THE METABOLISM AND HEPATOTOXICITY OF N-METHYLFORMAMIDE

by

Paul Gerard Pearson

A thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

in

ASTON UNIVERSITY, BIRMINGHAM

NOVEMBER 1985

`Be clever . . . and let who will be good´

Sean O´Casey, 1926

This thesis is dedicated to my parents

Aston University, Birmingham

The metabolism and hepatotoxicity of N-methylformamide

by

Paul Gerard Pearson

submitted for the degree of Doctor of Philosophy, 1985

Aspects of the metabolism and hepatotoxicity of the industrial solvent and investigational antitumour agent, N-methylformamide (NMF, $OHCNHCH_3$) have been evaluated in mice. NMF was metabolised extensively *in vivo*, depleted hepatic glutathione (GSH) stores, and caused centrilobular necrosis in the liver. Pretreatment of mice with N-acetylcysteine prevented the hepatic GSH depletion and hepatotoxicity. Conversely depletion of hepatic GSH prior to NMF administration lowered the hepatotoxic dose threshold of NMF.

Incubation of ^{14}C-NMF with mouse liver microsomes resulted in metabolic activation of NMF to a covalently bound species. The binding was dependent upon the presence of NADPH and was abolished by heat inactivation of the microsomal enzymes or by addition of GSH to the incubations. SKF 525A increased the binding of ^{14}C-NMF metabolites to microsomes and increased the severity of centrilobular necrosis following administration of NMF to mice. Pretreatment of mice with phenobarbitone did not increase the covalent binding *in vitro* or increase the severity of the hepatotoxicity. The parallels between the *in vitro* covalent binding and the *in vivo* hepatotoxicity indicate that NMF was activated to a reactive necrogenic species possible by a cytochrome P-450 independent pathway.

Administration of ^{14}C-NMF to Balb/C mice resulted in irreversible association of ^{14}C-NMF metabolite(s) with hepatic macromolecules. Similarities between the pattern of binding *in vitro* and the association with macromolecules *in vivo* suggested a role for covalent binding in the hepatotoxicity of NMF *in vivo*.

There were marked differences between strains of mice in their susceptibility to NMF hepatotoxicity. In the most susceptible strain (Balb/C) the extent of NMF metabolism, GSH depletion, and covalent binding was greater than in the less susceptible strains. These findings and the ability to potentiate or to protect against NMF hepatotoxicity by manipulation of hepatic GSH, implied a protective role of GSH in detoxifying the metabolically generated reactive species responsible for NMF hepatotoxicity.

Keywords: N-methylformamide hepatotoxicity, hepatic glutathione, covalent binding, metabolic activation, reactive species.

ACKNOWLEDGEMENTS

I wish to thank Dr. Andreas Gescher and Dr. Ernest Harpur for their encouragement and friendship shown to me throughout the course of this investigation.

I wish to thank Drs. Kestell and Threadgill for their assistance and helpful discussion.

I am particularly indebted to Dr. Iona Pratt who performed pathological examination of liver sections. I also wish to thank Beci Holt for her expert technical assistance in preparing the liver sections for examination.

I wish to acknowledge the Pharmaceutical Society of Great Britain, who provided the financial support for this investigation.

Finally, I wish to thank Caroline Etchells for kindly and efficiently typing this thesis.

CONTENTS

SECTION 4: RESULTS

List of Figures

List of Tables

ABBREVIATIONS

ALT	L-alanine aminotransferase
AP	aminopyrine.
AST	L-aspartate aminotransferase
BSO	D, L-buthionine S,R, sulphoximine
DEM	diethylmaleate
DMF	N,N-dimethylformamide
F	formamide
GLC	gas chromatography
GSH	reduced glutathione
GSSG	oxidised glutathione
HMF	N-hydroxymethylformamide
HNMF	N-hydroxymethyl, N-methylformamide
HPLC	High performance liquid chromatography
i.p.	intraperitoneal
NAC	N-acetyl-L-cysteine
NADP(H)	nicotinamide adenine dinucleotide phosphate (reduced form)
NAD(H)	nicotinamide adenine dinucleotide (reduced form)
NAPQI	N-acetyl-p-benzoquinoneimine
NEF	N-ethylformamide
NMF	N-methylformamide
NPT	Non-protein thiols
PB	phenobarbitone
p.o.	oral
s.d.	standard deviation
SDH	sorbitol dehydrogenase
s.e.mean	standard error of the mean

SKF525A	2-diethylaminoethyl 2,2-diphenyl valerate; proadifen
vitamin E	dl-α-tocopherol
^{14}C-DMF	^{14}C N,N-dimethylformamide (labelled in formyl moiety)
$^{14}CH_3$-NMF	^{14}C N-methylformamide (labelled in methyl moiety)
O^{14}C-NMF	^{14}C N-methylformamide (labelled in formyl moiety)

SECTION 1

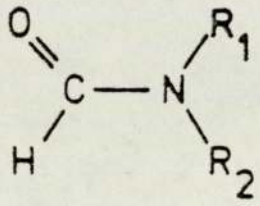
INTRODUCTION

1.1 Antitumour activity of N-alkylformamides

Interest in the biological properties of N-methylformamide NMF (Fig 1.1. I) arose in the 1950's when its antitumour activity was first demonstrated against the murine sarcoma 180 (1). A study of the structure-activity requirements in over 100 formamides and related compounds, demonstrated that NMF was the most potent inhibitor of the growth of Ehrlich ascites tumour cells (2). Exchanging the methyl moiety for an ethyl moiety (NEF, Fig 1.1. IV) completely abolished the activity. This structure-activity relationship has also been observed for the N-methyl containing antitumour agents hexamethylmelamine, aryldimethyltriazines and procarbarazine (3). Furthermore, the activity of the dimethylanalogue, DMF (Fig 1.1. V) and the N-desmethylanalogue, F (Fig 1.1. III) were marginal, indicating a precise structural requirement for antineoplastic activity.

More recently the antineoplastic structure-activity requirements have been evaluated against the mouse reticulum cell sarcoma M5076, and TLX5 lymphoma (4,5). The ability of NMF to retard the growth of two human colon cancer cell lines, HCT-15 and DLD-2, xenografted in nude mice has been reported (6). NMF also exhibited antitumour activity in MX1 mammary tumour, CX1 colon tumour and LX1 lung xenograft in the National Cancer Institute human tumour xenograft panel (7).

The mode of antitumour activity of NMF is unknown. Studies in the 1950's suggested that NMF is able to interfere with the metabolism of C_1 fragments by inhibition of dihydrofolate reductase, while the administration of either citrovorum factor or folic acid did not reduce the antitumour activity or host toxicity (1). Also, interference with the synthesis of nucleic acid bases by NMF has been



			R_1	R_2
I	N-methylformamide	(NMF)	CH_3	H
II	N-hydroxymethylformamide	(HMF)	CH_2OH	H
III	formamide	(F)	H	H
IV	N-ethylformamide	(NEF)	C_2H_5	H
V	N,N-dimethylformamide	(DMF)	CH_3	CH_3
VI	N-hydroxymethyl, N-methylformamide	(HNMF)	CH_2OH	CH_3

Figure 1.1 Chemical Structures of some formamide derivatives

reported (1,8). The requirement of a N-methyl group for the in vivo antitumour activity is of interest, as several other antitumour agents exhibit this feature. For example, dacarbazine (5- [3,3 - dimethyl - 1 - triazeno] imidazole - 4 - carboxamide), procarbazine (1- methyl - 2 - p - [isopropylcarbamoyl] benzylhydrazine) and hexamethylmelamine (HMM; 2, 4, 6 - tris [dimethylamino] - 1, 3, 5 - triazine) undergo metabolism to stable N-hydroxymethylmetabolites (3) which were considered to be the ultimate anti-neoplastic species as they may liberate cytotoxic formaldehyde or react directly with critical cellular nucleophiles. The N-hydroxymethyl moiety may also undergo dehydroxylation to a methylene iminium ion which is conceivably a reactive species (9). The formation of iminium species from carbinolamine intermediates has been proposed as the activation pathway for nicotine (10, 11), methapyrilene (12) and dimethylaminoazobenzene(13). Furthermore, the role of N-hydroxymethylpentamethylmelamine in the activation of HMM has been demonstrated quite clearly (14). An analogous scheme for the metabolic demethylation of NMF has been proposed (Figure 1.2) in which NMF undergoes C-hydroxylation to yield a stable carbinolamine (HMF) which can decompose to liberate either, formamide and formaldehyde, or form a methylene iminium species. This is an attractive hypothesis in view of the poor antitumour activity of NEF, the N-ethyl analogue that cannot be metabolised in this way. However, an investigation into the antitumour activity of HMF did not support the hypothesis that HMF is the active metabolite of NMF (15). HMF was inactive against the TLX5 lymphoma and sarcoma 180 in vivo, and had only marginal activity against the M5076 reticulum cell sarcoma. NMF on the other hand showed pronounced activity against all three tumour lines.

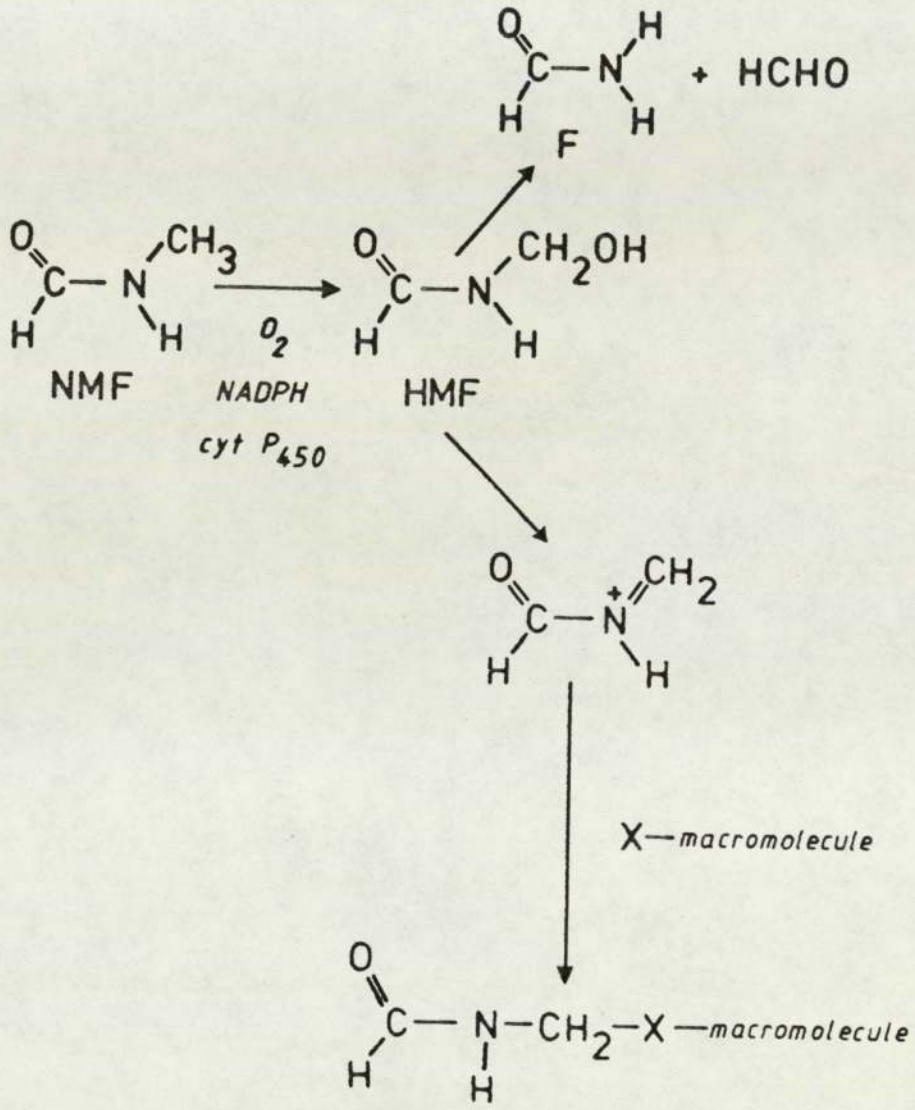


Figure 1.2 Proposed scheme for the demethylation of NMF to F and formaldehyde or to the iminium species which can react with nucleophilic moities (x) of macromolecules

Furthermore, no cross resistance was observed between NMF and dacarbazine or procarbazine when tested against the TLX5 lymphoma previously made resistant to the latter two agents indicating that they act via different mechanisms (4).

More recently, interest has focused upon the ability of NMF to induce terminal maturation of undifferentiated tumour cell lines in culture (16). The chemical induced conversion to a more benign phenotype is associated with altered morphology, loss of clonogenicity in soft agar, and a reduction of tumourigenicity (17). The ability of NMF to induce differentiation is a property shared by other polar solvents, including DMF and dimethylsulphoxide (16). For the induction of differentiation, concentrations of NMF in the range of 10^{-1}M are required. The peak plasma concentration achieved in mice after administration of an effective antitumour dose of 400mgkg^{-1} NMF is 7mM (18), and in a phase I clinical trial, peak plasma levels of NMF approached only 1mM (19). The disparity between the plasma concentration of NMF achieved *in vivo*, and those required to induce terminal differentiation *in vitro*, raises doubt as to whether NMF acts as an antitumour agent *in vivo* by inducing tumour cells to differentiate.

On the basis of the pronounced antitumour activity and the absence of myelotoxic properties (20, 21), an unusual feature of an antineoplastic agent, NMF has been evaluated in phase I (22, 23) and phase II clinical trials (24). In the first clinical study in 1956, all 5 patients receiving NMF exhibited symptoms of hepatotoxicity (25). In a recent phase I trial, hepatomegaly, reversible transaminites and an obstructive pattern of liver function disorder was observed (22). Hepatotoxicity was dose limiting in a phase II

clinical study in patients with advanced colorectal carcinoma. The patients experienced a reversible transaminites and hyperbilirubinaemia (24), pathological changes commonly observed in the phase I clinical trials of NMF. It is unclear if the hepatotoxicity is dose limiting, as many patients also experienced severe nausea (22). However, the clinical importance of the hepatotoxicity of NMF, and the lack of understanding of the underlying mechanism of toxicity suggested the need for further investigation.

