher et al, 1982). Therefore it was considered pertinent to investigate the effect of repeated dosing of NMF on the hepatic levels of total glutathione in mice (7.IV). A dose of 400mg/kg daily for 5 days (the optimum regimen against the TLX5 lymphoma) was used and the animals were killed 24 hours after the last dose of NMF. Levels of hepatic glutathione in mice which were depleted one hour after a single dose of 100mg/kg NMF returned to normal values 15 hours after the dose (Gescher, unpublished). It was hypothesised that repeated dosing of NMF could produce a cumulative depletion of glutathione levels probably with subsequent exacerbation of hepatotoxicity.

Several strains of mice were used in this study. The previous investigation in which single doses of NMF were administered had involved the use of CBA/Ca mice. For comparison with that study, CBA/Ca mice were used in this investigation. NMF is active against the murine tumours TLX5 (grown in CBA/Ca mice) and M5076 (grown in BDF_1 mice). The presence of a tumour can alter the metabolism of drugs (1.II) and possibly the levels of hepatic glutathione. It was considered useful to perform this experiment in animals with and without

tumours for two reasons. Firstly to determine if repeated doses of NMF affected hepatic glutathione levels in animals with tumours which are sensitive to the drug and secondly to determine if, in the case of CBA/Ca mice, the presence of the TLX5 tumour affected glutathione levels when compared with animals without tumours. A third strain of mice, BALB/c, was also used as this was the strain used for NMF toxicity studies and as the donor of hepatocytes (7.II).

8.III.ii Results

Levels of hepatic glutathione determined 24 hours after the fifth of five daily i.p. injections of 400mg/kg NMF to mice of all the strains investigated are shown in Table 10. It can be seen that in no strain did NMF at repeated doses cause a long-lasting disturbance in glutathione status. Similarly the presence of the TLX5 tumour in CBA/Ca mice did not appear to affect levels of hepatic glutathione when compared with animals without tumours.

8.III.iii Discussion

The lack of cumulative depletion of hepatic glutathione levels shown in this experiment does suggest that if the effect seen 1 hour after a single dose (Gescher et al, 1982) is due to an electrophilic metabolite of NMF which is conjugated with GSH in the liver, subsequent biosynthesis of new GSH fairly rapidly repletes non-protein thiol sites. Depletion of glutathione by NMF seems to be transient with levels being restored to normal within hours of dosing with no apparent long lasting disturbance in glutathione biosynthesis. This result is all the more remarkable in that this dose of NMF caused

extensive damage in some animals. It is also obvious that the presence of the TLX5 tumour did not affect liver glutathione levels in CBA/Ca mice. Differences in hepatotoxicity between strains are discussed in section 8.VIII. BALB/c mice appeared to be more susceptible to the toxic effects of NMF and most of the livers of NMF treated BALB/c mice showed damage. It has been shown previously that there appears to be a strain difference in toxicity to NMF as the LD10 value for BDF_1 mice is three times that of BALB/c mice (Langdon, submitted for publication). The lack of difference in glutathione levels between strains but the obvious increased susceptibility towards NMF induced liver toxicity seen in BALB/c mice suggests that glutathione depletion may be only one of several manifestations caused by biochemical events which lead to liver damage but are not the cause of the hepatotoxicity.

Mouse strain	Glutathione content (GSH + GSSG, μ moles/g liver)	GSSG content (μ moles/g liver)
BALB/c (male)		
control (3)	6.9 ± 0.5	0.65 ± 0.18
NMF treated (3)	7.0 ± 2.9	0.46 ± 0.19
CBA/Ca (female)		
control	6.3 ± 1.4	0.42 ± 0.06
NMF treated (6)	7.8 ± 1.0	0.26 ± 0.06
CBA/Ca (female with TLX 5 lymphoma)		
control (5)	5.5 ± 1.0	0.53 ± 0.06
NMF treated (6)	7.2 ± 1.5	0.50 ± 0.22
BDF_1 (female, with M5076 sarcoma)		
control (5)	4.3 ± 0.9	0.97 ± 0.11
NMF treated (5)	6.6 ± 1.2	0.67 ± 0.27

Table 10 :

Hepatic glutathione levels (mean ± S.D.) 24 hours after the fifth of five daily i.p. injections of 400mg/kg NMF (number of animals in brackets).

8.IV The Study of TARM in isolated mouse hepatocytes on incubation with NMF and related formamides

8.IV.i Introduction

There have been many reports on the relationship between glutathione levels and lipid peroxidation and their involvement in hepatotoxicity (Anundi et al, 1979; Younes and Siegers, 1981). Glutathione provides one of the most important defence mechanisms of the cell against oxidative stress (5.II) and for several toxins such as CCl_4 or adriamycin, depletion of glutathione and oxidation of lipids in the membrane have been associated with subsequent tissue damage, although the connection between the two processes is unclear (Babson et al, 1981). The hepatotoxin CCl_4 is considered to cause toxicity by initiating the peroxidative breakdown of polyunsaturated acids in the cell membranes following free radical formation (Slater, 1972). Therefore it was considered important to investigate if lipid peroxidative processes were initiated in hepatocytes following incubation with NMF and its analogues. The measurement of thiobarbituric acid reactive material (TARM) is considered to be a useful method of assessing lipid peroxidation (5.III). The formation of breakdown products of lipid peroxides was determined by this method in mouse hepatocytes which were incubated in the same way as described for the experiments in which levels of glutathione were determined (7.II and 7.VI). In order to ascertain if depletion of glutathione was directly related to the production of lipid peroxides in hepatocytes, cells were preincubated with diethylmaleate (DEM), a known depletor of GSH, prior to NMF addition. DEM 340μM added 30 minutes before NMF, was shown to decrease glutathione in the cells to 30% of the original value, in agreement with Babson et al (1981)

It was considered that if an oxidative metabolite of NMF was responsible for any lipid peroxidative effect in hepatocytes, induction of cytochrome P-450 dependent enzymes in the hepatocytes would possibly increase peroxidative damage with a corresponding elevation in TARM levels. For this reason, animals were pretreated with PB 50mg/kg i.p. for 4 consecutive days (7.VI) and hepatocytes isolated from these animals were incubated with either NMF 7mM or saline for 180 or 240 minutes.

8.VI.ii Results

Figures 17(a) shows the result of incubation of isolated mouse hepatocytes with or without NMF 7mM for a maximum of 240 minutes, on the production of TARM. At 80 minutes, when a significant reduction in GSH was shown previously (8.III), no evidence of lipid peroxidation was found. At 120 minutes, more TARM was measured in cells in both control incubations and incubations with NMF, but only at 180 and 240 minutes was any marked difference noted between the NMF and control incubations, NMF values being 2 to 3 times the control values.

Reports have suggested that lipid peroxidation may be inhibited by addition of free radical scavengers or antioxidants. α-Tocopherol (vitamin E) and ascorbic acid (vitamin C) have both been successfully used to inhibit the production of lipid peroxides by xenobiotics, (Fujiita et al., 1982; Myers et al., 1976). Following this result with NMF, hepatocytes were incubated with NMF 7mM plus either 0.5 or 1.0mM ascorbic acid for up to 4 hours. With both concentrations of ascorbic acid, TARM levels for NMF incubations were decreased to control levels at 240 minutes.

Figure 18 shows TARM levels in mouse hepatocytes incubated for 240 minutes with equimolar concentrations of NMF, DMF, HMF, F, D-NMF or saline. This time was chosen as it was the time of most marked elevation in TARM in hepatocytes incubated with NMF (Figure 17a). As was noted when glutathione levels were determined, it is evident that only NMF produces any marked elevation in TARM levels when compared with the other formamides and incubation with D-NMF produced an almost identical result to that obtained with NMF.

Incubation of hepatocytes with DEM before addition of NMF did not result in a difference in TARM levels when compared with control incubations. In experiments with hepatocytes isolated from animals pretreated with PB, all TARM levels were elevated over the levels in hepatocytes obtained from untreated animals. When expressed as the percentage of the levels obtained from identical incubations using untreated animals, TARM levels for saline incubations were increased by 50% and 33% at 180 and 240 minutes respectively. Expression of results for NMF incubations in a similar manner showed an increase by 154% and 94% at 180 and 240 minutes respectively.

8.IV.iii Discussion

Incubation of hepatocytes with NMF lead to marked lipid peroxidation but only after 4 hours incubation. Cell viability by this time had decreased considerably in both control and NMF incubations (to 50-60%), but to a similar extent. The long time span required for NMF to induce lipid peroxidation may reflect the fact that the pro-oxidant metabolite of NMF took a long time to be generated in sufficient quantities. Alternatively, this phenomenon may not be due

to the pro-oxidant activity of an NMF metabolite but may be a consequence of the NMF-induced depletion of hepatic glutathione, which in turn makes the hepatocytes more susceptible to endogenous oxidative stress (Smith et al, 1983). The fact that incubation with ascorbic acid prevented the elevation of TARM levels seen with NMF does suggest that free radicals may be implicated in this toxic response. The reversal of NMF-induced lipid peroxidation by vitamin C may also be relevant to the prevention of liver damage in the therapeutic use of NMF.

The experiments using hepatocytes from PB treated animals show further evidence for an oxidative metabolite of NMF being responsible for the increased lipid peroxidation. In these experiments, it was also shown that hepatocytes from PB treated animals are more prone to oxidative damage as both saline and NMF incubations of these hepatocytes exhibited higher TARM values than hepatocytes from untreated animals. The experiments with DEM seem to indicate that glutathione depletion is not directly related to the elevation of TARM levels in hepatocytes. Again it was shown that the effect on TARM levels is specific for NMF as no other formamide derivative elicited an increase. As in the glutathione experiments with hepatocytes, D-NMF produced a similar response to that of NMF.

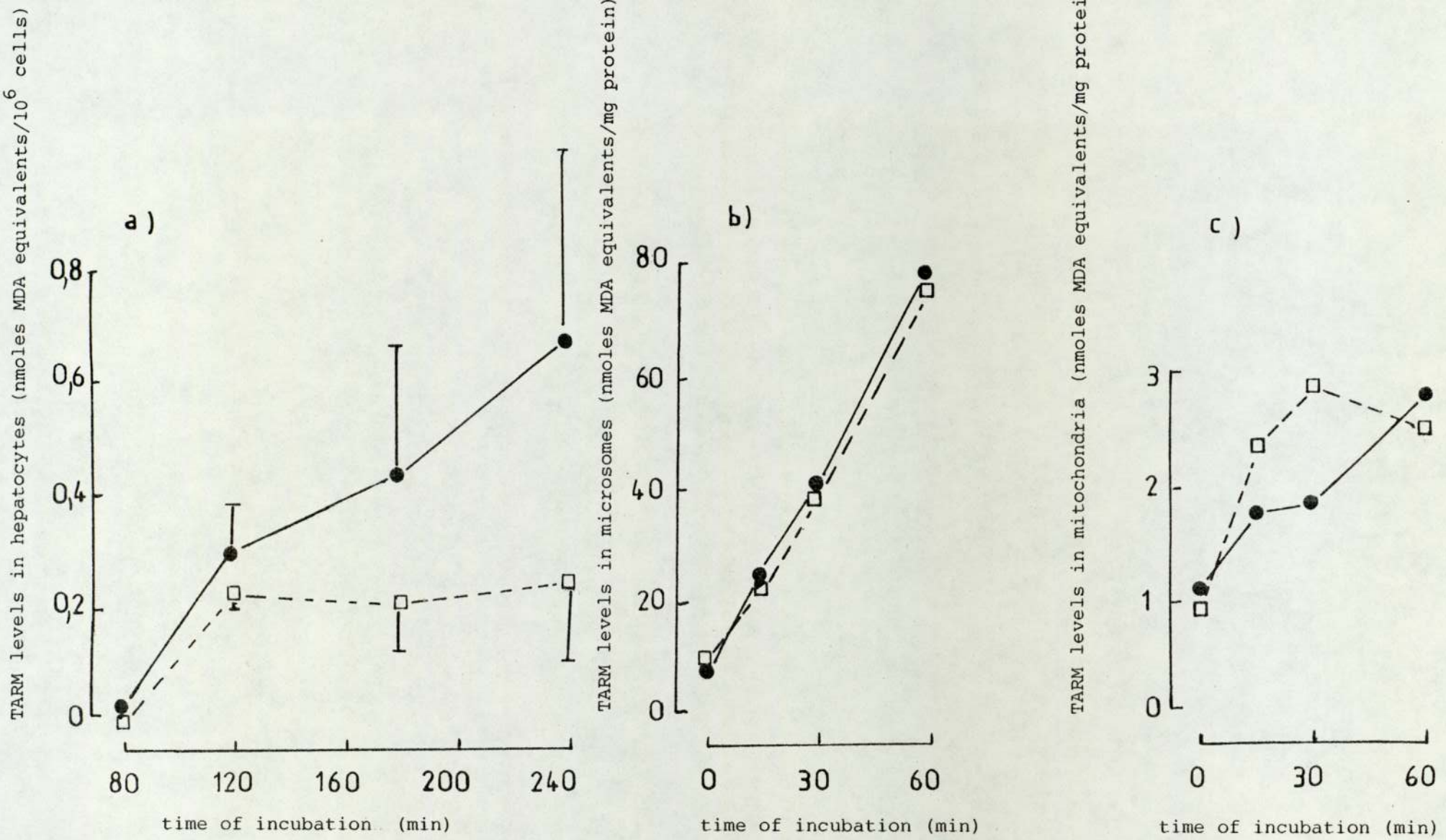


Figure 17 : TARM levels in a) hepatocytes, b) microsomes and c) mitochondria incubated with (●-●) or without NMF 7mM (□-□).