1.2 Hepatotoxicity of N-alkylformamides

During the course of the phase I and phase II clinical trials, the hepatotoxic potential of NMF has been an undesirable side effect (22-24). Since the first clinical trial, nearly 35 years ago (25) studies have been undertaken to characterise the nature of the hepatotoxicity. It has been established that NMF is hepatotoxic in mice (6, 20, 21, 26), rats (27, 29) and dogs (20). The dimethylanalogue of NMF, DMF is also hepatotoxic in experimental animals (28-31). DMF is an industrial solvent used extensively in the manufacture of polyacrylnitrile fibres and synthetic leather, and also in the purification of acetylene, and in the refinement and desulphuration of mineral oils (32). Workers occupationally exposed to DMF, either through inhalation of vapours or dermal contact, have developed symptoms of hepatotoxicity (33) and a disulfiram type reaction of alcohol intolerance (34, 35). The latter effect has been attributed to DMF induced alteration in aldehyde metabolism (36, 37), and non-competitive inhibition of liver alcohol dehydrogenase (38). It was found that after equimolar doses of DMF and NMF, blood acetaldehyde levels, in male rats following ethanol administration, were elevated compared with controls. The maximum elevation occurred 3h and 18h after NMF and DMF administration respectively (36), and no elevation was observed when ethanol was administered 3h after DMF. The latency required for DMF to produce this effect may indicate that metabolism to NMF or another unknown species is a pre-requisite for enzyme inhibition. A single case of NMF induced alcohol flushing was seen in a phase I trial, in a patient who drank a glass of wine after a course of NMF treatment (22).

The hepatotoxicity of NMF has been investigated in Balb/C

(21, 26) and BDF_1 mice (2). Plasma SDH activity, an indicator of liver cell injury, was measured and reached a peak 24h after administration of a hepatotoxic dose of NMF to mice. Plasma SDH activity, was markedly increased after a single i.p. injection of $150mgkg^{-1}$ in Balb/C mice or $800mgkg^{-1}$ NMF in BDF_1. This result indicates a marked strain difference in the susceptibility of mice to the hepatotoxicity of NMF. The increase in plasma SDH levels at doses of $200mgkg^{-1}$ or greater in Balb/C mice, was accompanied by histopathological evidence of damage, and necrosis in the centrilobular region (26). A correlation has been established between SDH elevation and histopathological evidence of damage in rat livers following NMF and DMF administration (28).

In Swiss mice doses of $374mgkg^{-1}$ or more of NMF administered i.p. daily for 21 days caused centrilobular necrosis and an increase in the number of mitotic cells in the liver (6).

Chronic administration of NMF to BDF_1 mice was less hepatotoxic than the same total dose administered as a single i.p. injection. Furthermore chronic low dose administration of NMF to M5076 bearing BDF_1 mice was therapeutically superior to acute high dose administration, no loss of antitumour activity was observed, and the hepatotoxicity was avoided. These considerations contributed to the rationale for testing this agent on a chronic alternate day schedule in a phase I clinical trial conducted in 1983 (22).

Co-administration of DMF ($479mgkg^{-1}$) and NMF ($387mgkg^{-1}$) delayed the onset of NMF induced hepatotoxicity (29) which was attributed to DMF inhibiting the metabolism of NMF. This is interesting in view of the suggestion that DMF may inhibit its own

biotransformation in vivo (42). (See section 1.3).

Until recently, the hepatotoxicity of DMF had been attributed to NMF, as a toxic metabolite of DMF (37, 39, 40). However, what was identified as NMF by G.L.C (27, 43), in the plasma and urine of animals exposed to DMF, has recently been unequivocally shown to be HNMF, the immediate product of C-hydroxylation of DMF (29, 44, 45). It has also been proposed that NMF is the metabolic precursor of another toxicant which is responsible for the hepatotoxicity of DMF (29). This suggestion would account for the greater potency of NMF than DMF as a hepatotoxin (26, 27, 28). Furthermore, LD_{10} values in tumour bearing BDF_1 mice administered formamide derivatives daily over a nine day period, indicated that the LD_{10} for NMF ($312mgkg^{-1}$) was lower than for DMF ($1080mgkg^{-1}$), suggesting that the whole body toxicity of NMF is much greater than DMF (15).

Many hepatotoxins of diverse chemical structure eg. thioacetamide, paracetamol, bromobenzene and carbontetrachloride require metabolic activation to a reactive species to exert their deleterious effects. The pattern of NMF induced hepatotoxicity suggests that a reactive metabolite may be involved. In an in vivo - in vitro bioassay, NMF was cytotoxic only at concentrations in the 10^{-1}M range (4). However, levels achieved in the plasma of mice, after the administration of a hepatotoxic dose of $400mgkg^{-1}$ did not exceed 10^{-2}M. In addition a single i.p. injection of NMF caused an acute depletion of hepatic non-protein thiols (NPT) in Balb/C mice (4). The depletion of NPT was dose related and specific to NMF when compared with a series of formamides. In a separate study in rats, doses of $1000mgkg^{-1}$ NMF caused a 32% depletion of GSH, compared with controls. DMF produced no depletion of hepatic GSH (28). Incubation

of 7mM, NMF with freshly isolated hepatocytes caused a depletion of intracellular GSH after 80 minutes (26), compared with controls. These observations, and the delay of greater than 12h for the appearance of indices of toxicity in Balb/C mice following administration of a hepatotoxic dose of NMF (26), suggest the involvement of a reactive species in the hepatotoxicity of NMF.

The NMF induced liver necrosis is restricted to the centrilobular region of the liver. Hepatocytes located in the central region of the liver lobule are particularly susceptible to injury caused by toxic metabolites formed by the microsomal cytochrome P-450 system (46). This is attributed partly to the increased cytochrome P-450 activity of the cells in the centrilobular region (47), and partly due to a lower GSH content in the centrilobular than periportal hepatocytes (48). Hence, the pattern of centrilobular necrosis induced by NMF, also supports the hypothesis that NMF is metabolically activated to a reactive species. The reactive species would deplete GSH and then covalently modify potentially critical nucleophilic sites to the detriment of the centrilobular hepatocytes.

Recent studies indicate that liver mitochondria may be a target for the reactive metabolites, as NMF potently and selectively inhibited Ca^{2+} sequestration by liver mitochondria isolated from Balb/C mice pretreated with hepatotoxic doses of NMF, 1 hour before mitochondria isolation. The effect was dose-related, and was not observed at non-hepatotoxic doses. Other formamide analogues, DMF, HMF or F did not inhibit the mitochondrial sequestration of calcium within 1 hour of administration of equimolar doses compared with NMF. The inhibitory effect of NMF on the mitochondrial Ca^{2+} pump was not

due to general damage of mitochondrial function as the mitrochondrial respiration was not adversely affected by NMF. This implies that the effect of NMF upon mitochondrial calcium sequestration may be a primary toxic event which subsequently leads to cell death and liver necrosis.

1.3 Metabolism of N-alkylformamides

It is only in the last 10 years that interest in the fate of both NMF and DMF in biological systems has emerged. The absorption, distribution, metabolism and excretion of both of these N-alkylformamides have been studies in experimental animals (18, 41, 42, 50) and in man (19, 51, 52). The fate of DMF is of interest due to the concern over occupational exposure to this agent. Interest in NMF is primarily related to its antitumour activity and hepatotoxicity, but also as a potential metabolite of DMF.

Occupational exposure to DMF may lead to uptake via the cutaneous and pulmonary routes, of which the cutaneous route appears to be the most important when no protective measures are taken (53). Following exposure DMF is rapidly metabolized and disappears from the plasma within a few hours of exposure (51). The major metabolite, HNMF, which until recently was considered to be NMF by G.L.C. determination (see Section 1.2) (28) is eliminated in the urine within 24 hours of exposure. The precise time course of urinary excretion of this metabolite is dependant upon the duration and extent of exposure (51). In several reports measurement of urinary HNMF (as NMF by GC) has proved a useful index of exposure to DMF (53-56).

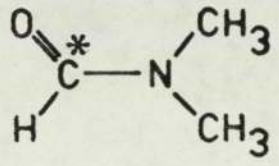
Studies in mice (44) and rats (28, 45) have suggested that the principle urinary metabolite of DMF is not NMF, but HNMF, a proposed carbinolamine intermediate formed by enzymatic C-hydroxylation of DMF. Upon G.L.C. analysis of urine samples HNMF decomposed to NMF. In a similar manner the carbinolamine HMF formed by C-hydroxylation of NMF, decomposed to yield formamide (18). Alkaline hydrolysis of the urine from mice administered DMF led to the liberation of formaldehyde, which was not present in the urine of

control animals (44). In the urine of rats administered DMF 1000mgkg^{-1}, the amount of formaldehyde liberated upon alkaline hydrolysis correlated well with the amount of HNMF present as measured by G.L.C. Authentic HNMF also released formaldehyde only upon alkaline hydrolysis (44). The presence of HNMF in the urine of rats administered DMF has been demonstrated unequivocally by direct chemical-ionization mass spectral analysis and by high field ^{1}H-NMR spectroscopy. The corresponding carbinolamine from NMF, HMF, has been identified tentatively in the urine of mice by TLC-autoradiography (41).

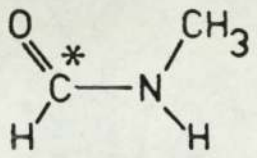
Administration of 496mgkg^{-1} ^{14}C-DMF labelled in the formyl moiety (Figure 1.3a), to mice indicated that ^{14}C-DMF or its metabolite(s) are rapidly excreted in the urine, more than 82% of the dose was excreted in 24 hours (44). Of the total dose administered 4.9% was excreted as unchanged DMF and 56% as HNMF.

The excretion profile in the urine of rats was qualitatively similar to that obtained in mice, but DMF was metabolised and eliminated at a slower overall rate in the rat (28) than in the mouse (44). Also the reduced rate of excretion of HNMF in the rat when the dose of DMF was sequentially raised indicated that DMF may inhibit its own biotransformation (42). The ability of DMF to increase pentobarbital induced sleeping time in mice may reflect inhibition of pentobarbital metabolism (28).

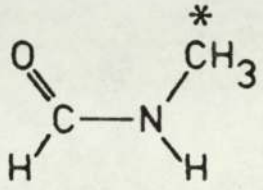
In mice, NMF is metabolised mainly to carbon dioxide, which is exhaled and to methylamine which is excreted in the urine (41). Following a dose of 400mgkg^{-1} NMF administered to CBA/CA mice, 26% of the dose was excreted unchanged in the urine (18), and 30% as the



OH^{14}C-DMF



OH^{14}C-NMF



$^{14}CH_3$-NMF

Figure 1.3 Structures of ^{14}C-DMF, and ^{14}C-NMF analogues. (* = position of ^{14}C label)

major metabolite methylamine (41). G.L.C. analysis of the urine of mice receiving NMF revealed that HMF and/or F, was a minor metabolite consisting of less than 2% of the dose (18).

$^{14}CH_3$-NMF was administered i.p. to CBA/CA mice and the plasma disposition of parent drug and ^{14}C metabolites were examined (18), when it was found that during the first 24 hours, the radioactivity-time curve and the plasma profile of unchanged NMF were superimposable. After 24 hours, NMF could not be detected in the plasma by G.L.C., however radioactivity derived from $^{14}CH_3$-NMF was still detectable in the plasma up to eight days post drug administration. This species was cleared from the plasma with an elimination half life of 71.1h (18).

After administration of 400mgkg^{-1} ^{14}C-NMF labelled in either the methyl moiety ($^{14}CH_3$-NMF) or formyl moiety (OH^{14}C-NMF), 39% and 14% of the dose was exhaled, respectively, in the breath of CBA/CA mice within 24 hours (44). The sequence of events giving rise to CO_2 from the methyl moiety is depicted in Figure 1.4.

A more detailed study of the pulmonary clearance of $^{14}CO_2$ derived from $^{14}CH_3$-NMF in CBA/CA revealed that 3% of the administered dose of 400mgkg^{-1} was cleared as $^{14}CO_2$ over seven hours at a constant rate of 0.007% per minute (18). This is unusual as the $^{14}CO_2$ exhalation profiles seen with other N-methyl containing xenobiotics eg. hexamethylmelamine, aminopyrine and caffeine, often reflect the plasma disposition of the parent drug (57). This difference has been accounted for by a saturable metabolic clearage of the N-C bond of HMF so that CO_2 is liberated at a rate which is independant of the plasma NMF concentration (18). Alternatively, the long terminal half-life and zero order exhalation of $^{14}CO_2$ may reflect elimination of

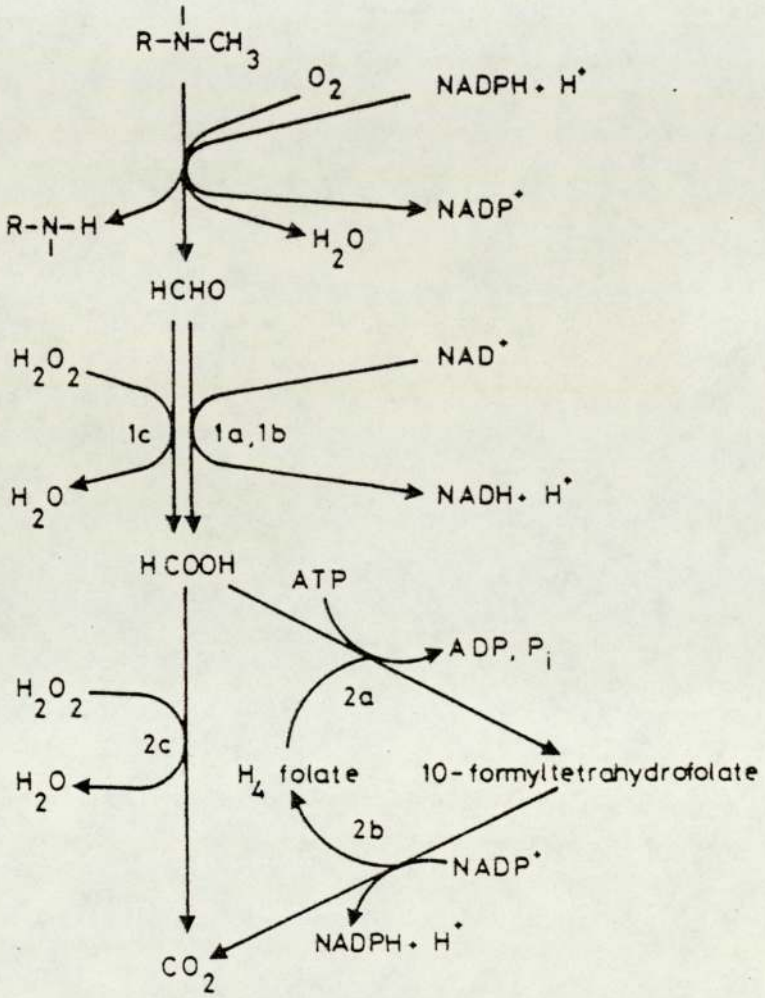


Figure 1.4 Simplified reaction sequence for drug N-demethylation (Cytochrome-P-450-dependant monooxygenase) to formaldehyde formate and CO_2-production.

Reactions are: 1a, formaldehyde dehydrogenase (GSH), 1b, aldehyde dehydrogenase; 1c, catalase (peroxidatic mode); 2a, 10-formyltetrahydrofolate synthetase; 2b, 10-formyltetrahydrofolate dehydrogenase; 2c, catalase (peroxidatic mode). (From reference 63).

radioactivity from C_1 pools.