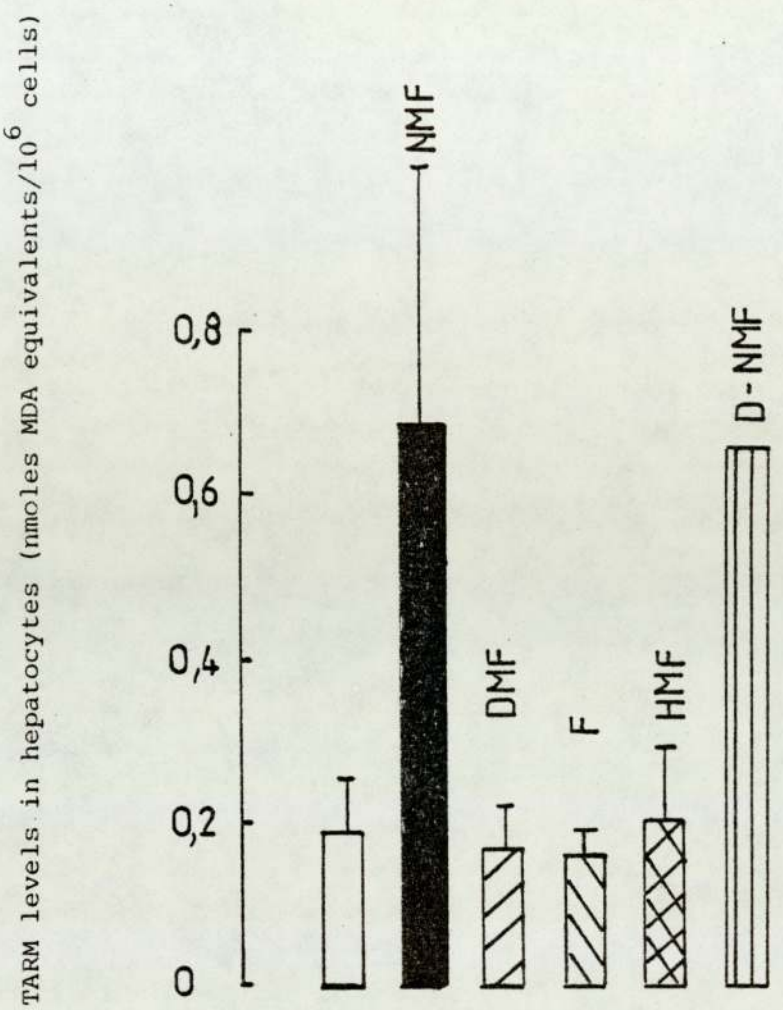


Figure 18 : TARM levels in mouse hepatocytes incubated with either saline, NMF, DMF, F, HMF or D-NMF 7mM for 240 minutes.

8.V The study of TARM in liver fractions on incubation with NMF

8.V.i Introduction

The pro-oxidant effect of NMF in hepatocytes (8.IV) suggested that examination of different compartments within the cell would be useful in order to identify the intracellular site of peroxide generation. The theory that a NMF metabolite could be responsible for the elevation of TARM in hepatocytes led to an investigation of the role of the endoplasmic reticulum and the other main organelles for cellular metabolism, the mitochondria, were also investigated. Microsomes or mitochondria from mouse livers, isolated as described in 7.VII, were incubated with and without cofactors for a maximum time of 60 minutes, with NMF 7mM or saline.

8.V.ii Results

Figures 17(b) and (c) show TARM levels determined in microsomes or mitochondria incubated with cofactors and 7mM NMF or saline. Although TARM levels were detectable in all incubations, incubation with NMF with either fraction did not lead to levels exceeding those of control incubations. In those experiments where cofactors were omitted from the incubations, similar results were obtained and TARM levels were not unlike those seen in Figures 17(b) and (c).

8.V.iii Discussion

The results of these experiments using microsomal or mitochondrial fractions may be interpreted in several ways. Firstly TARM is produced in all incubations, with and without NMF to a measurable

extent. It may be that the lack of difference between NMF and control incubations may be due to the fact that NMF is not metabolised in the fractions studied. However, if oxidation of the NMF molecule is important one would expect this to occur in the microsomal fraction, in which the majority of cytochrome P-450 dependent mixed function oxygenase enzymes, that are involved in oxidative metabolism, are located. It is possible that for the relatively short incubation time (1 hour, compared with 4 hours in hepatocytes), the quantities of pro-oxidant metabolite which had been generated from NMF were inadequate to produce a raised production of TARM in the incubate. The TARM levels produced in the mitochondrial incubations which were lower than those produced with microsomal incubations (Figure 17(b) and (c)) may not necessarily indicate an increased susceptibility of microsomes to oxidative stress. An alternative explanation may be that in the assay for TARM, one is measuring the reaction between the aldehydic breakdown products of lipid peroxidation with thiobarbituric acid (7.V) and these are known to be metabolised in the mitochondria (Högberg et al, 1975). It is therefore conceivable that aldehyde metabolism in the mitochondria may result in low TARM values.

8.VI The effect of NMF and related formamides *in vivo* and *in vitro* on Mg^{2+}-ATP dependent Ca^{2+} uptake into mitochondria and microsomes

8.VI.i Introduction

Disturbances of hepatocellular calcium homeostasis have been proposed by several authors to be an early and important manifestation of the hepatotoxicity of CCl_4 (5.III). Experiments by Lowrey *et al* (1981) and Pencil *et al* (1982) have shown that microsomal calcium pump activity is inhibited by CCl_4 *in vivo* and *in vitro*. It was proposed that this rapid toxic event may be ultimately responsible for the necrosis resulting from CCl_4 treatment. In analogy, the Ca^{2+} pump offered itself as a target for NMF or one of its metabolites to initiate events leading to hepatic lesions. Indeed, the effects on glutathione levels after 80 minutes, together with the delayed elevation of TARM levels in hepatocytes (8.II and 8.IV) suggest that some other toxic event must occur at an earlier time after NMF administration. Therefore it was decided to study the effect on the Mg^{2+}-ATP dependent Ca^{2+} pump in microsomes after pretreatment of animals with NMF 400mg/kg and also *in vitro*, following incubation of microsomes with NMF 7mM (7.VIII). As discussed in 5.III, the main organelles in the cell that regulate calcium homeostasis are the mitochondria and endoplasmic reticulum. Similar experiments with mitochondria were performed as with microsomes. In addition, HMF, DMF or F were administered to animals and their effect on the mitochondrial Ca^{2+} pump determined in order to check whether any effect after NMF was specific for this compound. As with the experiment on lipid peroxidation described in 8.IV, the possibility of a relationship between glutathione depletion and changes in $^{45}CA^{2+}$ sequestration was investigated by the use of DEM. Mice were dosed with

DEM 0.7ml/kg one hour before mitochondria were isolated and the $^{45}Ca^{2+}$ uptake into the mitochondria was determined.

8.VI.ii Results

Figure 19a shows the typical Ca^{2+} uptake curve produced with microsomes obtained from mice treated one hour prior to death with either CCl_4 2.5 ml/kg, NMF 400mg/kg or control injections. A marked depression of the ability to sequester Ca^{2+} was observed for CCl_4 but pretreatment with arachis oil, saline or NMF did not influence the $^{45}Ca^{2+}$ uptake. Uptake of $^{45}Ca^{2+}$ appeared to be linear with time up to 60 minutes for all mice irrespective of the pretreatments. For this reason, further experiments measuring $^{45}Ca^{2+}$ sequestration in microsomes were performed using two time points only, 30 minutes and 90 minutes. At 30 minutes, uptake would be on the linear part of the curve and at 90 minutes, uptake would be at the plateau (Figure 19a). Results for $^{45}Ca^{2+}$ uptake at these time points for microsomes from animals after 0.5, 1 and 2 hours after administration of NMF 400mg/kg or 1 hour after administration of saline are shown in Table 11. It is evident that there is no significant difference in $^{45}Ca^{2+}$ uptake by microsomes at various times after administration of NMF 400mg/kg to animals when compared with controls.

Figure 19b shows a typical $^{45}Ca^{2+}$ uptake profiles obtained from an in vitro experiment with microsomes incubated with NMF 7mM or saline and with or without cofactors. Table 12 shows data for 3 time points from 3 such experiments. The uptake of $^{45}Ca^{2+}$, expressed as cpm per mg protein, from individual experiments varied dramatically, resulting in a high standard deviation. However, within each experiment there was little difference between either NMF or saline, NMF with cofactors or saline with cofactors.

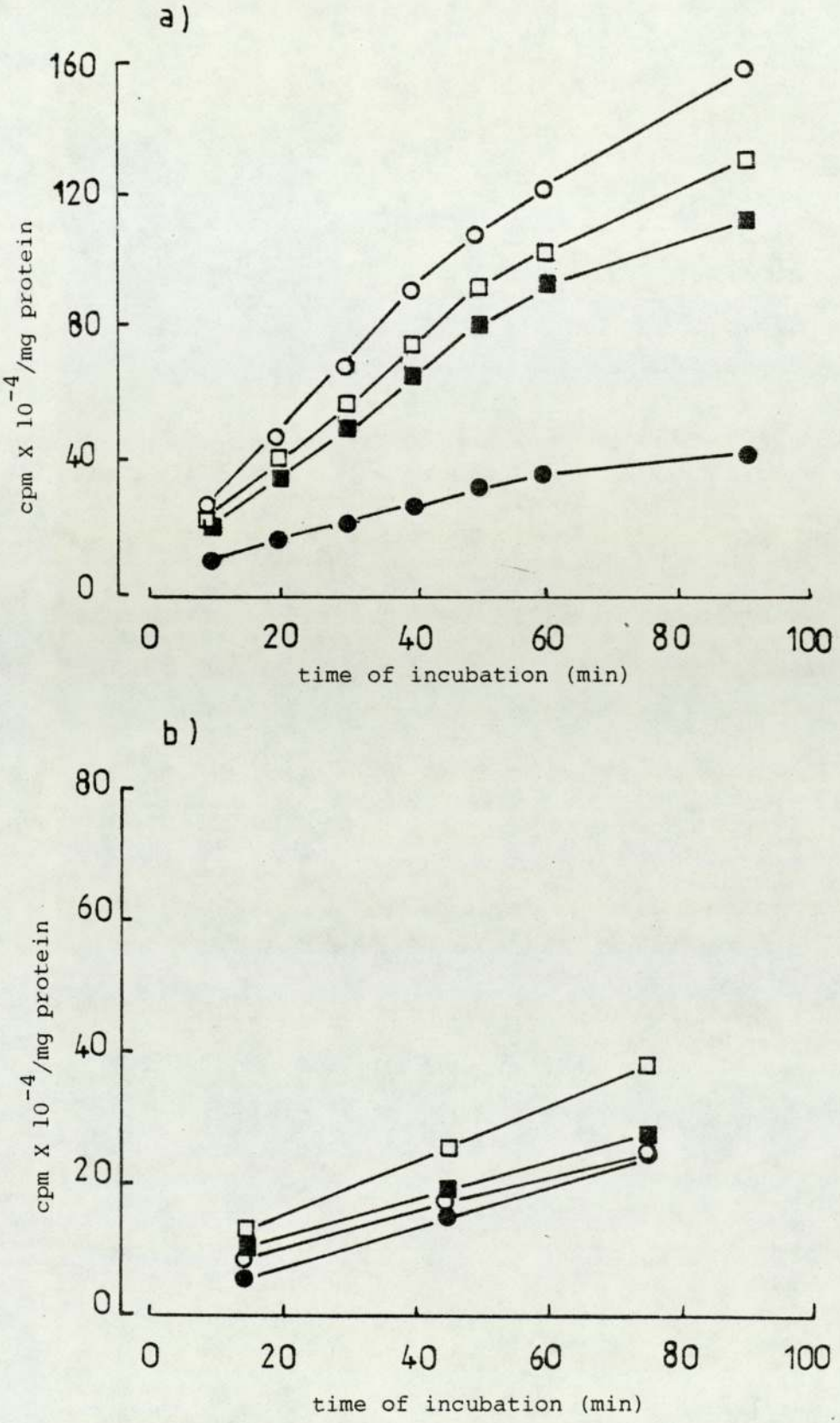


Figure 19 : Microsomal Ca^{2+} pump activity following a) NMF 400mg/kg (□), CCl_4 2.5ml/kg (●), arachis oil (○) or saline (□) treatment of mice 1 hour before and b) incubation with (■) and without NMF 7mM (●) and with (□) and without cofactors (○) <u>in vitro</u>

(graphs indicate values from one experiment, typical of 3)

	Time (hrs) after NMF administration			
Time point for $^{45}Ca^{2+}$ uptake (min)	CONTROL	0.5	1	2
30	28.07 ±1.76 (3)	31.4 (1)	29.53 ±5.45 (4)	32.03 ±4.3 (4)
90	79.37 ±11.58 (3)	73.5 (1)	86.75 ±23.46 (4)	92.85 ±16.69 (4)

Table 11 : $^{45}Ca^{2+}$ uptake (cpm x 10^{-4}/mg protein) into microsomes from mice treated with NMF 400mg/kg 0.5, 1, 2 hours or saline 2 hours previously.
(numbers in brackets denote number of experiments; values are means ± s.d.)

Time point for $^{45}Ca^{2+}$ uptake (min)	Contents of incubate			
	Saline	NMF	Saline/ Cofactors	NMF/ Cofactors
15	16.10 ±7.25	13.40 ± 4.91	16.60 ± 3.20	13.30 ± 8.98
45	30.70 ± 20.27	25.70 ± 11.95	37.47 ± 15.39	29.13 ± 26.66
75	46.27 ± 31.65	38.87 ± 18.67	57.80 ± 28.12	55.30 ± 46.76

Table 12 : $^{45}Ca^{2+}$ uptake (cpm x 10^{-4}/mg protein) into microsomes previously incubated with NMF 7mM or saline and with and without NADPH generating cofactor system

(values are means and s.d. of 3 experiments)

$^{45}Ca^{2+}$ uptake into mitochondria has been shown to be inhibited by addition of sodium azide 5mM to the buffering system when uptake was determined (Moore et al, 1975). Figure 20a shows the result of an experiment that confirms the inhibitory effect of azide on mitochondrial Ca^{2+} uptake. Figure 20b shows $^{45}Ca^{2+}$ uptake into mitochondria obtained one hour after administration of saline or NMF 400mg/kg to mice. Uptake is expressed as cpm X 10^{-4} per mg protein and there is a high standard deviation for controls at each time point. However, it is evident that NMF pretreatment of animals produced a dramatic inhibition of $^{45}Ca^{2+}$ sequestration into mitochondria ($p = 0.01 - 0.0001$). Table 13 gives the results of similar experiments following treatment of animals with DMF, HMF, F (6.8mMol per kg). Although only two experiments were carried out for each of these NMF analogues, the mean values are within the range obtained for the control experiments for each incubation time point. Only NMF pretreatment resulted in a severe inhibition of mitochondrial $^{45}Ca^{2+}$ uptake (Figure 20b).

Figure 20b also shows the values for experiments in which animals were pretreated with DEM 0.7ml per kg (mean of 2 experiments). This dose produced a depletion of total glutathione in the liver of 71.4%. It can be seen that the inhibition of the $^{45}Ca^{2+}$ uptake obtained for DEM was similar to that observed after NMF 400mg/kg.

Experiments with mitochondria incubated with NMF 7mM in vitro were attempted as described in 7.VIII. However, unlike similar experiments using microsomes, reliable uptake of $^{45}Ca^{2+}$ was unsuccessful, even in the control incubations and up to the time of this report being written, no meaningful results have been produced. According

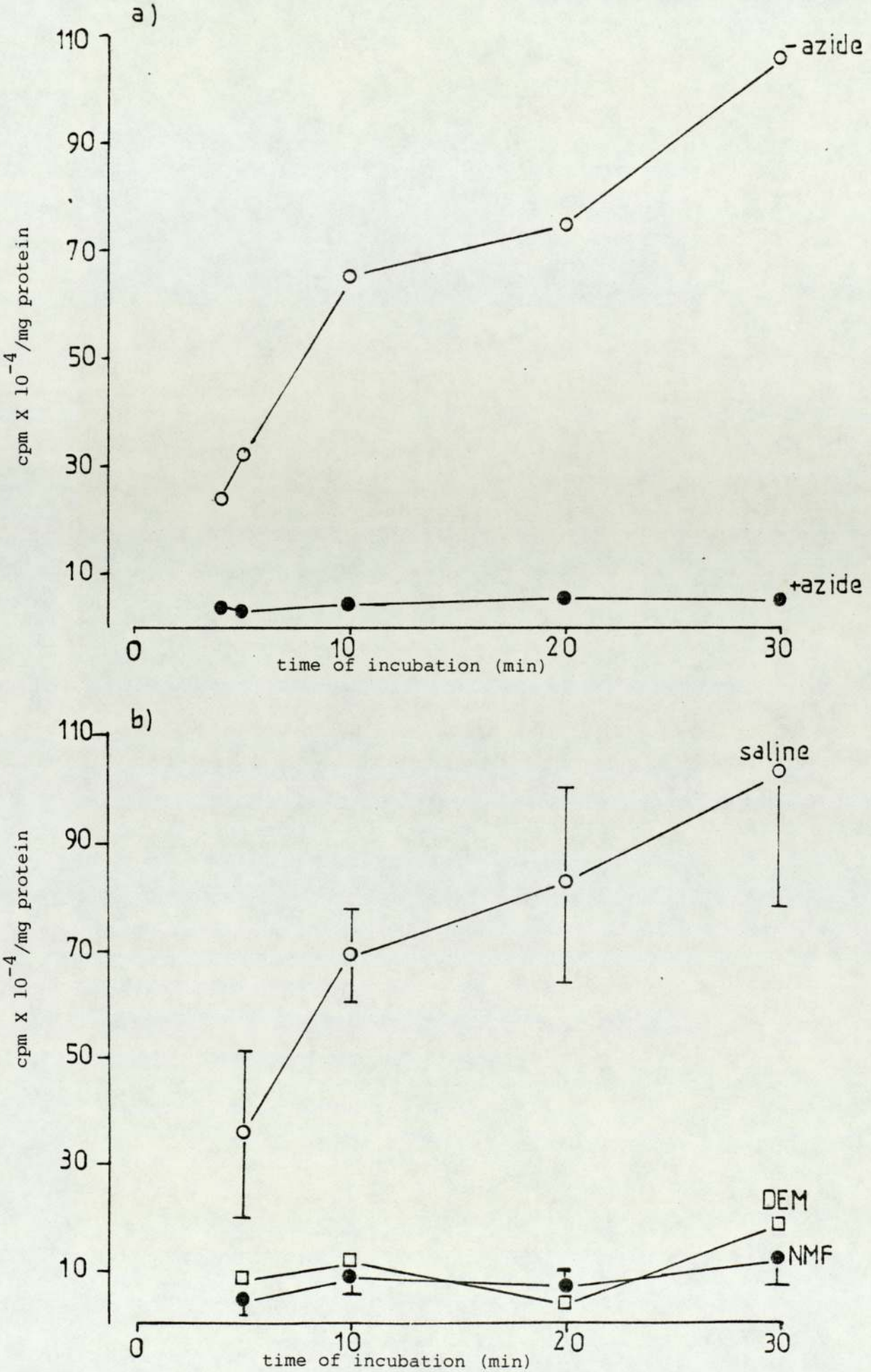


Figure 20 : Mitochondrial Ca^{2+} pump activity following a) addition of azide 5mM and b) administration of NMF 400mg/kg, DEM 0.7ml/kg or saline 1 hour before to mice.

Time of incubation for $^{45}Ca^{2+}$ uptake	SALINE	DMF	HMF	F	NMF
5	37.12 ± 16.80 (5)	17.80 (2)	21.15 (2)	32.55 (2)	4.10± 2.00 (3)
10	67.68 ± 10.79 (5)	48.50 (2)	45.35 (2)	42.95 (2)	9.47± 1.20 (3)
20	84.08± 19.75 (5)	60.20 (2)	40.65 (2)	48.60 (2)	7.43± 1.88 (3)
30	94.25± 17.78 (4)	-	-	-	12.07± 4.20 (3)

Table 13 : $^{45}Ca^{2+}$ uptake (cpm x 10^{-4}/mg protein) into mitochondria obtained from mice treated 1 hour previously with either NMF, DMF, F, HMF 6.8 mMoles/kg or saline.
(numbers in brackets are numbers of experiments; values are means ± s.d.)

to the literature, conditions for $^{45}Ca^{2+}$ uptake in *in vitro* experiments using mitochondria are similar to those for microsomes, with the omission of azide. However it may be that mitochondria from mice, as opposed to rats, may be more susceptible to changes induced by the metabolic incubation conditions. If the mitochondria are damaged during the working procedure, subsequent uptake of $^{45}Ca^{2+}$ may be compromised.

8.VI.iii Discussion

Using the procedures for assay of $^{45}Ca^{2+}$ uptake into rat microsomes that have been documented previously, a similar uptake into mouse microsomes was observed. CCl_4 was shown to inhibit this uptake in the mouse as was reported in the rat. However, unlike CCl_4, administration of NMF to mice did not appear to alter the uptake of $^{45}Ca^{2+}$ into microsomes. In addition, *in vitro* experiments did not reveal any toxic effect of NMF on the calcium pump of the microsomes. Therefore it may be concluded that, unlike CCl_4, the toxic effects of NMF on the liver, as evidenced by decreased liver glutathione levels and raised TARM levels were not preceded by a toxic effect on the microsomal calcium pump.

By contrast, however, the mitochondrial calcium pump appeared to be severely depressed by NMF 400mg/kg administered 1 hour prior to death of the animal. As with the other toxic responses evaluated in this study (8.II and 8.IV) the effect is seen to be specific for NMF when compared with the other formamide derivatives. On the basis of the effect of the GSH depleting agent, DEM, on the mitochondrial calcium pump it is tempting to suggest that glutathione depletion might also be

related to the inhibition of the mitochondrial calcium pump in the case of NMF. A relationship has been proposed between intracellular thiols and the microsomal calcium pump (Jones et al, 1983; Bellomo et al, 1982) and addition of physiological concentrations of GSH to rat hepatocytes was shown to protect the microsomal Ca^{2+} pump against oxidative damage induced by tbh (Jones et al,1983). However, in this context, two additional facts must be considered. Firstly, DEM is a chemically reactive compound and apart from its ability to undergo a reaction with glutathione, it affects many other intracellular processes (Plummer et al., 1981). A useful study to discover the relationship between GSH status and Ca^{2+} homeostasis would be to pretreat animals with certain sulphoximide derivatives (Griffith, 1981) Also, if an inhibition of $^{45}Ca^{2+}$ uptake is observed in in vitro experiments with mitochondria, it will be interesting to see if the addition of GSH, as described by Jones et al (1983) for microsomes, would possibly protect the pump from inhibition by NMF. Secondly, the hypothesis that glutathione may be important for the mitochondrial calcium pump in the liver contradicts the conclusions of Bellomo et al (1982) who suggested that only the microsomal calcium pump was thiol dependent and that the mitochondrial calcium pump was NADPH dependent. Perhaps experiments to investigate the changes in pyridine nucleotide redox states in mitochondria after NMF pretreatment would give further clues. It has been found previously that the ability of mitochondria to sequester and retain calcium is largely determined by this redox state (Lötscher et al, 1979; Lehninger et al, 1978.

Nevertheless it does appear that inhibition of the mitochondrial calcium pump following pretreatment with NMF is an early toxic effect of the drug and as changes in calcium homeostasis have been associated

with cell injury (Schanne et al, 1979), this effect may be related to the subsequent hepatotoxicity induced by NMF. Investigations to determine changes in the total intracellular calcium content after NMF administration may be useful to complement these results.

8.VII Ultrastructural changes in isolated mouse hepatocytes following incubation with hepatotoxins

8.VII.i Introduction

Several recent reports have demonstrated a correlation between biochemical effects induced in isolated hepatocytes by toxic substances with early morphological changes in the hepatocyte (5.IV). Using isolated rat hepatocytes, changes in cellular calcium concentration and intracellular glutathione were shown to be related to the appearance of swelling and protrusions or blebs of the hepatocyte surface membrane for menadione (Thor et al 1982a), bromobenzene (Thor et al, 1982b) and t-butylhydroperoxide (tbh) (Bellomo et al, 1982). These ultrastructural changes are thought to be an early indication of cytotoxicity. As changes in cellular glutathione have been noted on incubation of mouse hepatocytes with NMF 7mM after 80 minutes (8.I) and changes in calcium homeostasis in mitochondria of mice dosed with NMF 400mg/kg have been found 1 hour after treatment (8.VI), an investigation into the ultrastructural changes of the hepatocyte membrane was performed (7.IX), following incubation with NMF 7mM for 1 or 4 hours. In a control experiment, hepatocytes were incubated with t-butylhydroperoxide 4mM for 0.5 or 1 hour in order to produce a toxic effect on the hepatocyte membrane, as described by Bellomo et al (1982) for rat hepatocytes. In some experiments, animals were pretreated with PB prior to isolation of hepatocytes. If cytochrome P-450 dependent enzymes and hence drug oxidation were important for the toxic changes occurring within the hepatocyte, one might expect that an increase in the degree of ultrastructural change would occur with pretreatment of animals with PB.

8.VII.ii Results

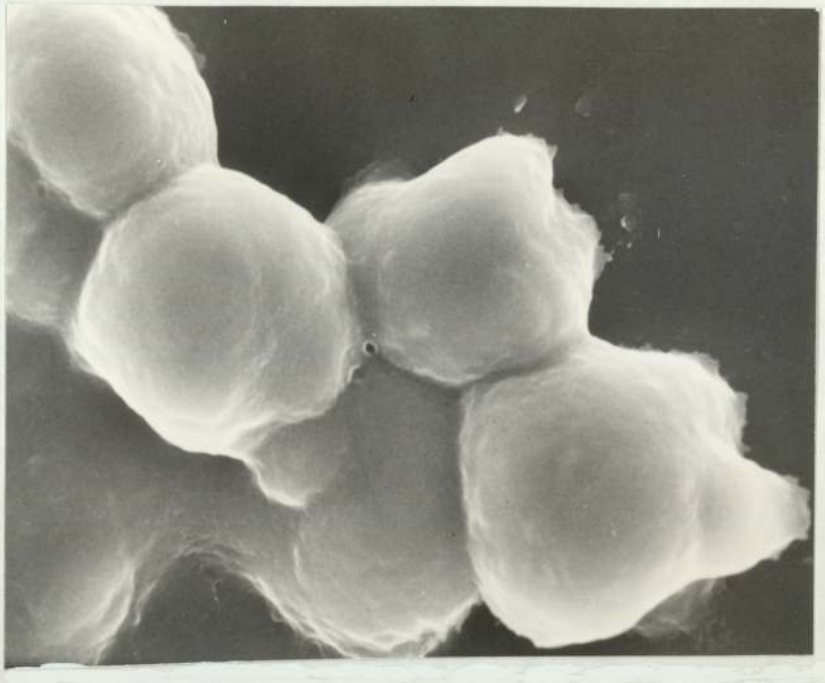
t-Butylhydroperoxide 4mM, which elicited dramatic changes on the outer membrane of rat hepatocytes (Bellomo et al, 1982), produced no noticeable blebbing or swelling when incubated with mouse hepatocytes for either 0.5 or 1 hour (Figure 21). Pretreatment of animals with PB before isolation of hepatocytes did not produce any ultrastructural changes for incubations with tbh. Similarly, incubations of hepatocytes with NMF 7mM for 1 or 4 hours from either PB or untreated mice did not lead to any evidence of structural changes in the hepatocyte membrane when compared with controls. (Figure 22).