As early as 1955, a series of events were described during the N-demethylation of dimethylglycine in which C_1 units were generated with the oxidation level of formaldehyde (60). These one carbon units which were described as 'active formaldehyde' were later shown to be N_5,N_{10}-methylenetetrahydrofolate (61). One carbon fragments generated in this manner are required for a variety of cellular reactions, including the denovo synthesis of purines and pyrimidines (62).

Free formaldehyde generated during cytochrome P-450 dependent drug N-demethylation may also enter one carbon pools (63) (See Figure 1.4). This arises by conversion of formaldehyde via formate to N_{10}-methyltetrahydrofolate "active formate", which can be reduced to N_5, N_{10}-methylenetetrahydrofolate (64), and be directly utilized for the biosynthesis of serine and thymine (62).

Despite extensive <u>in vivo</u> biotransformation of NMF, it has not been possible to demonstrate metabolism <u>in vitro</u> by liver fractions, using chromatographic techniques (4). Incubation of NMF with liver preparations, either whole homogenate, 9000g supernatant or microsomes failed to produce formaldehyde as a metabolite (4). Similarly G.L.C. analysis did not show disappearance of substrate or appearance of F, the product of N-demethylation. This is contrary to a previous report (39) that claimed 10% transformation of 1.7mM NMF to formaldehyde after incubation with a rat liver homogenate for 2 hours. However, the level of metabolically generated formaldehyde reported in this study (39) was close to the detection limit of the colorimetric assay employed (65).

The pattern of toxicity *in vivo* is consistent with metabolic activation of NMF by the liver (See Section 1.2). This theory is supported to a certain extent by tissue distribution studies. Following administration of $^{14}CH_3$-NMF ($400mgkg^{-1}$) to mice, radioactivity was distributed principally into the liver, then kidney, then lungs in descending order (139). The peak levels of ^{14}C-NMF metabolites of $^{14}CH_3$-NMF in liver occurred after 8 hours, which was considerably later than the plasma peak for parent drug and ^{14}C-NMF metabolites which occurred 2 hours after an i.p. dose of $400mgkg^{-1}$ $^{14}CH_3$-NMF (18). Measurement of NMF in various organs by G.L.C. indicated that the liver contained the lowest levels of NMF (50). The disparity between the levels of drug derived radioactivity and parent drug has been attributed to hepatic biotransformation of NMF (17).

It is possible that NMF is biotransformed at an extrahepatic site. However, incubation of NMF with kidney fractions, one of the principle organs into which NMF is distributed, failed to demonstrate significant metabolism (59).

In summary despite the extensive biotransformation of NMF *in vivo*, metabolism appears to occur not at all or only to a very minor extent *in vitro*.

1.4 Metabolism and function of glutathione in drug toxicity

The ubiquitous tripeptide glutathione (GSH, γ-glutamyl cysteinylglycine, Figure 1.5) was first described in 1885 as `philothion´ by De Ray Parlade. For nearly a century the metabolism and function of GSH has captured the imagination of many workers, and has been reviewed extensively (66-69).

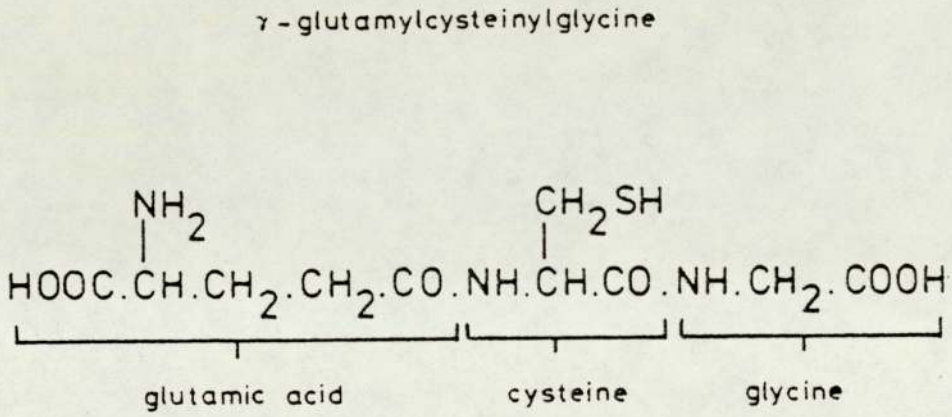


Figure 1.5 The structure of glutathione

GSH is found in most cells, often in millimolar concentrations, and at much lower concentrations in extracellular fluids such as blood and plasma. GSH functions as an intracellular reductant and plays important roles in catalysis, metabolism, amino acid transport and protects cells against free radicals, reactive oxygen species and toxic compounds of endogenous and exogenous origin.

The metabolism and functions of GSH are outlined schematically in Figure 1.6 (68). GSH is synthesised intracellularly

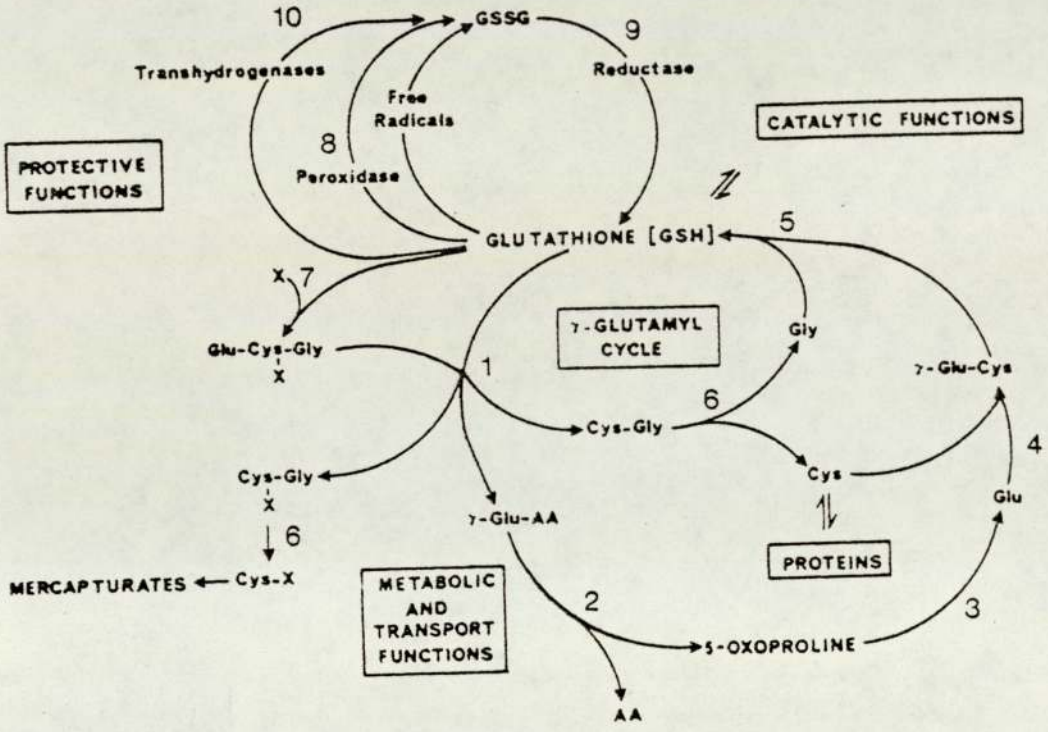


Figure 1.6 Outline of the metabolism and function of glutathione

Enzymes: 1, γ-glutamyltranspeptidase; 2, γ-glutamylcyclotransferase; 3, 5-oxoprolinase; 4, γ-glutamylcysteine synthetase; 5, glutathione synthetase; 6, dipeptidase; 7, glutathione S-transferase; 8, glutathione peroxidase; 9, glutathione reductase; 10, transhydrogenases; AA, amino acids; X, compounds that react with glutathione to form conjugates that lead to mercapturic acid formation (modified from reference 68).

in two stages by the consecutive action of γ-glutamylcysteine synthetase (enzyme 4) and GSH synthetase (enzyme 5) at the expense of ATP. The enzyme γ-glutamylcysteine synthetase is subject to feedback inhibition by GSH. The breakdown of GSH is catalysed by the membrane bound glycoprotein γ-glutamyltranspeptidase (enzyme 1). The enzyme interacts with GSH, and also GSSG and S-substituted glutathione, to catalyse the transfer of the γ-glutamyl moiety to amino acid acceptors. The cysteinylglycine liberated is cleaved by a dipeptidase (enzyme 6) to its constitutive amino acids. The γ-glutamyl amino acids produced by γ-glutamyltransferase are substrates for γ-glutamylcyclotransferase (enzyme 2) which converts these compounds to the corresponding amino acids and 5-oxo-L-proline. 5-oxo-L-proline is a substrate for ATP dependent oxoprolinase (enzyme 5) which liberates L-glutamate for the resynthesis of GSH. The reactions catalyzed by the six enzymes described above constitute the γ-glutamyl cycle which accounts for the intracellular turnover of GSH. Two of the enzymes in the cycle, γ-glutamyltransferase and dipeptidase function in the metabolism of S-substituted GSH derivatives, which may be formed non-enzymatically by reaction of GSH with certain electrophilic compounds or by GSH S-transferases (enzyme 7) (75-77).

The liver cytochrome P-450 monooxygenase system can generate a variety of reactive metabolites including reactive oxygen species ($O_2^{\cdot-}$ and H_2O_2) aldehydes (HCHO + CH_3CHO) and electrophiles. These are possibly detrimental to the integrity and function of the hepatocyte unless inactivated by further metabolism. GSH may detoxify such species in three ways (Figure 1.7):- 1). As a nucleophile in conjugation reactions (enzyme 7): 2). As a reductant in the reaction of GSH peroxidase (enzyme 8): 3). As a cofactor in aldehyde and α-

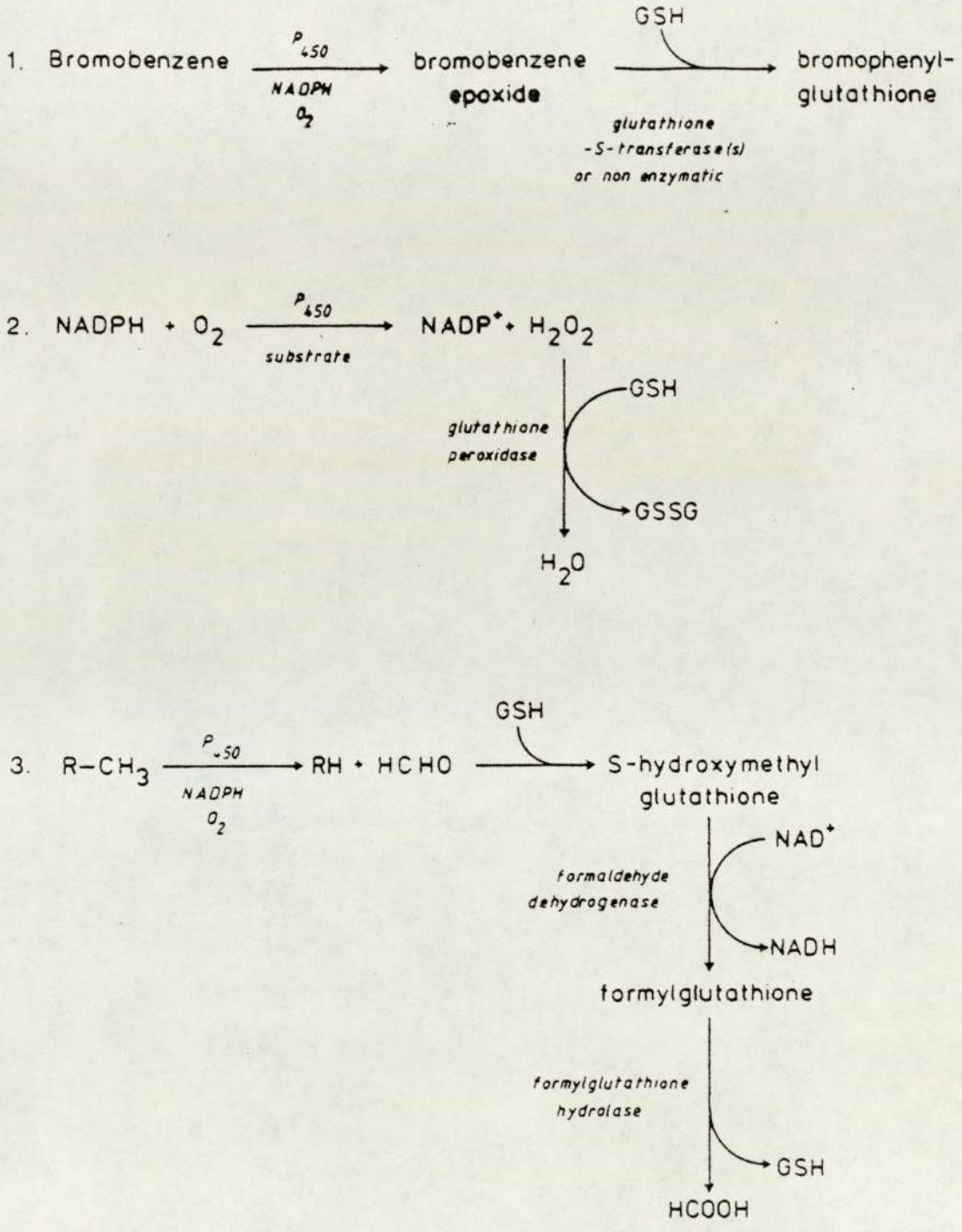


Figure 1.7 The role of glutathione in drug detoxification.

1) GSH conjugation reaction

2) GSH as a reductant with GSH peroxidase

3) GSH as a cofactor; aldehyde oxidation

ketoaldehyde oxidation.

An example of the first of the three reactions is the detoxification of bromobenzene (Figure 1.7.1). Studies with paracetamol and halogenated benzenes has shown that the hepatotoxicity of such compounds is preceeded by hepatic GSH depletion and prevented by either stimulation of GSH biosynthesis (71, 72), or by the presence of thiol containing amino acids (73, 74). In the case of bromobenzene, the 3,4-epoxide is detoxified by GSH transferase to bromophenylglutathione, which is subsequently excreted in the urine as the mercapturate (Figure 1.7).

In the second of the three protective roles of GSH (Figure 1.7.2), GSH peroxidase protects the liver from damage caused by H_2O_2 liberated as a by product of microsomal drug oxidation. The H_2O_2 arises from $O_2^{\cdot-}$ released from the oxy-complex of cytochrome P-450, which dismutates either spontaneously or under catalysis by superoxide dismutase to H_2O_2. GSH peroxidase utilizes the reducing properties of GSH to convert H_2O_2 to H_2O, with the concomitant oxidation of GSH to GSSG. The GSSG can subsequently be reduced by the NADPH-dependent flavoprotein GSH reductase (Figure 1.6, enzyme 9). Lipid peroxidation may also be prevented by the micronutrients selenium and α-tocopherol (Vitamin E). Two forms of GSH peroxidase exist, one of which is dependent upon selenium for activity (78-80).

The third example of the protective role of GSH, is as a cofactor for formaldehyde dehydrogenase (See Section 1.3), (Figure 1.4 and 1.7.3) N-Demethylation of drugs by the hepatic cytochrome P-450 system results in the production of formaldehyde. The general toxicity and hepatotoxicity of formaldehyde has recently been reviewed (81-83). The liberated formaldehyde reacts non-enzymatically with GSH

to form S-hydroxymethylglutathione, which is subsequently converted to S-formylglutathione by glutathione dehydrogenase. A second enzyme, S-formylglutathione hydrolase, converts S-formylglutathione into formic acid and GSH. It appears that under conditions in which S-hydroxymethylglutathione production is increased, the capacity of glutathione dehydrogenase may be exceeded. An increase in biliary efflux of GSH from rat liver has been observed in rats administered aminopyrine. It was hypothesised that formaldehyde formed in excess of its capacity to be metabolised is released into the bile as S-hydroxymethylglutathione, which then dissociates into GSH and HCHO (84).

In the context of this investigation, the reactions of GSH with xenobiotics and the role of the γ-glutamyl cycle are of great importance. The former are of interest as they provide an insight into the mechanism of reaction between GSH and metabolites of NMF. A clear understanding of the latter will facilitate selective manipulation of hepatic GSH status to explore the role of GSH in the hepatotoxicity of NMF. How this can be achieved is outlined in the following. It is possible to manipulate hepatic GSH levels by either depletion of GSH stores or by stimulation of GSH biosynthesis. In rats depletion of hepatic GSH increased the toxicity of bromobenzene (71). Conversley pretreatment of mice with N-acetylcysteine protected against the hepatotoxicity of paracetamol (72, 73).

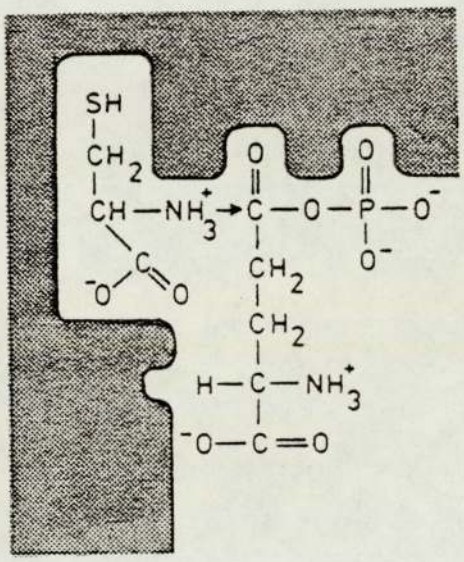
Hepatic GSH can be depleted by the administration of compounds which react with GSH either directly or under catalysis by glutathionetransferases, of these the most widely used is the α,β-unsaturated carbonyl compound diethylmaleate (DEM) (85).

DEM also produces effects unrelated to GSH depletion, including inhibition of some drug metabolising enzymes, but also stimulation of aniline and acetanilide hydroxylase activity (86). Clearly, the effects of DEM on drug metabolism are undesirable when evaluating the influence of DEM-induced GSH depletion on the toxicity of compounds which are metabolically toxified.

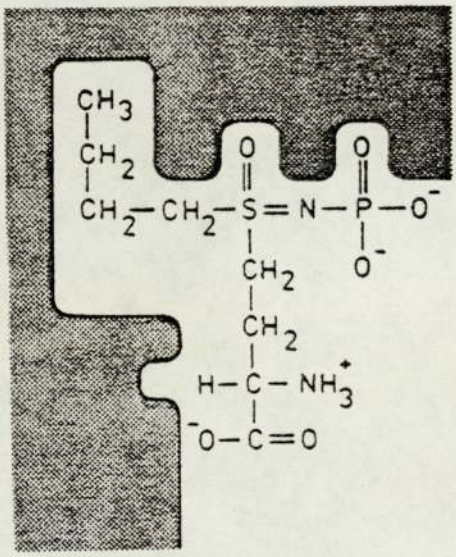
A different approach to depleting hepatic GSH is to employ inhibitors of GSH biosynthesis (67, 68, 87). GSH homeostasis is maintained by balancing the rate of GSH utilization with the rate of GSH biosynthesis. GSH biosynthesis is inhibited by buthionine sulphoximine (BSO) an inhibitor of γ-glutamylcysteine synthetase, (See Figure 1.6, enzyme 4). BSO is structurally similar to γ-glutamylcysteine (Figure 1.8), the enzymatic product of γ-glutamate and cysteine. BSO is phosphorylated by ATP to yield enzyme bound buthionine sulphoximine phosphate, the actual inhibitor. Buthionine sulphoximine phosphate mimics the transition state formed between γ-glutamyl phosphate, the natural enzyme intermediate, and cysteine.

BSO possesses two centres of asymetry (at the α-C and S), and consequently the synthetic product is a mixture of four isomers. Studies with methionine sulphoximine suggest that of the four isomers of DL-buthionine-S,R-sulphoximine, only the L, S isomer is phosphorylated by the enzyme (87).

Doses of 1600mgkg^{-1} BSO depleted hepatic GSH in C3H mice to 25% of control values within 4 hours, γ-glutamylcysteine synthetase activity and GSH levels returned to control values within 16 hours (82). The rate at which the level of GSH declines reflects its rate of utilization, which is comparable with the rate of GSH efflux from cells (68, 89). BSO rapidly depletes hepatic GSH to approximately 17-



CYSTEINE + γ-GLU-P



BUTHIONINE SULPHOXIMINE PHOSPHATE

Figure 1.8 The mode of action of BSO

BSO is phosphorylated and binds to the active site of γ-glutamylcysteine synthetase, and mimics the transition state between γ-glutamylphosphate the natural enzyme intermediate and cysteine.

20% of control, and further decreases occur more slowly. This finding is partly related to the relatively slow turnover of the mitochondrial pool of GSH, which does not rapidly equilibrate with cytosolic GSH (68, 90). The mitochondrial GSH pool also appears to be resistant to DEM induced hepatic GSH depletion (91).

BSO, unlike DEM, has no effect upon hepatic drug metabolism (88, 92), and is a useful probe to evaluate the role of GSH in the mechanism of hepatotoxicity of compounds that require metabolic activation as a prerequisite for toxicity.

The γ-glutamyl cycle may also be exploited to elevate intracellular GSH, by delivering prodrugs of cysteine. The enzyme 5-oxo-L-prolinase. (Figure 1.6, enzyme 3) catalyses the conversion of 5-oxoproline to L-glutamate. Replacing the 4-methylene moiety of 5-oxoproline with a sulphur atom produces L-2-oxothiazolidine 4-carboxylate (OTZ) (93, 94). OTZ is a good substrate for 5-oxoproline, which converts it via the unstable α-carboxy-L-cysteine to L-cysteine (Figure 1.9) (68). Administration of the thiazolidine to mice stimulated GSH biosynthesis and protected against the hepatotoxicity of paracetamol (95, 96). N-acetylcysteine also protected against paracetamol toxicity, however it was less effective in promoting GSH biosynthesis (95).

Administration of GSH alone to animals does not directly elevate hepatic GSH as it does not appear to be transported into cells. However, the monomethyl and monoethylester of GSH permeate cells and are subsequently hydrolysed to GSH (97), the GSH monomethylester has been shown to protect against the hepatotoxicity and GSH depletion caused by paracetamol (97).

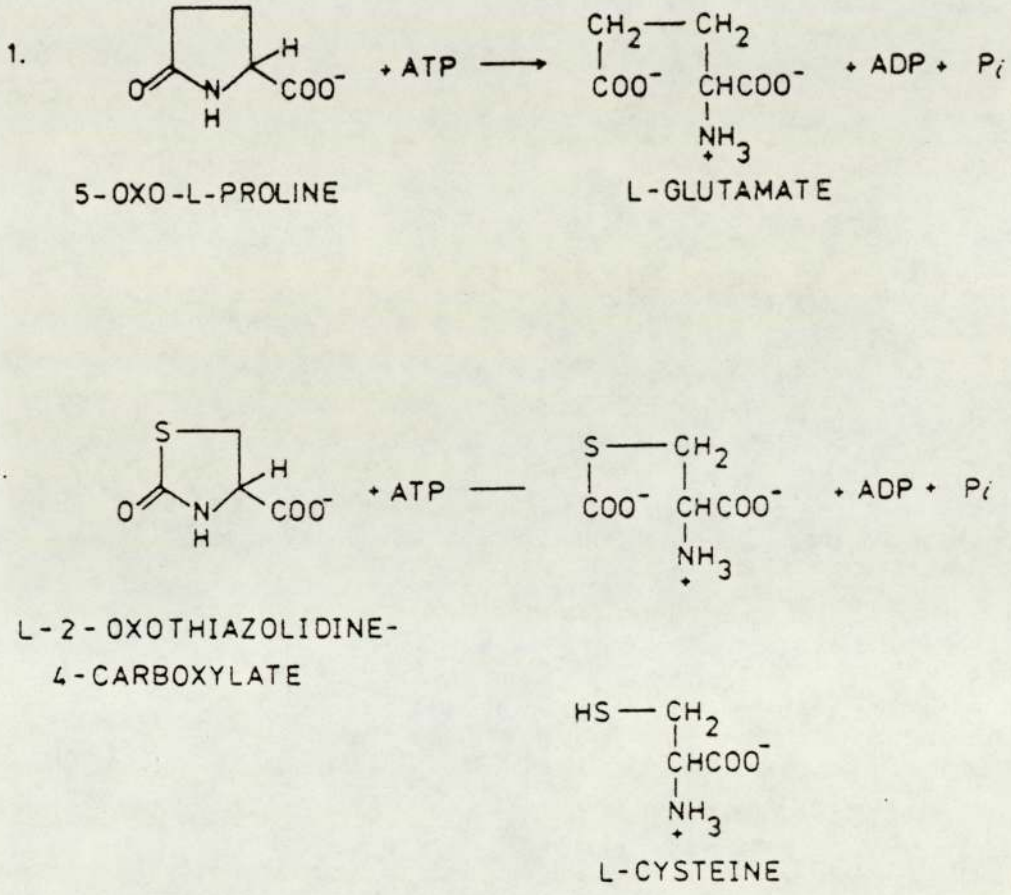


Figure 1.9 Reactions catalysed by 5-oxoprolinase

1) The normal substrate, 5-oxoproline is converted to glutamate.

2) The thiazolidine is converted via 5-carboxycysteine to cysteine (from reference 68)

This discussion, so far, has focused upon the central role of GSH in the detoxification of foreign compounds. However, there are examples of xenobiotics that undergo activation to toxic species following conjugation with glutathione. The carcinogen and mutagen, 1,2-dibromoethane binds irreversibly to DNA. The binding is mediated by conjugation with GSH catalysed by GSH S-transferases, to form S-(2-bromethyl)- glutathione a sulphur half mustard, which may form electrophilic episulfonium ions by the internal displacement of the second halogen atom by the sulphur atom, which then avidly binds to the N-7 position of guanine (98).

Another example of a compound that is activated by conjugation with GSH is Hexachloro-1:3-butadiene (HCBD) which is a byproduct in the manufacture of perchloroethylene by chlorination of hydrocarbons. HCBD is nephrotoxic and causes necrosis in the proximal tubules, but it is not hepatotoxic. HCBD causes a marked depletion of hepatic GSH, but not renal GSH, and undergoes GSH S-transferase mediated conjugation with GSH in the liver. The GSH : HCBD conjugate is transported to the kidney and metabolically activated by cysteine β-lyase to a reactive alkylating species, pyruvate and ammonia (99). Thus conjugation with GSH in the liver prior to renal activation plays a crucial role in the nephrotoxicity of HCBD.

Conjugation with GSH is therefore implicated in the activation of some toxins. However, it is clear that GSH plays a central role in the integrated defence of the liver against oxidative stress and reactive species. This protection is sometimes associated with depletion of GSH, which may preceed covalent binding of the reactive species to nucleophilic sites in cellular macromolecules. The consequences of such covalent modification will be discussed

further (see Section 1.5).

1.5 Covalent binding as a determinant of drug toxicity

Many chemically inert xenobiotics exert their mutagenic, carcinogenic or toxic effects following metabolic conversion to a reactive species (100, 101). The exact mechanisms by which these detrimental changes occur are unknown, but the irreversible interaction of the metabolites with critical nucleophilic sites is believed to be important (102-105). This is supported by the observation that parallel changes often occur between the incidence and severity of tissue damage and the covalent binding of metabolites to protein, lipid, RNA and DNA. Covalent binding studies *per se* have little predictive value in determining whether a given compound will evoke selective toxicity (104). However, for compounds such as frusemide, bromobenzene and paracetamol there is a direct correlation between the extent of covalent binding of their reactive metabolite(s) to liver macromolecules and incidence and severity of necrosis.

The role of covalent binding in the toxicity of a foreign compound can be assessed in a variety of ways. Binding can be determined either *in vivo* after administration of radiolabelled drug to animals, or *in vitro* in incubations of the drug with tissue homogenates, or tissue fractions, in the presence of cofactors of the activating system.

The role of covalent binding has been studies extensively in the case of the commonly used analgesic paracetamol. It is innocuous, when administered to human at therapeutic doses. However, large doses are hepatotoxic, and cause centrilobular necrosis in mice, which is attributed to a reactive metabolite (106-109). Dose response studies in mice revealed that the amount of covalent binding was negligable when the drug was given at doses up to $300 mg kg^{-1}$, which did not cause

necrosis (107). Pretreatment of mice with phenobarbitone, which induces drug metabolising enzymes, increased covalent binding of paracetamol metabolites to liver macromolecules, and increased the severity of necrosis. Conversley, pretreatment of mice with calbaltous chloride and piperonyl butoxide, inhibitors of paracetamol metabolising enzymes, reduced the severity of necrosis and also the extent of binding. Thus changes in the severity of the necrosis paralleled changes in the extent of the covalent binding of metabolites of radiolabelled paracetamol to liver macromolecules (107). Studies in vitro indicated that paracetamol was activated to a covalently bound species by liver microsomes (108). In vitro covalent binding was dependent upon NADPH and was abolished by CO, an inhibitor of cytochrome P-450, indicating a role for cytochrome P-450 in the metabolic activation of paracetamol. Significant covalent binding of the paracetamol metabolite(s) did not occur in vivo until the dose of paracetamol was large enough to deplete hepatic GSH by 75% (107, 109). Only when GSH was depleted by 75% did significant covalent binding occur, and likewise toxicity. Depletion of GSH with DEM markedly increased the covalent binding, which paralleled the change in paracetamol toxicity caused by DEM (109). Thus, at doses below the critical dose threshold of 300mgkg^{-1} in mice, the reactive metabolite appears to be conjugated with GSH without extensive depletion of hepatic GSH. However, above the critical dose threshold, GSH is significantly depleted (>75%) and the reactive species is bound to hepatic macromolecules (104).