8.VII.iii Discussion

The inability of tbh 4mM to elicit an alteration in the outer membrane of hepatocytes in this study is unexpected. Within 30 minutes of incubation of a similar number of rat hepatocytes with the same concentration of tbh, Bellomo et al (1982) observed dramatic changes in the hepatocyte surface structure. There could be several explanations for the lack of toxic response shown in this study. Firstly, the difference in species between the study here and those previously reported may be important. There have been many reports of differences in metabolism of drugs between species (Williams, 1971) and the metabolism of tbh may be no exception. However, it must be added that tbh, like most hydroperoxides, rapidly causes oxidative damage in various tissues (Lötscher et al, 1979) and it seems strange that it failed to cause any toxicity in the mouse hepatocytes. Secondly it is conceivable that the methodological differences between the experiments reported here and those reported previously (Thor et al, 1982a; Bellomo et al, 1982) may have

contributed to the unusual results. Following the lack of toxic response produced by tbh, it was not surprising that incubation of mouse hepatocytes with NMF 7mM did not produce a change in hepatocyte surface structure. However, it has been found that incubation of mouse hepatocytes with NMF at concentrations as high as 0.1M did not lead to a marked decrease in cell viability when compared with control incubations. Thor et al (1982b) in experiments with bromobenzene, found that the appearance of blebs on the hepatocyte outer membrane correlated with a decrease in cell viability. Consequently at concentrations of NMF 7mM, no ultrastructural changes in the hepatocyte surface would be expected.

a)



b)

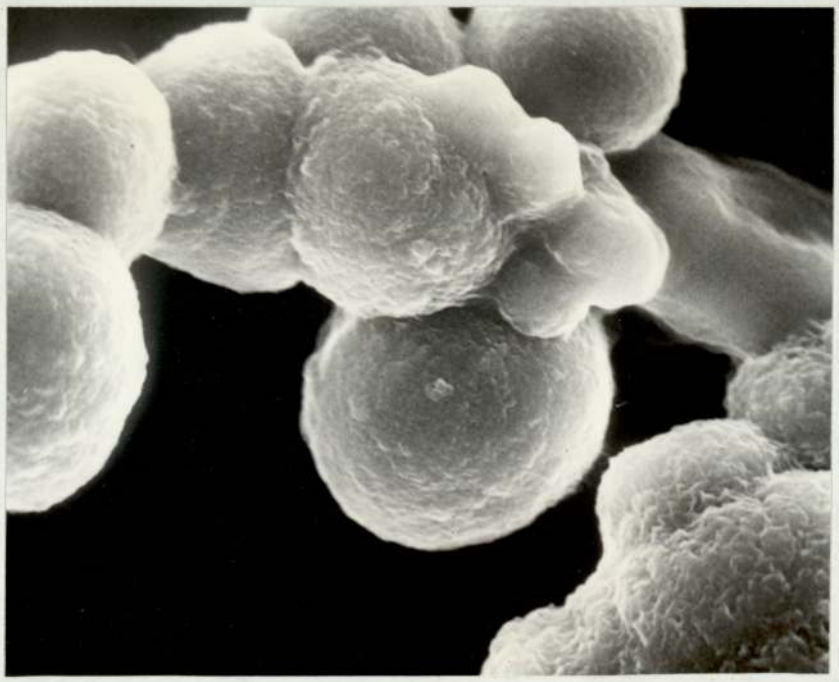
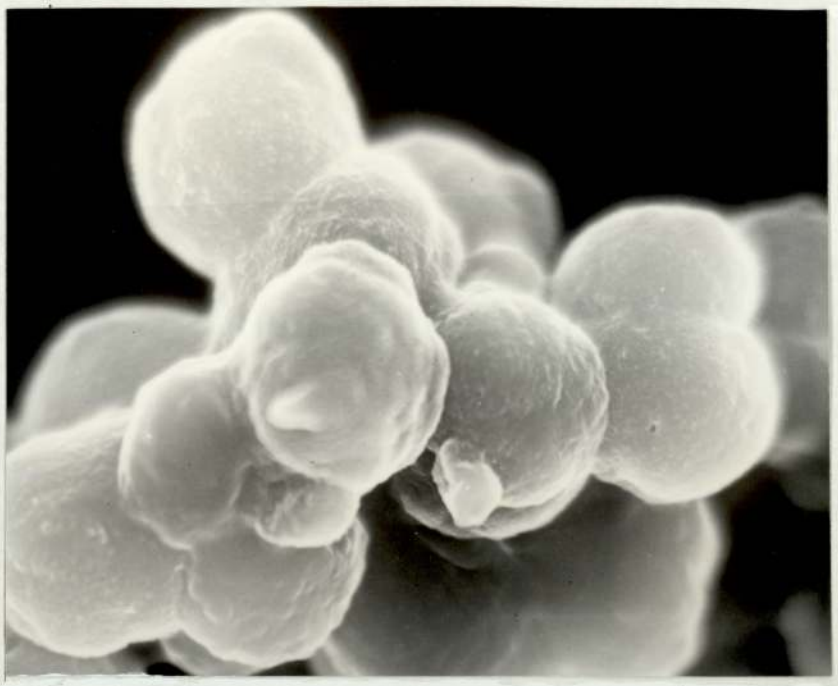


Figure 21 : Scanning electron micrographs of mouse hepatocytes incubated for 0.5 hours with either a) t-butyl-hydroperoxide 4mM or b) saline.

Magnification:- X 4000

a)



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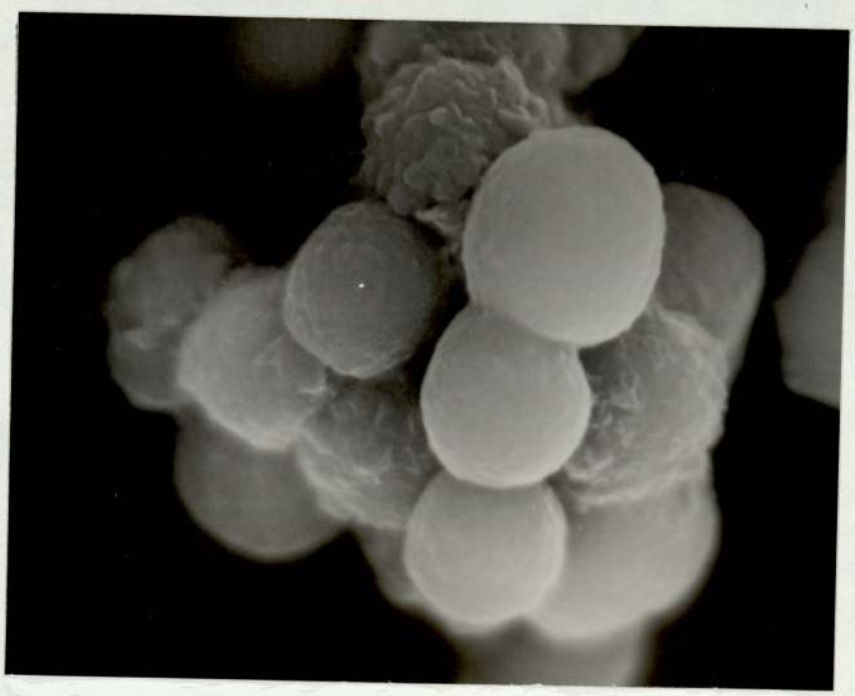


Figure 22 : Scanning electron micrographs of mouse hepatocytes incubated for 1 hour with either a) NMF 7mM or b) Saline.

Magnification :– X 3000

8.VIII Characterisation of NMF hepatotoxicity in vivo

8.VIII.i Introduction

Liver damage in vivo following administration of NMF has been demonstrated in several reports (5.II). In this study, characterisation of the nature of the damage in mice was investigated by several methods. Histopathological examination of livers of animals treated with NMF was used to evaluate the dose dependency and the time of onset of the hepatotoxicity after NMF administration (Table 7). In the dose dependency study, animals were given one of 3 doses either as a single dose or daily for 5 consecutive days. For the investigation to determine the time of onset of liver damage after a dose, a single dose of 400mg/kg was administered. It was considered useful to determine the time of onset of damage as this might reflect on the chemical species responsible for the damage by either the parent drug or a metabolite. It was also decided that information on the hepatotoxicity in animals bearing tumours sensitive to NMF would be valuable. For this reason, mice bearing either the TLX5 or the M5076 tumours were dosed with NMF 400mg/kg for 5 days and the livers examined (7.X.i). In order to assess if the liver damage induced by NMF in BALB/C mice was reversible, NMF 400mg/kg was administered i.p. to mice and the survivors were killed 7 days after the dose.

Toxicity was also evaluated in terms of the release of liver enzymes (SDH, ALT, AST) into the plasma, following NMF treatment (7.X.ii). Plasma levels of these enzymes have been used frequently in the assessment of the severity of drug induced liver damage (Korsrud et al, 1973; Korsrud et al, 1972) and also in the case of NMF

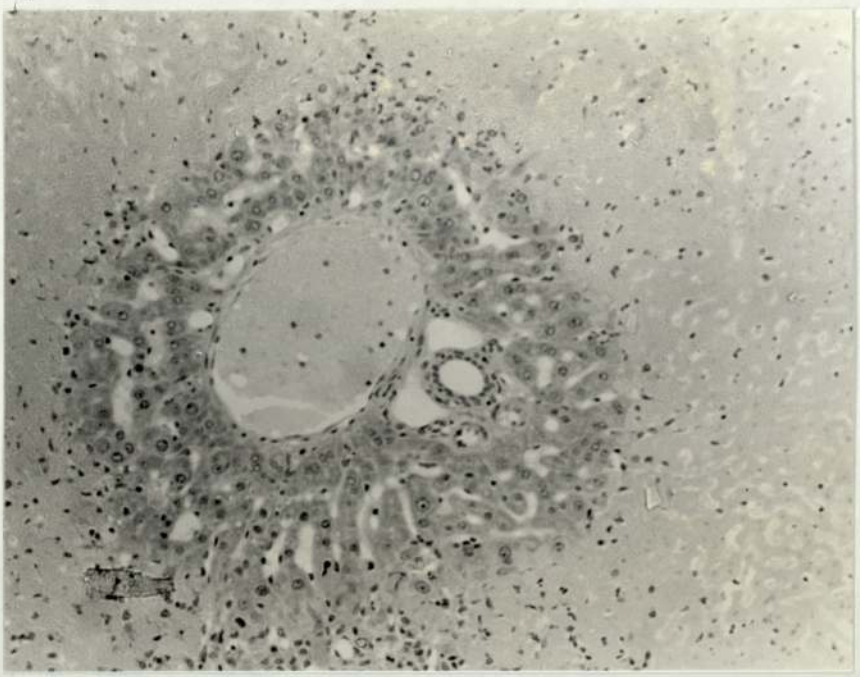
(Newman et al, 1981) and DMF (Scailteur et al, 1982; Lundberg et al, 1981). NMF was administered to mice as in those investigations on the dose dependency and the time of onset of hepatotoxicity (7.X.ii).

On the basis of the result in 8.IV, where elevated levels of TARM were found in mouse hepatocytes incubated with NMF 7mM for 4 hours, an investigation was considered to be useful to provide evidence of lipid peroxidation in vivo. Hepatotoxic damage in vivo, after NMF administration may be due to lipid peroxidative damage, following the results of the in vitro experiments with NMF (8.IV). TARM was determined in liver fractions obtained from mice as described in 7.X.iii.

8.VIII.ii Results

Gross inspection and histopathology of livers from BALB/c mice revealed damage invariably 24 hours after a single dose of 400mg/kg, 3 out of 8 mice exhibited damage after 200mg/kg and no damage was observed after administration of 100mg/kg NMF, when compared with control livers. At a dose of 400mg/kg NMF, animals lost between 1.5g and 2.0g in weight within 24 hours. At all of the doses, no consistent weight loss was observed. Animals exhibiting toxicity were lethargic with ruffled coats. Figure 23 shows photographs of sections of livers from animals having received a single dose of A 400mg/kg NMF, B 200mg/kg NMF, C 100mg/kg NMF and D saline, 24 hours previously. At a dose of 400mg/kg, NMF produced zonal necrosis, mainly of the periacinar or mid-acinar type and often damage was massive and confluent with evidence of cell debris, pyknotic nuclei and haemosiderin. Hepatocytes adjacent to the areas of severe damage