In a way similar to that outlined above for paracetamol frusemide, which causes midzonal and centrilobular necrosis, is only

hepatotoxic in mice at doses of greater than 150mgkg^{-1}. Below this dose there is little covalent binding and also no necrosis (104).

Considerable interest has centred upon the relationship between GSH depletion, covalent binding and hepatotoxicity of paracetamol. In particular the ability of thiol compounds to protect against the toxicity and covalent binding both in vivo and in vitro has been extensively studied (73, 110-112). These studies and also the identification of the bilary GSH conjugate (3-(Glutathion-S-yl)-4-hydroxyacetanilide)(113), (Figure 1.10) clearly demonstrate the ability of thiols to `capture´ the reactive metabolite of paracetamol. It has been demonstrated in vitro that the reactive metabolite of paracetamol can bind selectively to the cysteinyl thiol residue of bovine serum albumin (BSA) following metabolic activation (114). The binding characteristics of the reactive metabolite, compared favourably with those of the proposed reactive metabolite of paracetamol, N-acetyl-p-benzoquinoneimine (NAPQI, Figure 1.10). The adduct formed between BSA and paracetamol has been identified as 3-(cystein-S-yl)-4-hydroxyaniline, derived from the cys-34 residue of BSA (115).

The evidence discussed so far supports the view that covalent binding may play a role in the hepatotoxicity of foreign compounds. However, some evidence suggests that covalent binding may not be causally related to toxicity (116, 117). Following incubation of ^{3}H-paracetamol with isolated rat hepatocytes for various times, a percoll separation technique was used to allow covalent binding to be measured in viable and dead hepatocytes (117). The results indicated that after incubation for 4 hours and 22 hours the extent of binding was greater in viable cells, than in cells which were killed at 1 hour.

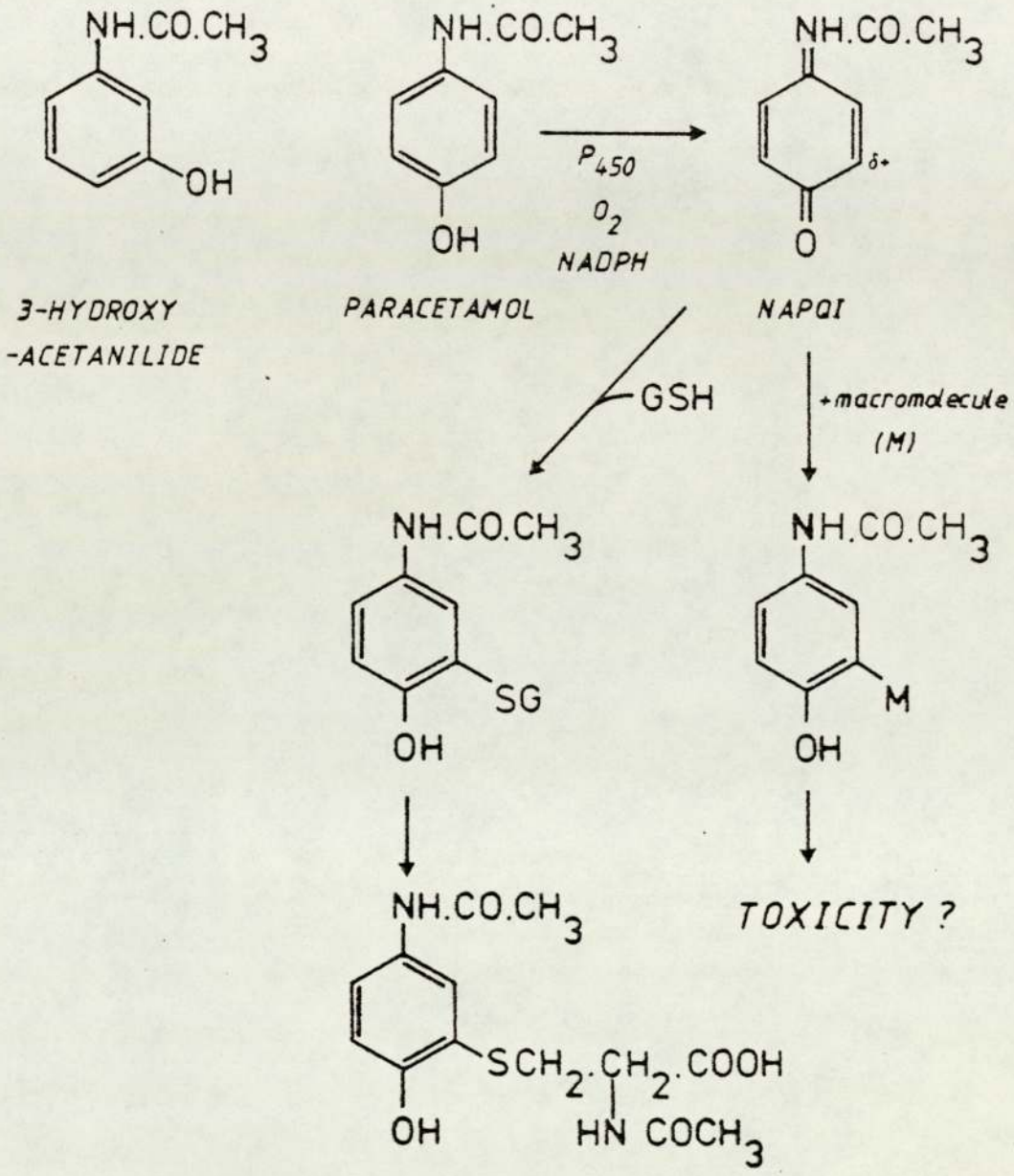


Figure 1.10 Metabolic activation of paracetamol

The conclusion drawn from this study was that covalent binding was only a measure of exposure to reactive species (117). Therefore the selectivity of the binding of reactive metabolites to certain nucleophilic targets may be more important in cell death than the extent of overall binding to macromolecules.

The binding of 3-hydroxyacetanilide (3-HAA) (Figure 1.10), a non-hepatotoxic, positional isomer of paracetamol, to microsomal protein and BSA has been evaluated. 3-HAA was found to bind more extensively to microsomal and soluble proteins than paracetamol (118). In addition GSH and ascorbic acid abolish the binding of the reactive metabolite of 3-HAA to protein, in a similar manner to which they abolish the covalent binding of the reactive metabolite of paracetamol, NAPQI, to protein. Therefore 3-HAA can be metabolised to a quinone or semiquinone species with similar properties to NAPQI, which may bind to microsomal protein, yet it is not hepatotoxic.

The results outlined above indicate that some controversy exists concerning the importance of covalent binding in the hepatotoxicity of paracetamol. Generally, caution is required when interpreting the data from covalent binding studies. Reactive species may be bound to macromolecules that are not essential to the life of the cell and thus deleterious effects do not ensue. Also metabolite-macromolecule complexes may undergo repair by cellular repair mechanisms. Studies on paracetamol induced centrilobular necrosis revealed that the covalent binding of the reactive metabolite initially was only slightly greater in the centrilobular hepatocytes than in the periportal hepatocytes (103). But after the total amount of covalent binding to liver macromolecules reached maximal values, the binding decreased in the periportal region until it was almost

entirely restricted to the centrilobular necrotic cells. This observation indicates that the decline in covalent binding is due to repair of the adduct in the periportal cells, and not due to loss of the adduct from the centrilobular hepatocytes.

The importance of binding to specific macromolecular target sites has been discussed in the context of Mcleans study in isolated hepatocytes (117). Another example which illustrates the importance of the specificity of binding is the drug isoniazid. Isoniazid is metabolised to acetylhydrazine, which is hepatotoxic and binds covalently to macromolecules in the liver and other organs in vivo (120). Acetylhydrazine does not produce detectable lesions in organs other than liver, despite covalent binding to proteins occuring in a number of organs. This finding implies that maybe only a small fraction of the binding to specific subcellular compartments is related to the subsequent tissue lesion (120).

Despite the lack of knowledge of the direct link between the binding and the genesis of the lesion, it is important to remember that studies on the covalent binding of paracetamol, bromobenzene and frusemide have shown unequivocally that there is a direct correlation between the extent of the covalent binding of the reactive metabolites to liver macromolecules and the incidence and severity of the liver necrosis.

1.6 Aims and scopes of the present investigation

The current state of knowledge on the metabolic fate, the antitumour activity, and the mechanisms of hepatotoxicity of NMF has been described in section 1.1 to 1.3. An underlying feature of both the antitumour activity and the hepatotoxicity is the proposal that NMF may exert its therapeutic or toxic effect following metabolic activation to a reactive species. In the present investigation, the mechanism of activation of NMF, and fate of the reactive species in the liver have been considered, together with the toxicological implications for NMF induced liver damage.

The hepatotoxicity of NMF in mice is characterized by a marked strain difference in susceptibility to NMF induced hepatotoxicity; the Balb/C mouse being more susceptible than the CBA/CA or BDF_1 mouse. It is also interesting that there is a strain difference in the antitumour activity of NMF against tumour lines which are syngerneic in these strains of mouse. The PC6A tumour which grows in the Balb/C mouse is resistant to NMF, whilst the TLX5 and M5076 which gorws in the CBA/CA and BDF_1 mouse, respectively, are sensitive to NMF. This observation tentatively suggests that the particular sensitivity of the Balb/C mouse to NMF hepatotoxicity, may reflect a different metabolic pathway than in other strains, which may also account for the lack of activity against the PC6A tumour.

NMF is reported to deplete GSH in isolated hepatocytes and in the livers of mice *in vivo* (Section 1.2), however, the extent of GSH depletion *in vivo* is inadequate to account for the severity of toxicity. There are a considerable number of compounds that deplete hepatic GSH prior to the onset of hepatotoxicity, for many of these it is possible to abolish the toxicity with GSH precursors and to

increase the severity of toxicity by depleting hepatic GSH. Here the role of GSH in NMF hepatotoxicity has been re-evaluated by selectively manipulating hepatic GSH levels prior to administration of NMF.

GSH depletion probably occurs secondary to generation of a reactive species. By pretreating mice with inducers and inhibitors of drug metabolism, whilst monitoring hepatic GSH levels, the role of metabolism in the generation of a reactive species may be established.

In Section 1.5 the potential role of covalent binding of reactive species, to nucleophilic sites of tissues, has been described as a determinant of drug toxicity. To establish the importance of covalent binding in NMF hepatotoxicity, the potential of NMF to interact with hepatic protein has been evaluated both in vivo and in vitro. The latter is of particular importance as it has not been possible to demonstrate in vitro metabolism of NMF (Section 1.3), despite extensive biotransformation in vivo. The measurement of covalent binding of NMF to microsomal protein, following incubation with ^{14}C-NMF, proved to be a particularly sensitive index of NMF metabolism in vitro. This approach provided information about the mechanism of metabolism of NMF, and by correlating changes of covalent binding to microsomes in vitro with hepatotoxicity in vivo it was possible to use this system as a model to determine the role of metabolically generated reactive species in the hepatotoxicity of NMF.

The significance of covalent binding in vivo, in the toxicity of NMF, has been evaluated by exploiting the strain difference in susceptibility to NMF toxicity in mice, to look at covalent binding in resistant and susceptible strains of mouse. In addition the extent of binding has also been measured in non-target tissue in order to

explain the organoselectivity of NMF toxicity. As an alternative approach, and to evaluate the role of metabolism, mice were pretreated with inhibitors and inducers of drug metabolism in order to evoke parallel changes between hepatotoxicity and covalent binding.

In addition to measuring GSH levels and covalent binding in different strains, it was thought necessary to support these investigations with a careful assessment of indices of liver dysfunction. The severity of hepatotoxicity was assessed by determination of activities in plasma of enzymic markers of liver damage, and by histopathological examination of liver sections.

The inter-relationships of changes in hepatic GSH status, and covalent binding, with changes in hepatotoxicity permits conclusions to be drawn as to their importance in NMF hepatotoxicity. By relating these findings to strain differences in NMF metabolism, the importance of metabolic activation of NMF can be established. From this series of investigations it has been possible to more fully understand the mechanism of NMF hepatotoxicity in murine species, and as a consequence action can be taken in the clinic to alleviate the hepatotoxicity of NMF in man.

SECTION 2

MATERIALS

2.1 Animals, maintenance and injections

Male Balb/C, CBA/CA and BDF_1 mice were purchased from Bantin and Kingman Limited, Hull, U.K. All animals were between 10 and 20 weeks old (20-25g), and were acclimatized for at least seven days under an alternating 12h light/dark cycle in an environmentally controlled animal house to synchronise circadian rhythms in hepatic non-protein thiol levels (121). Food (Heygate 41B Breeding diet) and tap water were available ad libitum.

Male Balb/C mice were used throughout, except when strain differences in metabolism (Section 4.1), glutathione depletion by NMF (Section 4.4), covalent binding (Section 4.8) and hepatotoxicity of NMF were evaluated (4.10).

All intraperitoneal injections of formamide analogues were performed between 8 and 10 a.m. in 200µl of saline, except when BSO pretreatments was performed, in which case BSO was administered between these times, and NMF was administered 4 hours later. All other compounds were administered in 200µl of isotonic saline as an i.p. injection unless otherwise stated.

2.2 Radiochemicals

[^{14}C]-Formic acid, Sodium salt, DL-[1-^{14}C] leucine, [^{14}C]n-hexadecane, and [^{14}C] methylamine hydrochloride were purchased from Amersham International. [^{14}C]-NMF labelled in the methyl or formyl moiety was prepared as described in Section 3.2.

2.3 Scintillation Fluids

Fisofluor mpc, multipurpose scintillation cocktail was purchased from Fisons Limited, Loughborough, United Kingdom; Beckman

Readysolve EP from Beckman, High Wycombe, United Kingdom; Dimilume 30 scintillation cocktail and Soluene 350 from United Technologies, United Kingdom. Ethanolamine (Scintran) and 2-ethoxyethanol (Scintran) were purchased from BDH Limited, Atherstone.

2.4 Chromatographic Materials

The following materials were obtained from the source given in brackets.

GLC columns and packing materials (Phase Separations, Queensferry, United Kingdom),

Analar grade acetone (BDH Limited, Atherstone),

Radial compression HPLC columns and pre-columns (Waters Associates, Northwitch, United Kingdom),

HPLC grade methanol (Fisons Limited, Loughborough).

2,4-Dinitrobenzene Sulfonic Acid (Eastman Kodak, Rochester, New York, USA.).

Silica gel 60 F_{254} 0.2mm coated thin layer plates or 2mm coated plates for preparative chromatography (Merck AG, Darmstadt, West Germany).