A



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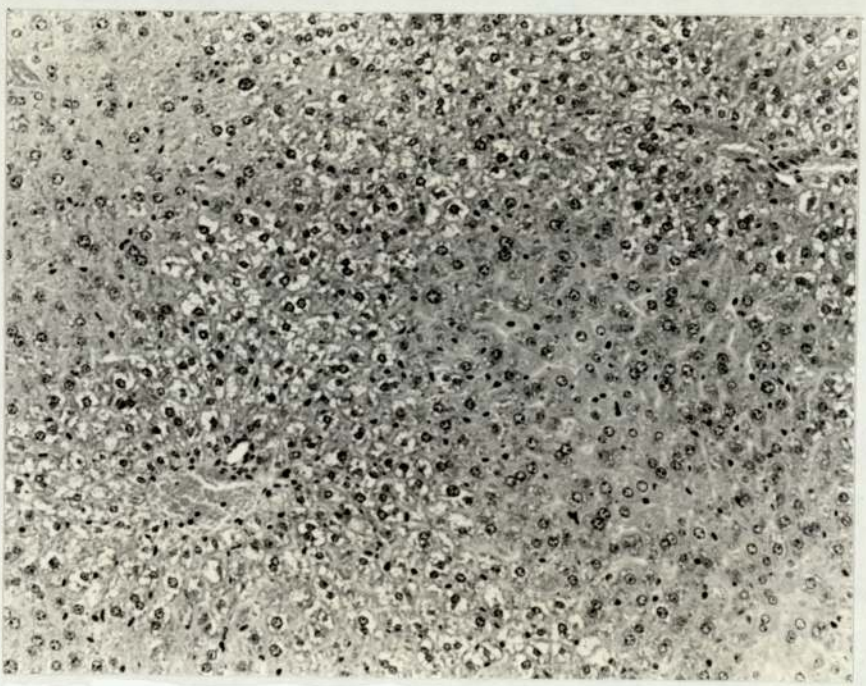
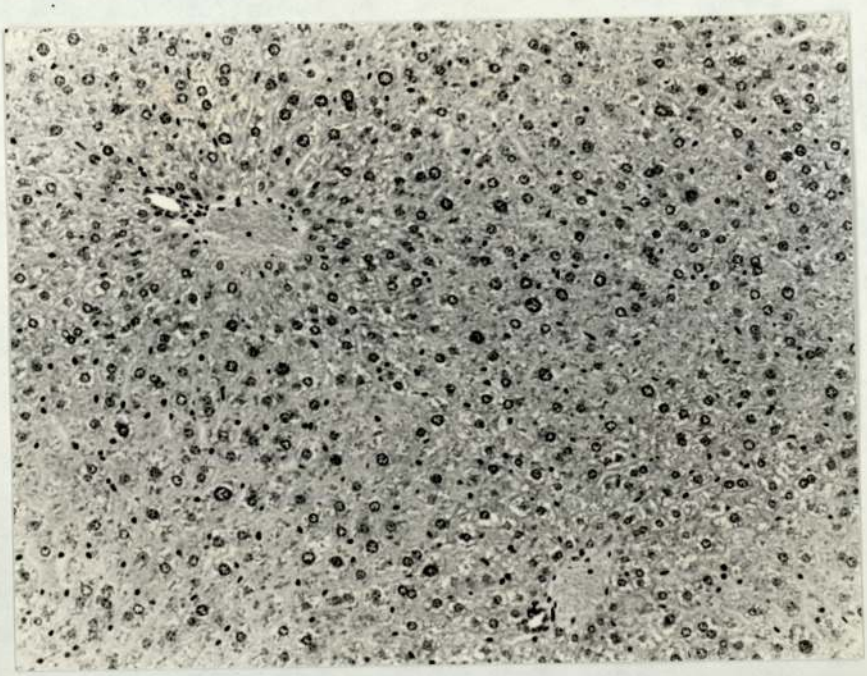


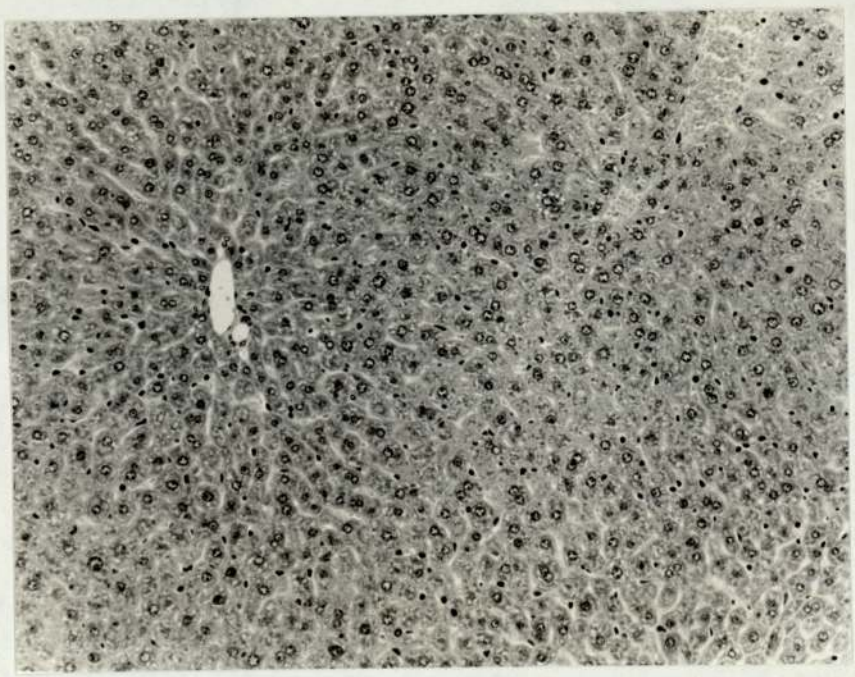
Figure 23 : Sections of livers from BALB/c mice after a single dose of NMF in saline; A 400mg/kg; B 200mg/kg; C 100mg/kg and D saline (control) 24 hours previously.

Severe periacinar necrosis was demonstrated in A and mild periacinar necrosis in B, with glycogen pallor. C showed no significant changes from control. D. H. and E. X 160.

C



D

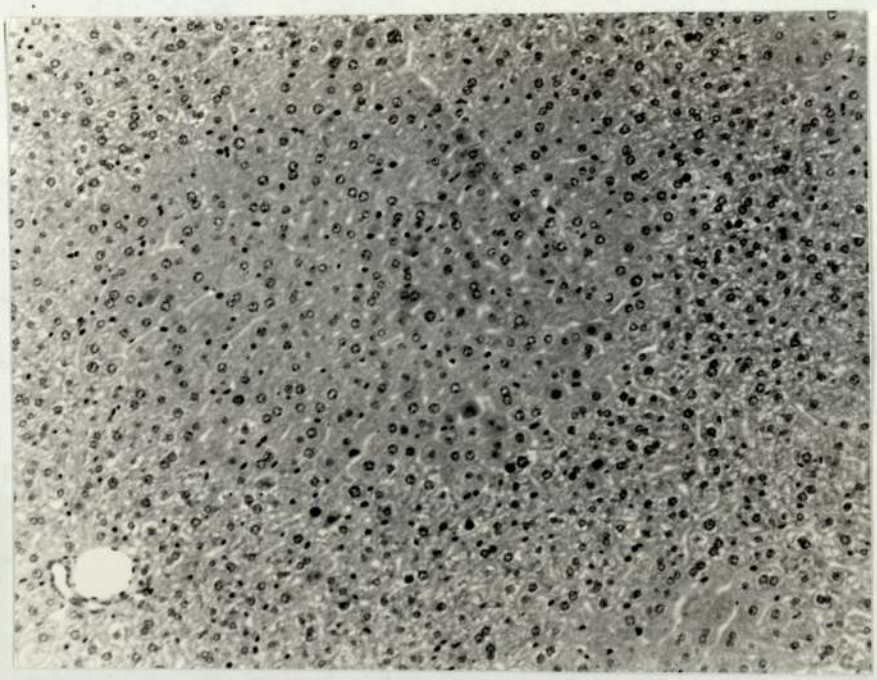


were observed to be enlarged with an increased abundance of mitotic figures and Küpffer cells. Some cells at the periphery showed fatty droplets and there was a general decrease in glycogen pallor. In livers of animals dosed with 200mg/kg NMF, that showed damage, periacinar necrosis was observed, but this was rarely confluent. As with the higher dose, pyknotic nuclei and cell debris were noted.

Repeated administration of 100, 200 or 400mg/kg NMF led to a pattern of toxicity similar to that described for the single doses. Animal weight loss was similar after 5 doses of 400mg/kg as for a single dose and 5 doses of 200mg/kg produced a loss of 0.5-1.0g in weight in 3 out of the 6 animals treated. As before, multiple doses of 100mg/kg produced no obvious toxicity. It is therefore evident that a total dose of 500mg/kg (given as 5 x 100mg/kg) NMF did not produce toxicity similar to that of 400mg/kg NMF (given as a single dose). On histopathological examination, multiple doses of NMF produced no difference in response than single doses.

In those experiments to investigate the time of onset of hepatic damage, gross inspection of livers did not reveal severe changes before 12 hours after injection of NMF. Histopathological examination at 4 hours after injection of NMF 400mg/kg showed that livers were identical to livers from control mice. At 6 hours, of the 6 livers examined, 5 were similar to control animals, but these appeared to have a reduced glycogen content and the periacinar area had a slightly increased abundance of Küpffer cells. The remaining liver examined showed more severe changes with evidence of fatty droplets and some haemorrhagic damage. At 8 hours and also at 10 hours after the dose of NMF, livers were similar to those examined at 6 hours,

A



B

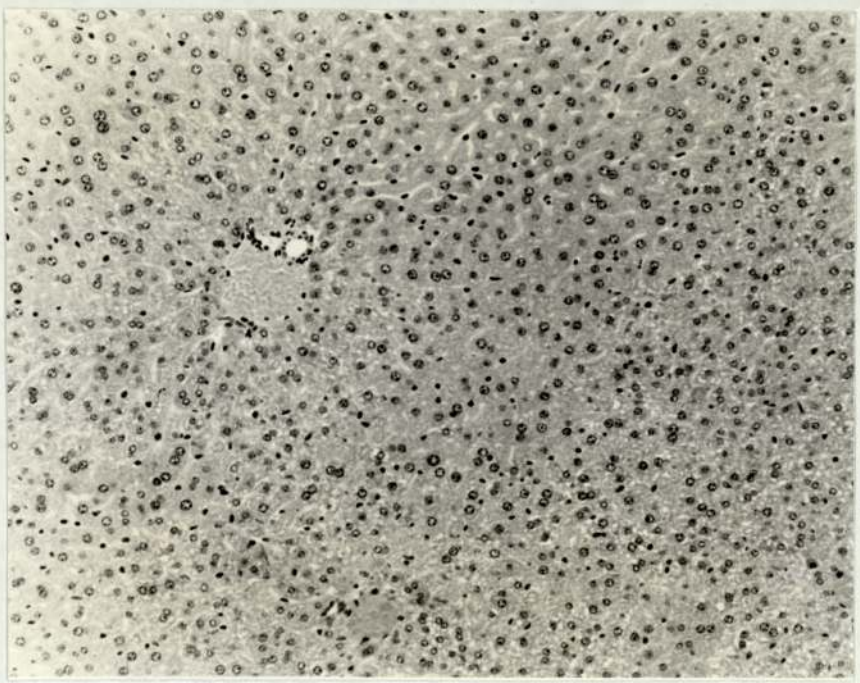
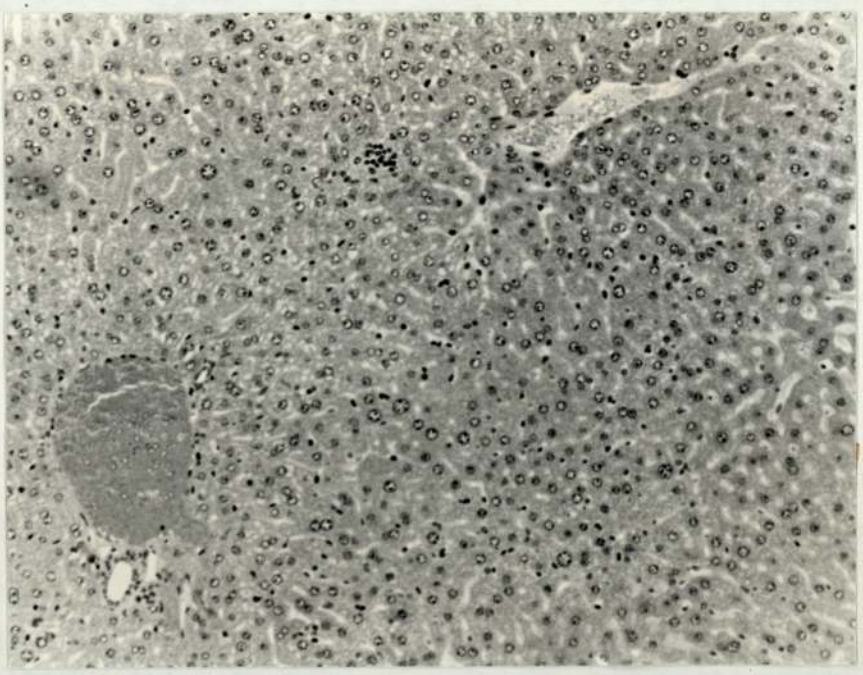
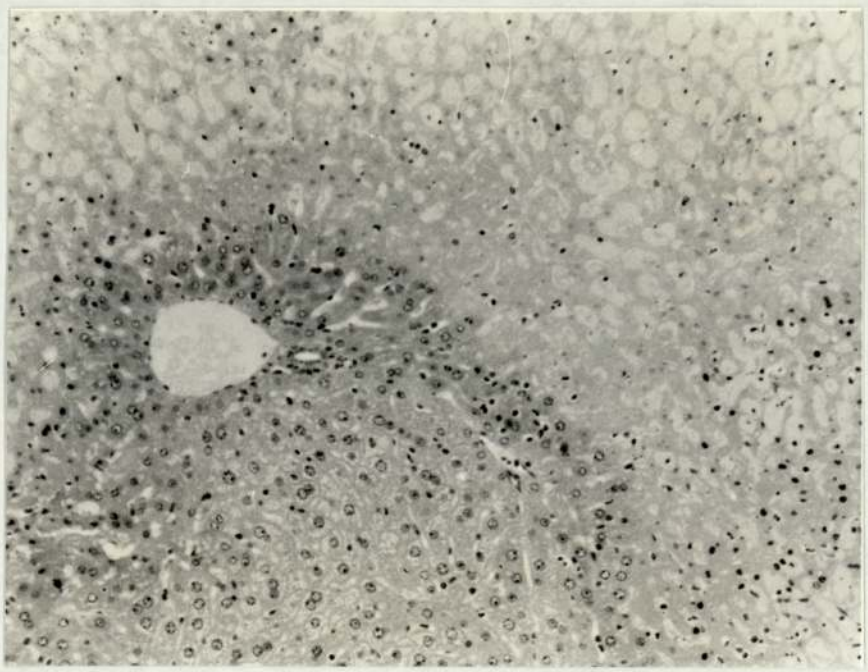


Figure 24 : Sections of livers from BALB/c mice at different times after a single dose of NMF 400mg/kg : A 4 hours, B 8 hours, C 12 hours and D 24 hours.
Minor changes only were seen in A, B and C, including glycogen pallor.
However, severe periacinar necrosis was demonstrated in D. H. and E. X 160.

C



D



showing decreased glycogen content in the periacinar area. Of the 2 livers examined histopathologically after 12 hours, one was similar to those described after 6, 8 or 10 hours. The other liver, however, showed more severe damage with pyknotic nuclei, mitotic figures and haemosiderin and diffuse periacinal degeneration. At 24 hours after the dose, livers showed damage similar to that found in the previous study of a single dose of 400mg/kg NMF after 24 hours. Photographs of sections of some of the livers at various times are shown in Figure 24.

In the study of the recovery of animals after a single dose of NMF 400mg/kg, of 15 mice treated, 2 died after 24 hours, 6 died between 24 and 48 hours and 2 died between 48 and 72 hours after administration of NMF. Of the 5 survivors, gross inspection of the livers revealed small areas of damage in 2, larger areas of necrosis in 2 others and an apparently normal liver in the remaining mouse, 7 days after the dose of NMF. As with all the damaged livers in this study, livers showing the most marked external damage also weighed between 50 and 100% more than control livers. The weights of the surviving animals are shown in Figure 25. The greatest weight loss appeared at approximately 48 hours after administration of NMF and weight was almost restored to pre-dose levels by day 7 after treatment. Of the 5 livers of NMF treated animals examined, one showed no evidence of damage. This was the liver which appeared normal on gross inspection on post mortem. This animal had shown signs of NMF toxicity in the days after treatment, including a significant weight loss which was gradually regained by 7 days after treatment. Of the 4 other livers studied, there were a few areas of necrosis but in the periacinal areas, where damage at this dose had been noted previously, signs of

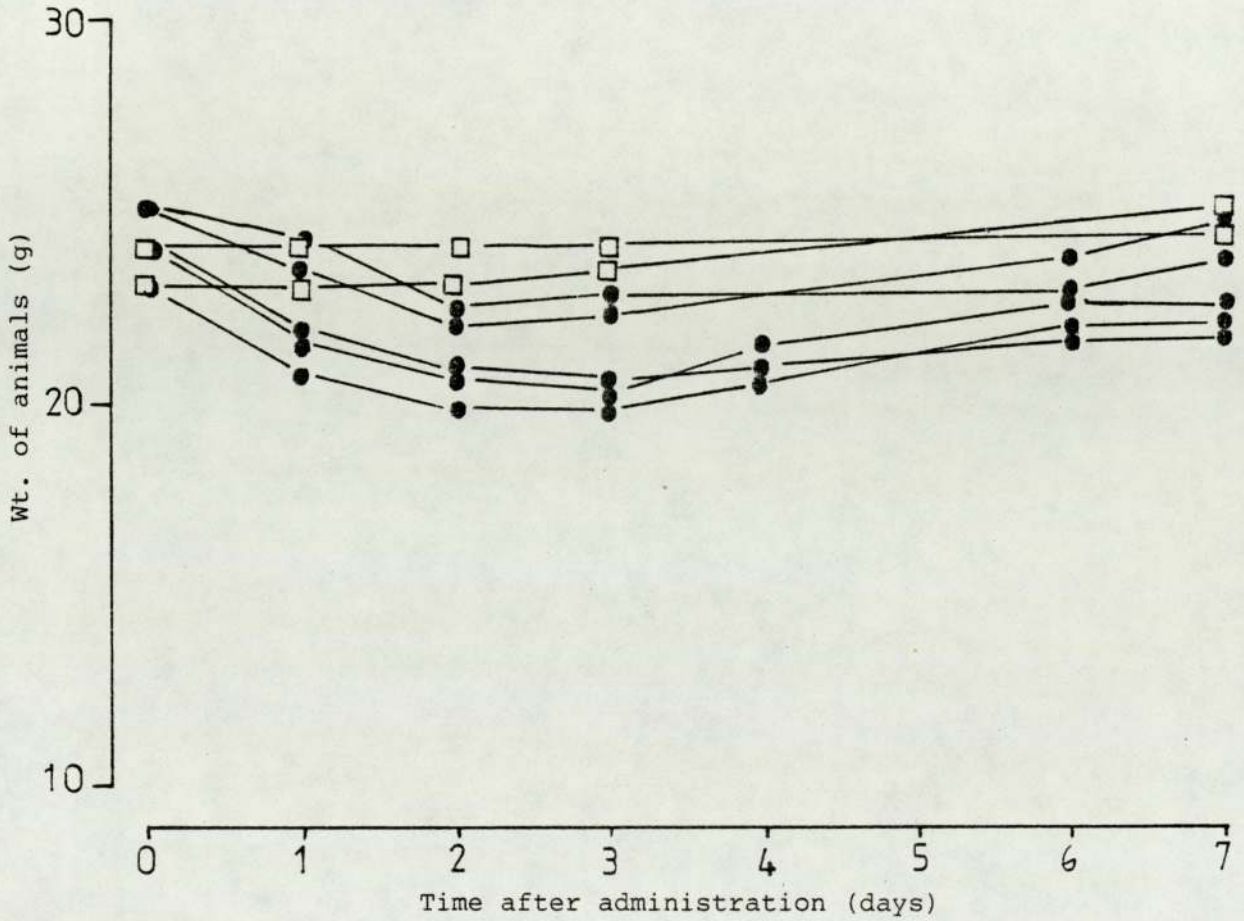
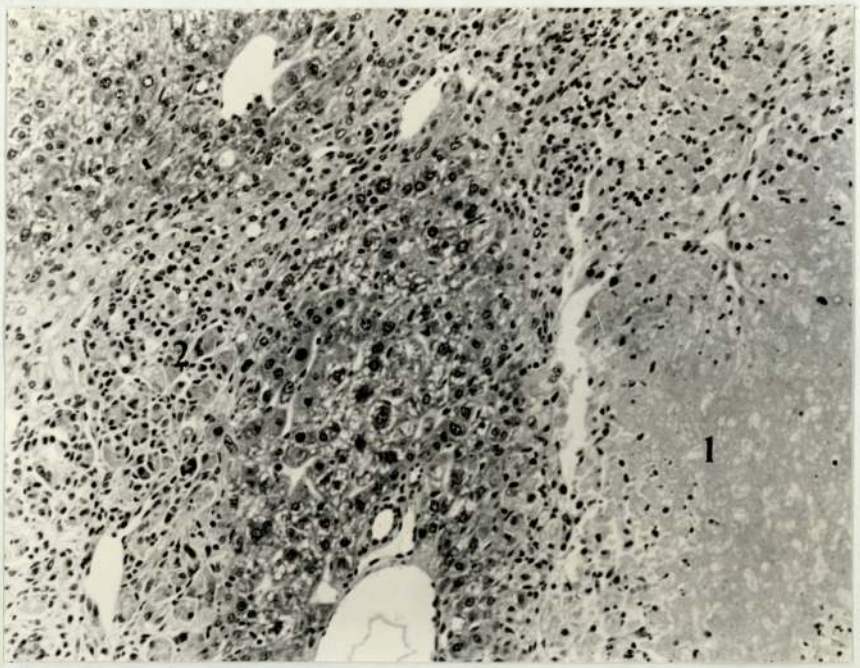


Figure 25 : Weights of BALB/c mice (g) for several days after administration of NMF 400mg/kg (●) or Saline (□).

regeneration were observed with an increase in the number of mitotic figures. In these areas, there were many Küpffer cells and many populations of mixed inflammatory cells. Bile duct hyperplasia was also evident. In those areas of apparently normal cells adjacent to areas of regenerating cells, hepatocytes seemed to be highly vacuolated with some evidence of fatty infiltration. Figure 26 shows photographs of sections of a liver from an animal dosed with NMF and one with saline, 7 days after treatment.

Female CBA/Ca mice with TLX5 tumours and female BDF_1 mice with M5076 tumours exhibited no outward signs of drug induced toxicity when NMF 400mg/kg was administered for 5 days. Gross inspection of livers revealed some damage in only 2 of 6 CBA/Ca mice treated with NMF. Livers of CBA/Ca mice bearing TLX5 tumours and treated with saline were similar to livers from CBA/Ca mice with no tumour, but livers of the former group did possess several clumps of oddly shaped hepatocytes which stained a deeper colour with haemalum, when examined histopathologically. These groups of cells were also found in thoselivers of CBA/Ca mice with TLX5 tumour treated with NMF. It is tempting to suggest that their existence may be related to the presence of the TLX5 tumour in the animals. No difference was observed in livers from BDF_1 mice with or without the M5076 tumour. All the livers from all 5 CBA/Ca mice with TLX5 tumour treated with NMF showed widespread periacinar degeneration of hepatocytes. Unlike the livers of BALB/c mice treated at this dose, no necrosis was evident. In the areas of degeneration, vacuolisation and glycogen pallor were reduced and there was an increase in the number of phagocytic cells. In those areas adjacent to the degenerative areas, vacuolisation appeared to be

A



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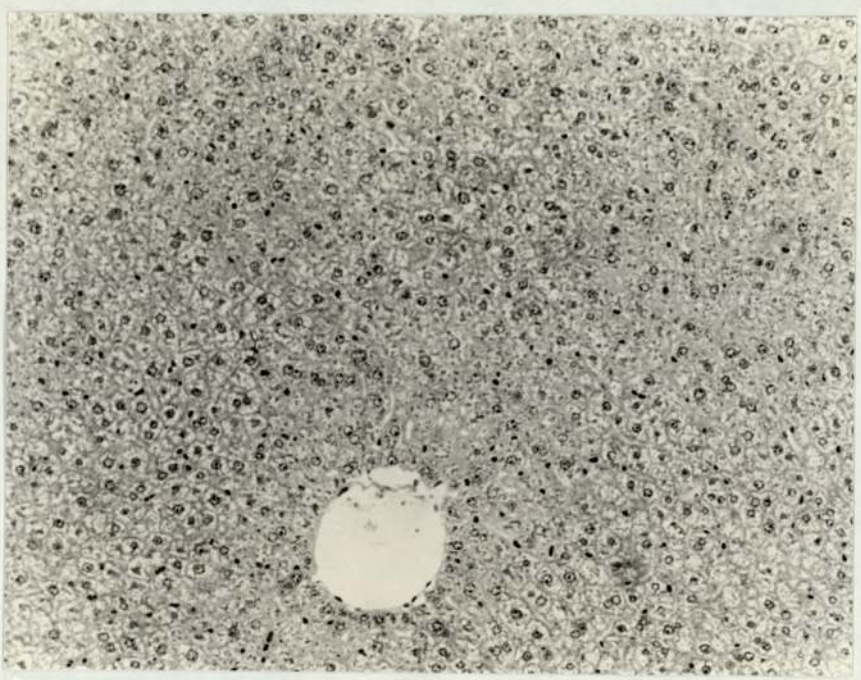


Figure 26 : Sections of livers from BALB/c mice 7 days after administration of NMF 400mg/kg, A or saline, B. Although necrosis was demonstrated in the periacinar region (1), there was evidence of regeneration in some areas (2) with increased numbers of mitotic figures. H. and E.X 160.

increased, with some evidence of fatty infiltration. In those livers of BDF_1 mice with M5076 tumour, treated with NMF 400mg/kg for 5 days there appeared to be no marked change in hepatocellular structure. In one liver only, a slight devacuolisation in the periacinar region was noted.

As with the histological examination of livers, plasma SDH levels in mice 24 hours after a single dose of NMF showed that damage was related to the dose. Figure 27a shows plasma SDH levels plotted against the dose. Plasma ALT and AST values showed similar trends when compared with SDH levels with values after 400mg/kg NMF being as much as 100 X control values. Multiple doses of 100mg/kg or 200mg/kg NMF produced similar SDH levels to those after single doses. Figure 27b shows plasma SDH levels at different time intervals after administration of NMF 400mg/kg. It is seen that SDH levels were similar to control levels for up to 10 hours after administration of NMF and only after this time were elevations of SDH levels, consistent with incidence of liver damage, apparent. In the experiment in which the recovery of livers was investigated after a single dose of NMF 400mg/kg, SDH levels were similar to control levels 7 days after administration of NMF.

TARM values, an indicator of lipid peroxidation, obtained 24 hours after injection of a single dose of 100, 200 or 400mg/kg or 24 hours after the 5th dose of 100mg or 200mg/kg were only markedly elevated in mitochondria and cytosol after the high dose of NMF (Table 14). Values were so variable that statistical comparison was not possible. TARM values obtained 1 hour after NMF 400mg/kg to BALB/c mice were similar in all liver fractions to those from control animals.