2.5 Formamides analogues

N-Methylformamide was purchased from Aldrich Chemical Company, Gillingham, and dimethylformamide and formamide from BDH Chemicals Limited, Atherstone. All were purified further by distillation. N-Hydroxymethylformamide was synthesised by Dr E.N. Gate according to a published method (122).

2.6 Miscellaneous Chemicals

2.6.1 Purchased

The following chemicals were purchased from the sources indicated:-

Aldrich Chemical Company, Gillingham

Tetramethylurea

Maleic acid diethylester (Diethylmaleate)

BDH Chemical Company, Atherstone

Ammonium Acetate

Acetyl Acetone

Sigma Chemical Company, Poole

L-alanine	A7627
Aminopyrine (4-Dimethylaminoantipyrine)	D8015
L-aspartic acid	A9256
Bovine Serum Albumin, Fraction V	A2153
Cysteine, dihydrochloride	C1276
β -D-(-)Fructose	F0127
D-Glucose-6-phosphate	G7879
Glutathione	G4251
β -Nicotinamide adenine dinucleotide phosphate (NADP)	N0505
β -Nicotinamide adenine dinucleotide phosphate, reduced form (NADPH)	N1630
β -Nicotinamide adenine dinucleotide reduced form (NADH)	N8129
N-Acetyl-L-Cysteine	A7250
Promethazine, hydrochloride	P4651
Pyridoxal-5-phosphate	P9255

dl-α-tocopherol T3251

2.6.2 Gifts

SKF525A (2-diethylaminoethyl 2,2-diphenylvalerate) was kindly donated by Smith, Kline and French Limited, Welwyn, United Kingdom.

2.6.3 Synthesised

DL, Buthionine S,R sulfoximine (BSO) was kindly prepared by Dr M.D. Threadgill according to a published method (87).

2.7 Enzymes

The following enzymes were purchased from Sigma Chemical Company.

Glutathione reductase	G4751
Glucose-6-phosphate dehydrogenase	G8878
L-Lactate dehydrogenase	L2500
Malic dehydrogenase	M9004

2.8 Buffers, reagents and cofactor solutions

2.8.1 Glutathione assay

Phosphate 125mM and EDTA 6.3mM buffer, pH 7.5

11.12g $NA_2\ HPO_4\ .\ 2H_2O$

1.17g Na_2 EDTA

were dissolved in distilled water, the pH adjusted to pH 7.5 (Philips PW9410 pH meter), and the volume made up to 500ml).

Trisodium Orthophosphate solution

Na_3PO_4 12.5g was dissolved in 10ml of distilled water by application of heat. When dissolved, the volume was adjusted to 25ml, and maintained in solution by constant stirring on a hot plate.

DTNB solution

5´5-dithiobis- (2-nitrobenzoic acid), Ellman´s Reagent, (23.8mg) was dissolved in phosphate -EDTA buffer, pH 7.5 (10ml). The solution was prepared immediately prior to use and stored on ice in the dark.

NADPH solution

NADPH (6.3mg) was dissolved in 25ml of phosphate -EDTA Buffer, pH 7.5, immediately prior to use.

Glutathione reductase

Glutathione reductase (Type III, from yeast) was diluted with phosphate -EDTA buffer, pH 7.5, to produce a solution with a final enzyme activity of 50iu. per ml).

2.8.2 Microsome preparation and incubation buffer

Phosphate buffer 100mM, pH 7.4

A. KH_2PO_4 (1.70g) was dissolved in 250ml of distilled water.

B. Na_2HPO_4 (1.77g) was dissolved in 250ml of distilled water.

A was added to B with constant stirring until pH 7.4 was reached.

2.8.3 Plasma enzyme assays

Measurement of Sorbitol dehydrogenase

Tris-HCl buffer 100mM pH 6.6

Tris (hydroxymethyl) Aminomethane (6.05g) was dissolved in 400ml distilled water and the pH adjusted to pH 6.6 with dilute HCl, the volume was then made up to 500ml.

NADH solution (355μM)

NADH, disodium salt (12.58g) was dissolved in 50ml of Tris-HCl buffer pH 6.6.

β -D-Fructose solution (2.5M)

Fructose (4.5g) was dissolved in 10ml of Tris-HCl buffer pH6.6.

Measurement of Alanine and aspartate aminotransferase

Tris base stock (1M)

Tris (hydroxymethyl) Aminomethane (12.11g) was dissolved in 100ml of distilled water.

Tris buffer (100mM) pH 7.8

Tris base (50ml) was diluted with 300ml of distilled water and 5.5ml of HCl (6N). The pH was adjusted to pH 7.8 with 1N NaOH at 30°C and the final volume adjusted to 500ml.

L-aspartate solution (228mM)

L-aspartic acid (7.59g) was dissolved in a solution containing 25ml of tris base stock, 175ml of distilled water and 25ml of 2.5N NaOH. The pH was adjusted to pH 7.8 at 30°C with NaOH

solution (1N) and the final volume made up to 250ml with distilled water.

2-Oxoglutarate solution (225mM)

2-Oxoglutaric acid (1.65g) was dissolved in 25ml of distilled water and 5ml of tris base and the pH adjusted to pH 7.8 at 30°C with 1N NaOH solution. The final volume was made up to 50ml with distilled water.

NADH solution (6.5mM)

β-$NADHNa_2.4H_2O$ (25mg) was dissolved in 5ml of Tris buffer pH 7.8.

Malate dehydrogenase

The commercial solution was diluted with glycerol : water (1:1) to provide a final activity of 36,000 i.u. l^{-1}.

Lactate dehydrogenase

The commercial solution was diluted with glycerol : water (1:1) to provide a final activity of 72,000 i.u. l^{-1}.

Pyridoxal-5´-phosphate (4.5mM)

Pyridoxal-5´-phosphate (11.1mg) was dissolved in 10ml of tris buffer, pH 7.8.

SECTION 3

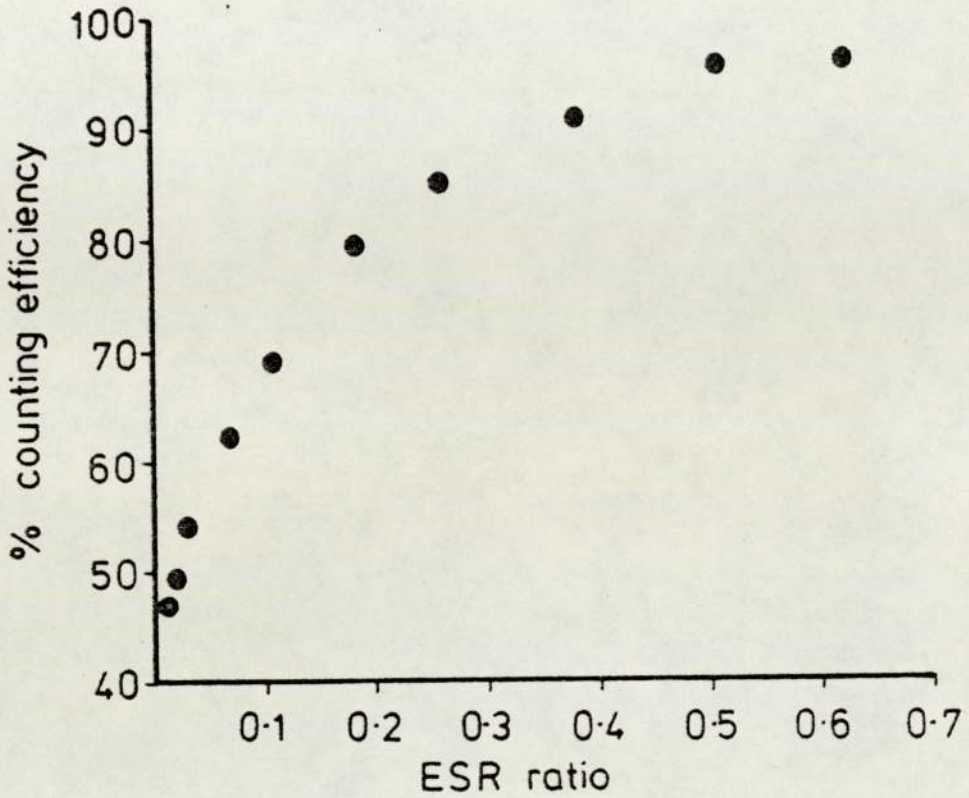
METHODS

3.1 Liquid scintillation counting and quench curves

Liquid scintillation counting has been used extensively throughout this investigation to quantify ^{14}C-NMF metabolites (Section 3.4) and to measure covalent binding of NMF to liver macromolecules *in vivo* (Section 3.7) and *in vitro* (Section 3.8). In all these procedures radioactivity was quantified in a Packard Tricarb 2660 scintillation counter operated in the external standardization mode (123). For each of the three applications the sample preparation conditions were different and a separate quench curve was constructed to permit the counting efficiency of each sample to be determined.

The quench curves were prepared using the following general procedure; 5µl of ^{14}C-hexadecane standard of known activity (standard dpm) was added to ten scintillation vials. Sequentially increasing volumes of chloroform (0-800 µl), the quenching agent, was then added. The counting efficiency of the prepared samples, defined as the ratio of the observed counts per minute to the disintegrations per minute, was determined from the count rate using the external standard ratio (ESR) from the calibration (quench) curve (Figures 3.1 to 3.3).

For all samples the counting efficiency was greater than 80%, and within the ESR range of the quenched standards.

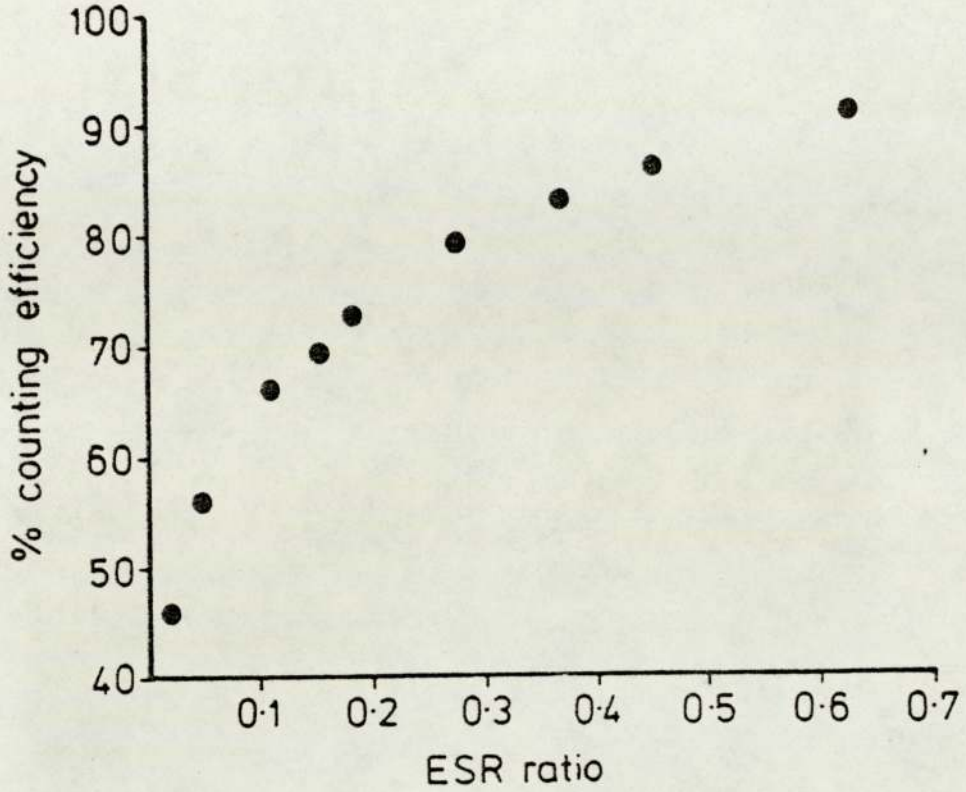


Sample preparation: 1ml of Soluene 350 + 10ml Beckman Readysolve EP (with 1% glacial acetic acid) decolourized with 200µl propan-2-ol and 600µl hydrogen peroxide solution (100vol). (Reference 124).

Counting Conditios:

PROGRAM	1	
TIME (MIN)	10.00	
2 SIGMA % (R G)	.2	.2
RADIONUCLIDE	CE	
LL REJECT (R G)	0	
BKG CPM (R G) OR FV	FV	
COUNTS/SAMPLE	1	
CYCLES/PROG	1	
DIVIDE CONSTANT	1	
# OF COUNTS FOR % STD	0	
STD DPM	4548	
PLOT (Y;N)	Y	

Figure 3.1 Quench Curve 1; correlation curve between the sample counting efficiency and the count rate of the external standard (External standard ratio; ESR)

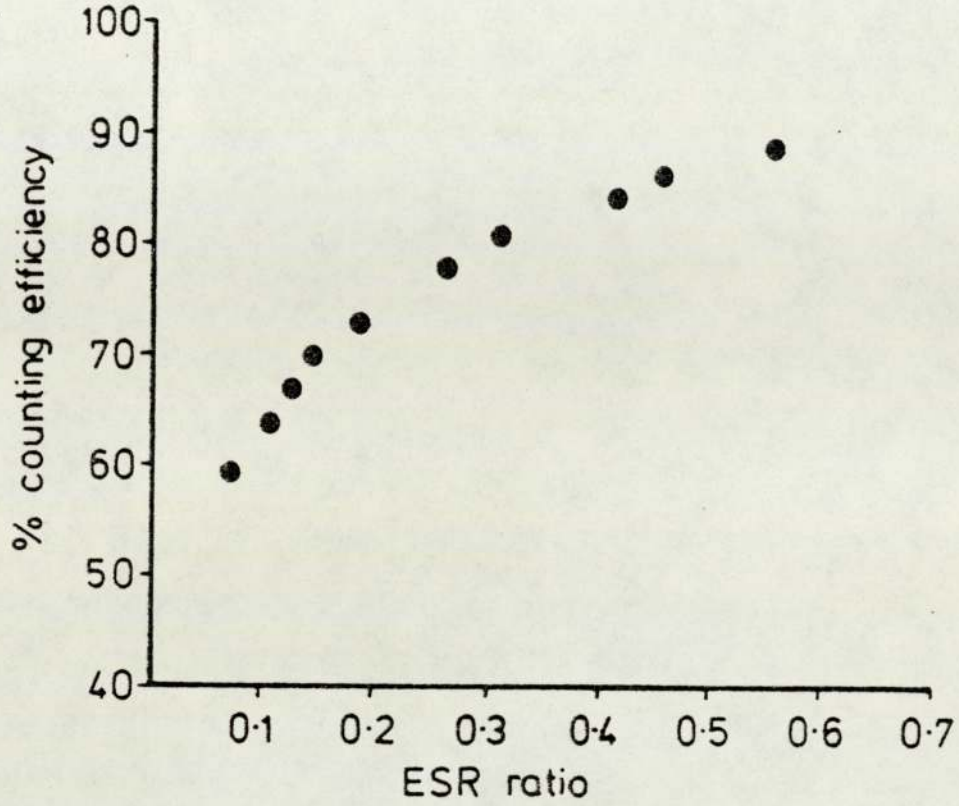


Sample preparation: 1 ml of aqueous sample + 10ml Fisofluor mpc.