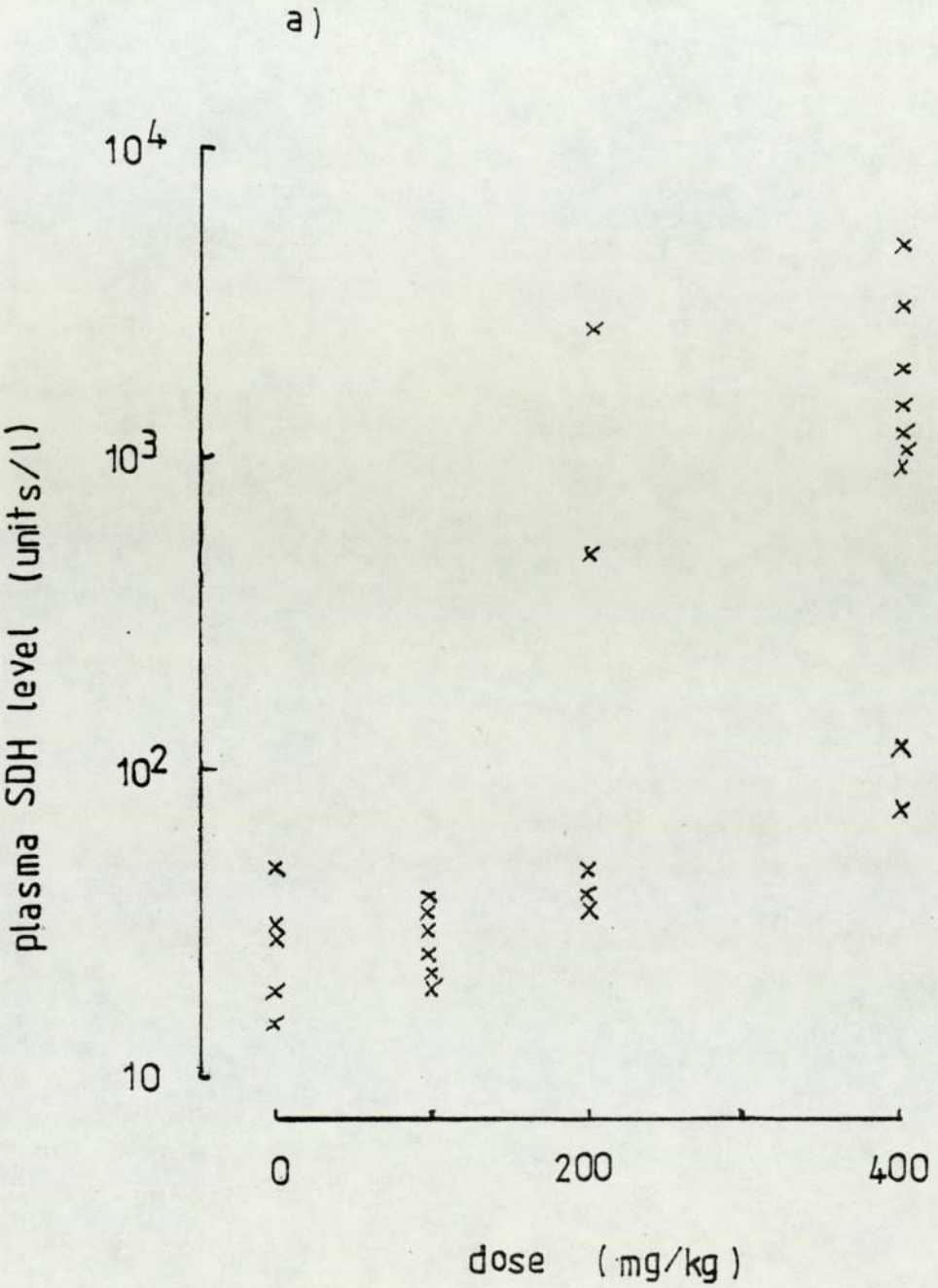
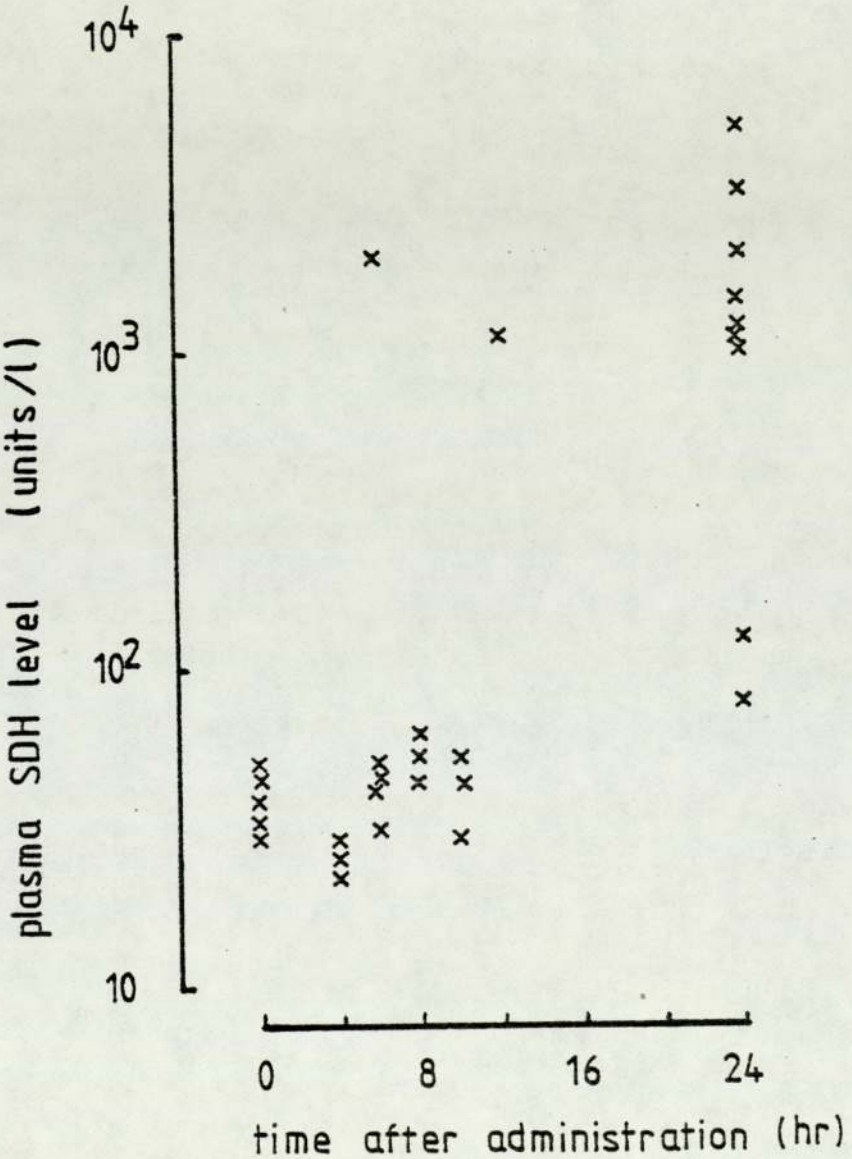


Figure 27 : Plasma SDH levels in BALB/C mice

a) 24 hours after i.p. saline (zero dose values 100, 200 or 400mg/kg NMF;

b) (overleaf) at different time intervals after NMF 400mg/kg.

b)


plasma SDH level (units/l)
10^4
10^3
10^2
10
0
8
16
24
time after administration (hr)

Cell fraction	TARM content (nmoles MDA/g liver equivalent
microsomes	
control mice (6)	0.50 ± 0.38
NMF treated mice (6)	0.79 ± 0.38
cytosol	
control mice (5)	0.89 ± 0.37
NMF treated mice (2)	2.99 ± 1.29
mitochondria	
control mice (6)	0.75 ± 0.10
NMF treated mice (6)	2.21 ± 1.40

Table 14 :

TARM content of liver fractions (mean ± S.D.) 24 hours after administration of 400mg/kg in BALB/c mice (number of experiments in brackets).

8.VIII.iii Discussion

In BALB/c mice, these results show a definite dose response of hepatotoxic damage induced by NMF, as evidenced by both liver plasma enzyme levels and histopathology (Figure 27a and Figure 23). In this strain of mice, the dose threshold above which a single dose is likely to cause necrosis is 200mg/kg. Interestingly, multiple doses of NMF did not produce a significantly different response to single doses and administration of five doses of 100mg (total dose 500mg) did not result in changes like those shown after a single dose of 400mg/kg (total dose 400mg). This result tends to confirm that of Langdon et al (submitted for publication), in which NMF administered in several doses proved to be less toxic than a single high dose administration. The enzyme levels tended to parallel the degree of damage induced by the different doses of NMF.

The livers that showed evidence of necrosis had damage in the periacinar area. This confirms the results shown previously in rats (Lundberg et al, 1981) and in mice (Dexter et al, 1982). As the monoxygenase enzymes which catalyze the oxidation of xenobiotics are concentrated in this area of the liver (Gooding et al, 1978), the tissue distribution of the NMF induced injury seems to mirror the distribution of cytochrome P-450. The area of damage induced by an agent may reflect the involvement of the enzymes located there in the biodisposition of the agent (5.IV). A similar distribution in the case of liver damage caused by CCl_4 has been suggested to reflect a major role for these enzymes in either the activation of the toxicant or the propagation of lipid peroxidation (Slater, 1972). By analogy, the involvement of cytochrome P-450 and/or lipid peroxides in the genesis of the damage induced by NMF is suggested. It is of interest that the

hepatocytes in this area of the liver have been shown to contain less glutathione than other regions (Smith et al, 1979) and it is tempting to suggest that the cells in the periacinar area were more susceptible to attack by toxic metabolites formed from NMF.

In the experiment on the time course of the hepatotoxic effect of NMF 400mg/kg, SDH levels again paralleled the damage seen histopathologically. No significant damage was observed until 10-12 hours after the dose of NMF. This length of time again suggests that NMF itself is not directly hepatotoxic and that metabolism to a necrogenic species is required. This species may not be produced in sufficient quantitites to elicit a toxic response until several hours after administration of NMF. From these experiments it is clear that NMF may be described as an intrinsic hepatotoxin.

From the result in 8.IV, it is possible that NMF causes hepatotoxicity by forming a metabolite that initiates peroxidative breakdown of membrane polyunsaturated fatty acids. Products of lipid peroxidation were found in liver mitochondria 24 hours after administration of a hepatotoxic dose but not after 1 hour (Table 14), the time at which glutathione depletion occurred. Also no change in lipid peroxide production in microsomes was found. This finding has to be interpreted with caution as the necrosis was already established at the time at which TARM was determined, so that an increase in levels of TARM in the livers may have been a consequence of the damage rather than the cause. This view has been postulated as the involvement of lipid peroxidation in several hepatotoxicities (Smith et al, 1983).

In those experiments examining the recovery of livers after treatment, it was obvious that NMF 400mg/kg to BALB/c mice produces

over 50% mortality, and in those animals that survived, outward toxic signs were evident. At this dose, massive periacinar necrosis was observed after 24 hours (Figure 23) and after 7 days, livers were seen to be gradually regenerating (Figure 26), indicating that the damage appeared to be reversible after a single toxic dose. The levels of SDH in the plasma confirmed this. It would be interesting to monitor animals for a longer time than 7 days after a similar dose of NMF, in order to check if the tissue was fully repaired. It would also be useful to assess liver function after this time as scarring of liver tissue may result in reduced tissue function. Utilisation of the $^{14}CO_2$ exhalation test (see Part One), using ^{14}C-AP may prove to be of value in such a study.

In those experiments in which animals bearing tumours were treated with NMF, the striking result was the obvious difference in the hepatotoxicity induced by the drug in each of the 3 strains of mice. Together with earlier evidence of a strain difference (see 8.III on hepatic glutathione content in each strain) in toxicity, it appeared that liver damage differed in BALB/c, CBA/Ca and BDF_1 mice by diminishing degrees. It is of interest that the GSH content of livers of these strains did not differ before or after NMF treatment (8.III) and one may infer that total glutathione content of the liver may not be as relevant to the NMF hepatotoxicity as first thought.

SECTION 9

DISCUSSION

OF

PART TWO

In Part 2 of this thesis, experiments are described which were performed *in vitro* and *in vivo* with the main aim of characterising and elucidating the biochemical mechanisms of the liver damage induced by NMF in mice. In Section 8, the results of these experiments are discussed. In this section, several overall conclusions that arise from the results in Section 8 will be discussed, together with some ideas for future work.

The proposed interrelationship between glutathione content, lipid peroxide production and intracellular calcium homeostasis and their role as biochemical mechanisms of xenobiotic hepatotoxicity were discussed in 5.III. Abnormal changes from controls in each of these three features were noted after exposure to NMF administration either *in vivo* or *in vitro* (Section 8). However, the mechanism by which NMF induces liver damage does not appear to be as convincingly explained by these changes as the mechanisms by which the classical hepatotoxins such as CCl_4 cause hepatotoxicity. Hepatic glutathione depletion after drug administration has been correlated with liver damage (Mitchell, 1974). A depletion of intracellular total glutathione caused by NMF has been shown in isolated hepatocytes (8.I) and a depletion of non-protein thiols has been demonstrated in BALB/c mice one hour after NMF 400mg/kg administration (Gescher *et al*, 1982) The importance of glutathione in NMF hepatotoxicity was therefore tentatively implicated. However, after multiple doses of NMF to BALB/c mice, hepatic total glutathione levels were unchanged despite clear manifestations of hepatoxicity at the time when glutathione was determined (8.III). In addition, comparison of levels of hepatic glutathione

between the three strains of mice (CBA/Ca, BALB/c, BDF_1) revealed no difference following multiple doses of NMF, even though the difference in hepatotoxicity between the strains was highly significant (8.III and 8.VIII). The latter results tend to suggest that the glutathione changes seen *in vitro* and after a single dose of NMF are transient and are probably not causally related with the mechanisms of NMF hepatotoxicity.

On the other hand, the results of pretreatment of mice with the glutathione depletor, DEM, when a marked inhibition of mitochondrial Ca^{2+} uptake similar to that produced by NMF was observed, seem to confirm the importance of GSH in the early events leading to toxic changes in the liver cell (8.VI). Apart from GSH depletion however, DEM produces other biological effects (Plummer *et al*, 1981) and these could have been responsible for the inhibition of calcium uptake. Indeed, the dependence of mitochondrial Ca^{2+} uptake on glutathione would be incongruent with the suggestions of Bellomo *et al* (1982) according to which extra mitochondrial (and not mitochondrial) Ca^{2+} compartmentation is thiol dependent.