Counting Conditions:

PROGRAM	12	
TIME (MIN)	10.00	
2 SIGMA % (R G)	.2	.2
RADIONUCLIDE	CE	
LL REJECT (R G)	0	
BKG CPM (R G) OR FV	FV	
COUNTS/SAMPLE	1	
COUNTS/PROG	1	
DIVIDE CONSTANT	0	
# OF COUNTS FOR % STD	0	
STD DPM	17600	
PLOT	Y	

Figure 3.2 Quench Curve 2; correlation curve between the sample counting efficiency and the count rate of the external standard (External standard ratio; ESR)



Sample preparation: 1ml NaOH Solution (1N) + 10ml Dimilume 30

Counting Conditions:

PROGRAM	3	
TIME (MIN)	10.00	
2 SIGMA % (R G)	.2	.2
RADIONUCLIDE	CE	
LL REJECT (R G)	0	0
BKG CPM (R G) OR FV	FV	
COUNTS/SAMPLE	1	
CYCLES/PROG	1	
DIVIDE CONSTANT	1	
# OF COUNTS FOR % STD	0	
STD DPM	21623	
PLOT (Y;N)	Y	

Figure 3.3 Quench Curve 3; correlation curve between the sample counting efficiency and the count rate of the external standard (External standard ratio; ESR)

3.2 Synthesis and purity of ^{14}C-NMF

N-Methylformamide labelled with ^{14}C in the methyl moiety ($^{14}CH_3$-NMF) or in the formyl moiety (OH^{14}C-NMF) was prepared by Dr M.D. Threadgill (125), the former by treating ^{14}C-methylamine hydrochloride with ethylformate and sodium carbonate, the latter by heating ^{14}C-formic acid, sodium salt with an excess of methylamine hydrochloride.

Following synthesis, ^{14}C-NMF was purified by preparative layer chromatography on silica gel 60 coated plates (2mm) developed in chloroform:methanol (4:1). Radioactive bands were localized by autoradiography (Section 3.3). The band migrating at Rf 0.71, was quantitatively removed and exhaustively extracted with double distilled acetone (Analar, BDH). Following flash evaporation of the acetone, the residue was dissolved in 2ml of sterile isotonic saline and passed through a millipore filter (0.45μ) to remove residual silica. An aliquot of solution was taken and the specific activity determined by liquid scintillation counting (Quench curve 2, Section 3.1).

At regular intervals throughout the investigation the purity of ^{14}C-NMF was checked by thin layer chromatography and was consistently greater than 99%

3.3 Autoradiographic techniques

Thin layer chromatography plates were sandwiched together with Singul-X-RP X-ray film (A.B. Strangas, Sweden) in an X-ray cassette, and stored at -20°C for at least one week. Following exposure the X-ray film was developed with Kodak D-14 developer and fixed for 5 minutes with Kodafix (Kodak, Hemel Hempstead, UK).

In the case of the preparative layer plates, 30 minutes exposure at room temperature was sufficient to visualize radioactive bands.

3.4 Metabolism of NMF in mice : Mass balance study

3.4.1 NMF administration and sample collection

Quantitative aspects of the metabolism of NMF were evaluated in Balb/C mice in the following way. A dose formulation prepared to deliver 10μCi of ^{14}C-NMF and a dose of either 400mgkg^{-1} or 100mgkg^{-1} in 200μl of isotonic saline. 300μl of the formulation was drawn into a 1ml syringe and the weight of the syringe was recorded. After administration of an i.p. injection of approximately 200μl to Balb/C mice the syringe was reweighed and the precise dose calculated from the specific activity of the dose formulation.

After NMF administration, the mice were placed in an airtight mouse metabolism cage (Jecons, UK) designed to facilitate continuous trapping of expired $^{14}CO_2$ and collection of urine and faecal samples at 12 hour intervals over a period of 72 hours (See Figure 3.4).

At 12h intervals the metabowl was washed with 5ml of distilled water and the urine and faecal samples were collected into preweighed vials. The volume of ethanolamine:2-ethoxyethanol (1:4) in the CO_2 traps was recorded and aliquots were taken for determination of radioactivity. All samples were stored at 0-5^{o}C prior to analysis. 72 hours after NMF administration the mouse was killed by cervical dislocation, weighed and frozen at -20^{o}C. The metabolism cage was washed with approximately 50ml of distilled water to recover residual radioactivity.

3.4.2 Determination of ^{14}C-NMF excreted and residual NMF

Total radioactivity excreted in the urine of Balb/C mice was determined by weighing a 100μl aliquot of urine into a tared scintillation vial, to which 900μl of distilled water and 10ml of

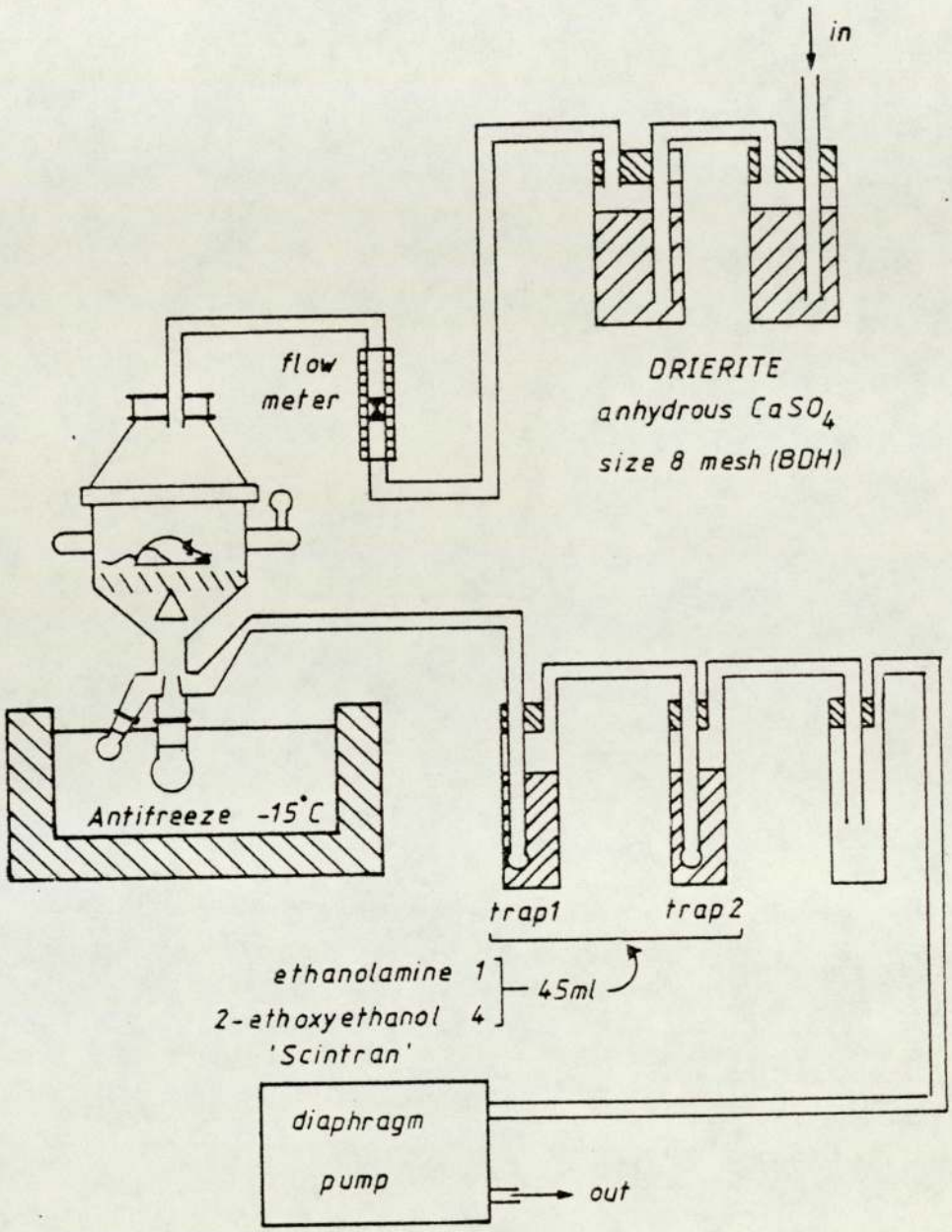


Figure 3.4 Metabowl arrangement used in Mass Balance Study

fisofluor mpc were added. Radioactivity was determined by liquid scintillation counting (Quench curve 2; Section 3.1). The residual activity remaining in the cage at 72 hours was estimated by treating an aliquot of the final wash in the same way as a urine sample.

Expired $^{14}CO_2$ in the breath of mice was determined by weighing a 100µl aliquot of ethanolamine:2-ethoxyethanol trapping cocktail into a preweighed vial. 900µl of distilled water and 10ml of fisofluor mpc were added and the radioactivity determined in the same way as for the urine samples.

Total radioactivity in the faeces was determined by addition of 2 volumes of distilled water to the faecal sample to produce a slurry. An aliquot was weighed into a scintillation vial and solubilized with soluene 350 at 37°C for 30 minutes. Following solubilization, 10ml of Readysolve EP was added and the sample was decolourized and counted using Quench curve 1 as described in Section 3.1.

Residual radioactivity in the carcass was determined by mincing the carcass and producing a 33% w/v homogenate in distilled water. An aliquot of the homogenate was then processed in the same way as the faecal samples.

Finally, the data was collected and the Mass Balance calculated (see Appendix I).

3.4.3 GLC assay for N-methylformamide and formamide

NMF and F were quantified from urine samples by a gas chromatographic assay described by Kimmerle and Eben (40) and modified by Brindley et al (4, 18) 100µl of urine was diluted with 400µl of acetone (Analar grade, BDH) containing 1,1,3,3-Tetramethylurea as an

internal standard. After removal of protein by centrifugation, analysis was performed on a Pye Unicam 204 gas chromatograph fitted with a glass column (1.5M long x 4mm internal diameter) packed with 100-120 mesh Chromasorb W AWDMCS coated with a stationary phase of 8% Carbowax 20M + 2% KOH. The column was conditioned at 210°C with a nitrogen flow rate of 50ml min^{-1} for at least 24h prior to analytical use. For analysis the column was maintained at 190°C, the nitrogen-phosphorous detector at 250°C, and the injector at 200°C. The gas flow rates were 40ml min^{-1}, 30ml min^{-1} and 300ml min^{-1} for nitrogen, hydrogen and air respectively. Under these conditions 1,1,3,3-tetramethylurea (TMU), NMF and F were resolved in the stated order in less than 10 minutes (see Figure 4.1). The recoveries of NMF was 106 ± 10% and Formamide 109 ± 12% (4).

TMU was present at a final concentration of 4µgml^{-1} in the diluted urine sample, and standards of F and NMF were prepared within the concentration range of 2 to 20µgml^{-1}.

3.4.4 HPLC assay for methylamine

3.4.4.1 Reagents and standards

Methylamine has been quantified by HPLC in the urine of mice administered NMF, following derivatization with 2,4-dinitrobenzene sulfonic acid (130). The following reagents were employed in the derivatization procedure.

Calibration methylamine solution

1.088g methylamine hydrochloride (= 500mg CH_3NH_2) (purum, Fluka AG, Switzerland > 98%) was dissolved in 100ml of double distilled water. A 500µl aliquot of this solution was taken and diluted to 100ml with water to give a 25µgml^{-1}

solution.

Internal standard solution

100µl of 4-amino-1-butanol (98%, Aldrich) was dissolved in 500ml of double distilled water.

Dinitrobenzene sulfonic acid (DNBS) solution

2.84g, 2,4-dinitrobenzene sulfonic acid was dissolved in 100ml saturated borax solution (di-sodium tetraborate, Analar BDH) and was extracted with 3 x 100ml diethylether (Analar, BDH).

3.4.4.2 Sample preparation

The DNBS solution (2ml) was placed in centrifuge tubes and heated at 95° for one hour (Beckman Dri block), after cooling the solution was extracted with diethylether (5ml) to remove small amounts of CH_3NH_2.

A calibration curve was constructed by addition of internal standard solution (25µl) and methylamine standard solution (20, 40, 60, 80 or 100µl) to centrifuge tubes containing DNBS solution. For urine samples, a 100µl aliquot of urine and 25µl of internal standard was added to DNBS solutions.

The DNBS solution, with the added amines, was heated at 95°C for one hour, cooled and extracted with 2 x 5ml of diethylether. The pooled ether extracts were dried with Na_2SO_4 (anhydrous) and the solvent evapourated under a stream of nitrogen.

The residues were stored at 0-5°C and were dissolved in 1ml of HPLC grade methanol immediately prior to HPLC analysis.

3.4.4.3 Chromatography conditions

Separation and quantification of the derivated monoamines were performed on a Waters Trimodule system equiped with a WISP 710B auto injector, 6000A pump, Waters RCM 100 and a Waters Lamda max 480 U.V detector. Separation was achieved using the following conditions:-

Column	C_{18}, 5µ radial compression cartridge
Mobile phase	MeOH: H_2O (60:40)
Flow rate	1ml min^{-1}
Injection volume	20µl
Detector	0.05 AUFS λ = 346nm
Run time	23 minutes

A calibration curve was constructed by determining ratios of peak areas of standard compound to the peak area of the internal standard.

3.4.5 Strain differences in the metabolism of NMF in mice

In order to compare strain differences in NMF metabolism in mice a mass balance study was also performed after administration of a dose of 400mgkg^{-1} NMF to CBA/CA mice. Sample collections were performed at 12 and 24h after NMF administration, and subsequently at 24h intervals for a further 48 hours.

3.5 Thioether excretion in the urine of mice following administration of N-alkylformamides

Male Balb/C mice were administered 6.8mmol kg^{-1} of the following N-alkylformamide analogues; NMF, HMF, F or DMF as a single i.p. injection.

Following injection the mice were housed in mouse metabolic cages (Jencons, U.K.) for 24h, at which time the cages were washed with 5ml of distilled water and the urine sample collected. Control 24h urine collections were also obtained after administration of 200µl of saline.

Thioethers in the urine were determined according to the method of van doorn (129),. modified to eliminate the preliminary reduction step. Essentially, the thioether concentration was estimated by measuring free sulphydryl groups, with 5´5-dithiobis -(2-nitrobenzoic acid) after alkaline hydrolysis of the urine.