Evidence of lipid peroxidation, by measurement of MDA, was found in isolated mouse hepatocytes only after 4 hours incubation with NMF and was not evident at earlier times when glutathione depletion was pronounced (8.IV). In livers of mice dosed with NMF, amounts of MDA were only markedly different from controls in those livers showing considerable damage 24 hours after administration (8.VIII). One hour after NMF administration to mice, when changes in non-protein thiols (Gescher *et al*, 1982) and when severe inhibition of mitochondrial Ca^{2+} uptake occurred (8.VI), no lipid peroxidation was evident. It therefore appears that lipid peroxidation is a late

event in NMF hepatotoxicity and is a consequence rather than a cause of the damage. This is similar to the result of Smith et al (1983) obtained with bromobenzene when lipid peroxidation was considered to be a secondary toxic effect of the compound. These results are different from results reported for CCl_4 where lipid peroxidation was an early toxic event (Smith et al, 1982). Mitchell et al (1982) also concluded, in experiments with paracetamol, that lipid peroxidation frequently occurs as a result rather than a cause of hepatocellular necrosis, independently of the mechanism that initiates the damage. The authors critically review the opinions published over the years on the mechanisms of chemically induced tissue injury and suggest that many toxic effects could not be explained by the proposed peroxidation or alkylation reactions. Many toxins produce a combination of both types of reaction. The theory of glutathione depletion always leading to peroxidation was also criticised as incorrect. No difference in lipid peroxidation between NMF or saline incubation of mitochondria or microsomes in vitro was observed (8.V), unlike CCl_4 which produced marked lipid peroxidation under similar incubation conditions (Cheeseman, 1982).

In mitochondrial or microsomal fractions from livers of mice administered with NMF 1 hr prior to death, inhibition of mitochondrial calcium uptake was observed, but microsomal calcium uptake remained unchanged. In vitro preparations of microsomes incubated with NMF showed no inhibition of calcium uptake (8.VI). These results are unlike those obtained after administration of CCl_4, which caused marked inhibition of microsomal but not mitochondrial Ca^{2+} very shortly after administration (Moore et al, 1976; Lowrey et al, 1981).

Therefore it appears that the mechanisms of NMF liver damage are dissimilar from those believed to be involved in the toxicity of another intrinsic hepatotoxin, CCl_4 , which was often used as a model in this study. CCl_4 has been shown not to deplete hepatic GSH (Docks and Krishna, 1976) but the role of lipid peroxidation in the toxicity of the compound is well established (Slater, 1972). Farber and El-Mofty (1975) described the change in opinion as to the mechanism of CCl_4 induced hepatotoxicity over the years. In early hypotheses,it was proposed that toxic cell necrosis was fundamentally a matter of mitochondrial degeneration with uncoupling of oxidative phosphoylation, loss of respiratory control and ATP activation. However these changes were discovered to be late phenomena and could be accounted for as secondary changes due to cell death rather than primary causative factors. According to a more recent theory, the primary target is the plasma membrane, followed by an increase in intracellular Ca^{2+} concentration. Moore et al (1976) have since shown that the microsomal calcium pump, rather than the mitochondrial pump, is severely inhibited within one hour of CCl_4 administration to rats and Lowrey et al (1981) and Waller et al (1983) have both correlated lipid peroxidation and covalent binding of CCl_4 to the endoplasmic reticulum with the inhibition of the microsomal calcium pump. Although the evidence from the study presented in this thesis is very incomplete, it might be supposed that, unlike CCl_4, the mitochondria were the primary site for NMF change, with functional disturbance occurring (e.g. Ca^{2+} pump inhibition) within one hour of administration. It could be considered that intracellular glutathione depletion occurs as a consequence of metabolite conjugation but

glutathione resynthesis is rapid and the initial depletion of GSH was presumably not relevant to the overall hepatotoxicity of NMF. Finally, it could be proposed that lipid peroxidation occurs as a sequela in the cell, following on from several other unknown intermediate toxic events.

The depletion of hepatic non-protein thiols in vivo one hour after a single dose of NMF observed by Gescher et al (1982) suggested that an electrophilic metabolite was formed from the parent compound. This hypothesis was confirmed by several of the results of the experiments described in this thesis. The in vitro experiments with isolated mouse hepatocytes, in which intracellular glutathione was reduced after 80 minutes incubation with NMF (8.I) is consistent with this view, together with the delayed production of metabolites of lipid peroxides (8.IV). A similar conclusion was drawn from histological examination of livers of animals dosed with NMF. The most consistent manifestation in these tissues was periacinar necrosis and this damage did not manifest itself until at least 10 hours after administration of NMF (8.VIII). Plasma levels of liver enzymes correlated well with histological damage.

As described in 5.I, there is evidence that metabolism of NMF does occur in vivo (Brindley et al, 1982b) and recent work has identified the hydroxymethyl derivative of NMF, HMF, as present in the urine after NMF administration (Kestell, in preparation). It must be noted that previous experiments with liver fractions in vitro have not shown any chemical analytical evidence that NMF is metabolised. (Brindley et al, 1982b; Gescher et al, 1982).

The effects of NMF in isolated hepatocytes, described above, were found to be specific for NMF and no other formamide derivative that was tested, including HMF, caused any significant change when compared with controls (8.I). The identity of the reactive metabolite of NMF therefore remains a mystery. It is conceivable that minute amounts of an extremely reactive and unstable metabolite could induce these effects. Chemically it is conceivable to produce isocyanates (R-NCO) following oxidation of formamides (RNHCHO) (Richter and Ulrich, 1977). Isocyanates are highly reactive and toxic species that have been implicated in the cytotoxicity of nitrosourea compounds (Gibson, 1982). These anti-tumour agents decompose under physiological conditions to produce two major reactive intermediates, one of which is an isocyanate which is capable of forming covalent bonds with nucleophiles, the reaction being known as a carbamoylation reaction. Butyl-isocyanate has been shown to inactivate the alcohol dehydrogenase enzyme of yeast (Twu and Wold, 1973) by covalently binding to sulphydryl groups on the enzyme. Babson and Reed (1978) also observed the inactivation of glutathione reductase by the isocyanate derived from bis(2-chloroethyl)nitrosourea. The evidence of the ease of formation of isocyanates from formamides and their reactivity, especially with SH groups, could suggest that production of probably very small amounts of isocyanate from NMF could account for the reactions with glutathione and also for the inhibition of aldehyde dehydrogenase observed after NMF exposure (5.I).

The obvious strain differences in susceptibility to NMF induced hepatotoxicity (8.VIII) raises several interesting points.

Firstly, if a metabolite of NMF was indeed responsible for the liver damage, it would appear that there was a qualitative or quantitative difference in metabolite formation between the different strains and that in BALB/c mice, the toxic metabolite is produced in larger amounts or at a faster rate. Species differences in drug metabolism are well known (Williams, 1971), but strain differences are rarely recorded. It is interesting to note that NMF is active against the TLX5 tumour (in CBA/Ca mice) and the M5076 (in BDF_1 mice) but showed no activity against the PC6A tumour in BALB/c mice. The antitumour activity of NMF - which is also unexplained - is believed to be due to a metabolite (I.I). It is conceivable that the difference in sensitivity of these three tumours to NMF may not be due to differences inherent in the tumours but may be due to differences in the metabolism of NMF between strains. It is possible that in BALB/c mice, metabolism of NMF leads to production of a hepatotoxic intermediate whereas in other strains, a larger quantity of a metabolite responsible for the antitumour activity is formed.

There are many experiments that could be performed to expand on the results described in this thesis. If NMF metabolites do react with glutathione, isolation and identification of the conjugates in the urine or plasma following NMF administration are undoubtedly valuable. To investigate further the route of C^1 unit metabolism for NMF by searching for intermediate substances, such as S-hydroxymethyl-glutathione, would also be useful. It would be advantageous to evaluate the theory that carbamoylation reactions are involved in NMF hepatotoxicity, but the high reactivity and short

half life of isocyanates *per se* would prevent any direct experiments on their ability to cause hepatotoxicity.

To support the theory that metabolic conversion of NMF to a reactive electrophilic species occurs *in vivo*, it is also necessary to investigate the irreversible binding of NMF or its metabolites to tissue macromolecules, such as protein, RNA or DNA, as discussed by Gillette (1974) and Pohl and Branchflower (1981). Using radiolabelled NMF, observation of covalent binding in the liver, together with a correlation between the changes in this feature with the severity of toxicity would provide evidence that reactive metabolites of NMF were involved. Such an approach was recommended by Mitchell *et al* (1982).

The results for the calcium uptake by mitochondria and microsomes requires further investigation. Firstly, measurements should be made of any change in total intracellular calcium ion content of the liver following administration of NMF. An increased intracellular calcium level would be consistent with the inhibition of uptake by mitochondria shown in these experiments. Secondly, to complete the experiments *in vitro* using isolated mouse hepatocytes, measurement of changes in intracellular calcium content after a short incubation period of cells with NMF would perhaps confirm the results found *in vivo*. Pencil *et al* (1983) assayed calcium uptake with microsomes isolated from hepatocyte suspensions. A similar assay for microsomes and mitochondria after incubation with NMF could yield interesting results. An investigation into the other mitochondrial functions such as uncoupling of oxidative phosphorylation, under the same conditions under which calcium

uptake was inhibited, would also yield further information on the mechanisms of the effect on Ca^{2+} uptake.

Following on from such a study using isolated hepatocytes, it would be interesting to investigate if NMF induced changes in calcium homeostasis in isolated tumour cells which were sensitive to the drug. An increased calcium content in the cells could be important in the explanation of the mechanisms of antitumour activity of NMF.

Possibly one of the most interesting experiments would be to determine any change in pyridine nucleotide status following NMF administration *in vivo* and *in vitro*, as suggested in 8.VI. Lehninger *et al* (1978) predicted that the pyridine nucleotide redox state was involved in the control of mitochondrial Ca^{2+} and this was later confirmed by Bellomo *et al* (1982). The action of glutathione reductase in the reduction of GSSG to GSH occurs with a parallel oxidation of NADPH to NADP+. The results of experiments with isolated mouse hepatocytes described here (8.I), following NMF incubation, showed a decrease in intracellular GSH, with a corresponding increase in extracellular GSSG. Orrhenius and Jones (1978) observed similar results following incubtion of rat hepatocytes with ethylmorphine and initially suggested that GSH oxidation may have been due to a depletion of NADPH. Subsequent measurement of NADPH concentration proved this assumption to be incorrect and the authors concluded that oxidation of GSH, catalysed by glutathione peroxidase, coupled to reduction of H_2O_2 was responsible. Chance *et al* (1978) described how hydroperoxide reduction by the action of GSH and glutathione peroxidase was coupled to NADPH oxidation by the activity of NADPH dependent glutathione reductase. Therefore

it is evident that measurement of the pyridine nucleotide redox state after NMF administration *in vivo* and *in vitro*, under the same conditions as described in the experiments in this thesis, might yield valuable information.

Lastly it might well prove useful to investigate further the structural changes in the liver and the ultrastructural changes in the cell following NMF administration. S.E.M. studies of liver cells after varying doses of NMF and times of administration could show the intracellular sites of damage. An indication of whether the mitochondria were indeed the initial areas of damage, following NMF treatment would therefore be possible. A preliminary experiment on the recovery of livers after NMF administration to mice has been described (8.VIII). It might also be informative to examine the state of livers several weeks after NMF administration to investigate if scarring of tissue or longer term degenerative changes occurred. A comparison of single with multiple doses of NMF might ultimately prove valuable in the clinical management of NMF induced hepatoxicity. Linked with this type of investigation, it would also be useful to investigate methods of prevention of damage, perhaps by co-administration or pretreatment of animals with substances such as α-tocopherol, which have been shown to reduce oxidative damage (Myers *et al*, 1976).

APPENDIX

Calculation of parameters for $^{14}CO_2$ exhalation experiments

1. Half life ($T_{\frac{1}{2}}$)

$$T_{\frac{1}{2}} = \frac{0.693}{\alpha \text{ or } \beta}$$

α = Slope of initial phase of profile (min^{-1})

β = Slope of terminal phase of profile (min^{-1})

2. Percentage of administered radioactivity exhaled per minute as $^{14}CO_2$ (% dose exhaled)

X c.p.m. = activity of sample vial (containing 2ml of trapping fluid)

y ml = total volume of trapping fluid in vessel at sampling time.

z μCi = activity of dose administered to mouse.

t min = time during which trapping fluid was collected.

1 nCi is assumed to be equivalent to 2.22×10^3 cpm

Activity in vessel collected over time, t, = $\frac{X \times y}{2}$ cpm

Activity exhaled per minute = $\frac{X \times y}{2 \times t}$ cpm

$$= \frac{X \times y}{2 \times t \times 2.22 \times 10^3} \text{ nCi}$$

Z μCi = $Z \times 10^3$ nCi

Percentage of original dose exhaled per minute as $^{14}CO_2$ = $\frac{X \times y}{2 \times t \times 22 \times Z \times 10^3}$

3. Total percentage of dose administered exhaled as $^{14}CO_2$ (total % of dose exhaled)

$\frac{X \times y}{2}$ cpm for all collections = $\leqslant \frac{X \times y}{2}$

Total % dose exhaled = $\leqslant \frac{X \times y}{2}$ / cpm injection

$$= \leqslant \frac{X \times y}{2} / Z \times 2.22 \times 10^6$$

4. Rate of $^{14}CO_2$ exhalation (nmoles per minute) at peak exhalation time (peak rate)

a mg = weight of drug weighed for injection

b ml = volume of original injection

c ml = volume of injection administered

d = molecular weight of the drug

p = % dose exhaled per minute at peak time

q = number of potential moles CO_2 exhaled from one mole of drug

$$\text{Peak rate} = \frac{a \times c \times q \times p \times 10^4}{b \times d}$$

q values for labelled compounds studied		d values
AP :	2	231
PCZ :	1	258
HMM :	6	210
Caffeine citrate:	1	386
NMF :	1	56
DMF :	1	70
Sodium formate :	1	68

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