N-acetylcysteine was used as a reference standard, and the urinary creatinine concentration was determined by the alkaline-picrate method (131). The results are expressed as µmolSH/µmol creatinine.

The dose related nature of NMF induced elevation of urinary thioethers was investigated after administration of doses of 100, 200 or 400mgkg^{-1} (6.8 mmol kg^{-1}) NMF to Balb/C mice.

3.6 The effect of NMF upon hepatic GSH homeostasis

3.6.1 Determination of total hepatic GSH

Total hepatic glutathione (GSH + GSSG) was determined using a kinetic assay (133, 134) in which catalytic amounts of GSH or GSSG bring about the continuous reduction of 5,5´ dithiobis (2-nitrobenzoic acid) (DTNB) (132) at the expense of NADPH according to the following scheme (134).

$$2\,GSH + DTNB \xrightarrow{\text{non enzymatic}} GSSG + 2TNB$$

$$GSSG + NADPH + H^{+} \xrightarrow{\text{GSSG reductase}} 2GSH + NADP^{+}$$

$$NADPH + H^{+} + DTNB \xrightarrow[\text{GSSG reductase}]{\text{GSH/GSSG}} 2TNB + NADP^{+}$$

The reaction rate is proportional to the concentration of glutathione and the formation of 5-thio-2-nitrobenzoate (TNB) was followed spectrophotometrically at 412nm.

To perform the determination, livers were excised from mice, weighed and homogenised in 10% metaphosphoric acid ($40ml\,g^{-1}$ liver). The precipitated protein was sedimented by centrifugation at 3000 rpm for 5 min (Hareus, Labofuge). The acidic supernatant was taken for the glutathione assay.

The acidic supernatant was (300µl) neutralized (pH 7-7.5)

with hot trisodium orthophosphate solution (120µl) (Section 2.8.1). 140µl of this mixture was transferred to a reduced volume (1cm pathlength) quartz cuvette in a heated cuvette holder (30°C) of a Beckman DU7 U.V. spectrophotometer. The following solutions were added to the cuvette; 700µl NADPH solution (Section 2.8.1) and 100µl of DTNB solution (Section 2.8.1). The reaction was initiated by addition of 10µl of GSH reductase solution (Section 2.8.1) and the change in absorbance at λ = 412nm was monitored for 5 minutes.

A series of GSH standards (5-50µM)were prepared in 10%w/v metaphosphoric acid solution and subjected to the same analytical procedure to produce a calibration line (Table 3.1). The rate of enzyme reaction was proportional to GSH concentration within the range of standards prepared.

Concentration (µM)	5	10	20	30	40	50
Δ Abs 412nm (units/min)	0.097	0.165	0.305	0.440	0.537	0.605

correlation coefficient = 0.993

slope = 0.014

intercept = 0.067

Table 3.1 Calibration of glutathione concentration (µm) against initial rate of enzyme reaction (Δ Abs 412nm)

3.6.2 Depletion of hepatic glutathione

3.6.2.1 Inhibition of GSH biosynthesis

GSH biosynthesis in the livers of Balb/C mice was inhibited with DL-buthionine S,R sulphoximine (BSO). BSO was dissolved in 0.1 N NaOH to provide a dose of 1600mgkg^{-1} in a volume of 300µl (pH 8.5) and administered as a single i.p injection to Balb/C mice (88). Hepatic GSH levels were determined, as described in 3.6.1 at 2 hour intervals for up to 6 hours after BSO administration.

3.6.2.2 Chemical depletion of GSH

Depletion of hepatic glutathione by chemical reaction with an electrophile was achieved by administration of diethyl maleate (DEM; 0.3, 0.5 or 0.7mlkg^{-1}) to Balb/C mice. DEM was administered in 200µl of arachis oil as an i.p. injection (150), and hepatic glutathione content was determined one hour later (see Section 3.6.1)

Control mice received 200µl of arachis oil.

3.6.3 Depletion of hepatic GSH by NMF in Balb/C, CBA/CA and BDF_1 mice

The dose related nature of NMF induced depletion of hepatic GSH was evaluated in Balb/C mice, one hour after administration of a single i.p. injection of 100, 200 or 400mgkg^{-1} NMF.

The time course of depletion was evaluated after a dose of 200mgkg^{-1} NMF, by measuring hepatic GSH levels at 30 minute intervals for 2 hours, and subsequently at hourly intervals for a further 2 hours.

Differences between strains of mice in NMF induced depletion of hepatic GSH were evaluated in Balb/C, CBA/CA and BDF_1 mice by measuring hepatic GSH levels (Section 3.6.1) after administration of a

dose of 200mgkg^{-1} NMF.

3.6.4 Pretreatments to effect the NMF induced depletion of hepatic GSH

3.6.4.1 Inhibition and induction of drug metabolism

The role of drug metabolism in NMF induced depletion of hepatic GSH was evaluated by pretreating mice with phenobarbitone (PB) or SKF525A. PB (50mgkg^{-1}), as the sodium salt, was administered daily to Balb/C mice for four days between 9 and 10 A.M. (136). On the fifth day mice received 200mgkg^{-1} NMF, and hepatic GSH levels were determined one hour later (Section 3.6.1). SKF525A (60mgkg^{-1}) was administered to mice 1 hour before 200mgkg^{-1} NMF. One hour after NMF administration hepatic glutathione levels were determined.

3.6.4.2 Influence of GSH precursors

Cysteine or NAC were administered to mice in order to protect against NMF induced depletion of hepatic GSH.

Cysteine (300mgkg^{-1}), as the dihydrochloride, was administered as an i.p. injection in 100µl of 0.64N NaOH (pH 7) to mice 10 minutes before and 20 minutes after an i.p. injection of 200mgkg^{-1} NMF (150). NAC was administered to mice either as an i.p. injection of 100mgkg^{-1} 1h before NMF, or an oral dose of 1200mgkg^{-1} in 200µl of saline 20 minutes before 200mgkg^{-1} NMF (73).

3.6.4.3 Influence of radical scavenging agents

Promethazine (24mgkg^{-1}), as the hydrochloride salt, was administered to mice 10 minutes before a dose of 200mgkg^{-1} NMF (138).

Vitamin E (dl- α -tocpherol) 100mgkg^{-1}, was administered to mice 15h before 200mgkg^{-1} NMF. Vitamin E was dissolved in 1 part of 95%

ethanol to which was added 9 parts of 16% Tween 80 (polyoxyethylene sorbitan monooleate) in isotonic saline. The final dose was administered as an i.p. injection in 200µl of this formulation (137). In control experiments, mice received vitamin E or promethazine with 200µl of isotonic saline.

3.7 The covalent binding of ^{14}C-NMF to hepatic and renal macromolecules in vivo

3.7.1 Determination of covalent binding

The covalent binding of ^{14}C-NMF to liver macromolecules was determined at 1,4,8 or 16h after coadministration of 5μCi ^{14}C-NMF with 20, 100 or 400mgkg^{-1} NMF to Balb/C mice. At the allotted time points, livers were excised, weighed and deep frozen until analysis.

Liver homogenates (25%w/v) in distilled water were prepared and the macromolecules, principally but not exclusively proteins, were precipitated with 3 volumes of acetone (see Figure 3.5). The precipitated proteins were extensively washed with water and water:methanol (1:1), due to the high water solubility of NMF and its metabolites (140), until no further radioactivity could be removed. The tissue pellet was solubilized in 2ml of soluene 350 and 2x1ml aliquots were transferred to scintillation vials and the residual radioactivity was determined as described in Section 3.1 (Quench Curve 1). Protein loss during the procedure was less than 5% and the results were expressed as ng NMF equivalents bound mg^{-1} protein (141). The covalent binding of ^{14}C-NMF metabolites to kidney macromolecules was also determined in the way described above.

3.7.2 Pretreatments to influence covalent binding to hepatic macromolecules in vivo

The effect of inhibition of drug metabolism on the covalent binding of 400mgkg^{-1} ^{14}C-NMF was evaluated by pretreating mice with SKF525A as described in Section 3.6.4.1.

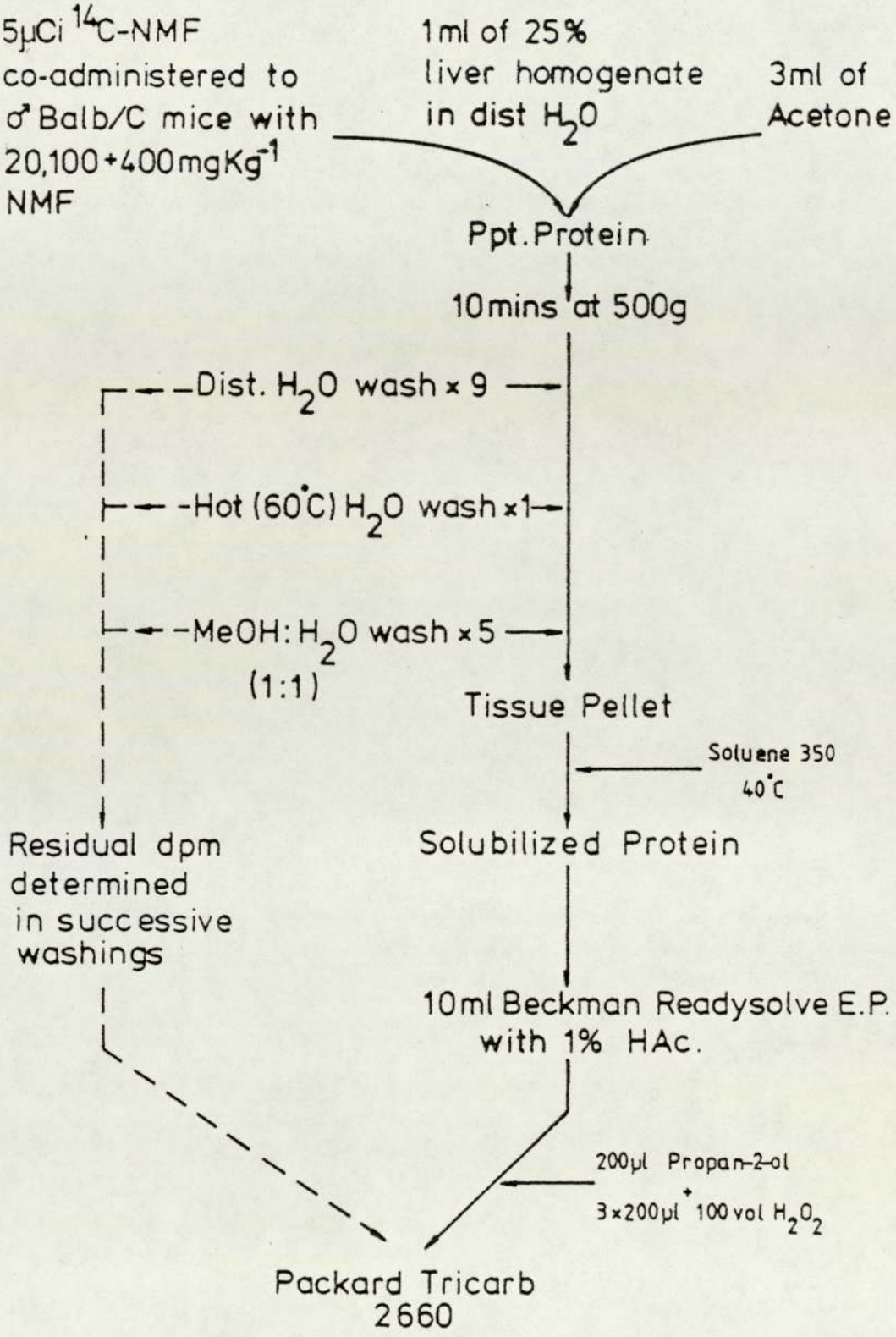


Figure 3.5 Procedure for measuring covalent binding of ^{14}C-NMF to hepatic macromolecules in vivo

GSH was depleted to less than 30% of control values prior to administration of 400mgkg^{-1} ^{14}C-NMF by treating mice with DEM (0.3 or 0.7 mlkg^{-1}) 1 hour prior to NMF, or with BSO (1600mgkg^{-1}) 4h before NMF administration (see Section 3.6.2).

The potential for NMF to undergo incorporation into hepatic macromolecules during denovo protein synthesis was evaluated by pretreating Balb/C mice with methotrexate or cycloheximide. Methotrexate (titrated to pH7) was administered as a single daily i.p. injection over a 6 day period according to the following schedule: Day 1, 20mgkg^{-1}; Days 2-5, 10mgkg^{-1}; Day 6, 10mgkg^{-1} followed by 400mgkg^{-1} $^{14}CH_3$-NMF (142).

Cycloheximide (1mgkg^{-1}) was administered as a single i.p. injection 15 minutes before administration of 400mgkg^{-1} ^{14}C-NMF (142). The extent of inhibition of protein synthesis achieved was evaluated by administration of 2µCi [1 ^{14}C] leucine to mice, with and without cycloheximide pretreatment.

The extent of covalent binding of ^{14}C-NMF and the incorporation of [1^{14}C]-leucine was determined as described in Section 3.7.1.

3.7.3 Strain differences in covalent binding

The extent of covalent binding of OH^{14}C-NMF and $^{14}CH_3$-NMF metabolites to hepatic and renal macromolecules of BDF_1 and CBA/CA mice 8h after a single i.p. injection of ^{14}C-NMF was determined as previously described (see Section 3.7.1).

3.8 The covalent binding of ^{14}C-NMF to liver microsomes in vitro

3.8.1 Microsomal incubations

Livers were excised between 8 and 10 a.m., weighed and rinsed in ice-cold phosphate buffer (50mM, pH 7.4). A 20% w/v homogenate was prepared in phosphate buffer by 8 strokes of a Camlab 563 C homogeniser (speed 6) fitted with a teflon pestle. The resulting suspension was centrifuged at 9000g for 20 minutes in a MSE pegasus centrifuge at 4°C. The 9000g supernatant was further centrifuged at 100,000g for 60 minutes at 4°C. The cytosolic supernatant was discarded and the microsomal pellet was resuspended in phosphate buffer (5mlg^{-1} liver) (143). The microsomal suspension (750µl) was transferred to a 20ml pyrex beaker, containing phosphate buffer and cofactors, to produce a protein concentration of 2mg ml^{-1} in a final volume of 2ml. The incubation mixture consisted of the following components in the final concentration stated: NADP (1mM), glucose-6-phosphate (5mM), glucose-6-phosphate dehydrogenase (2 units ml^{-1}), $MgCl_2$ (5mM) and ^{14}C-NMF (7mM).

Incubations were initiated by addition of substrate to the beaker in a shaking water bath maintained at 37°C. The incubations were terminated after 2 hours by precipitation of the microsomal protein with 4ml of acetone or 2ml of trichloracetic acid solution (12.5%w/v). Incubations were also performed in the absence of NADP or with heat denatured (100°C for 2 minutes) microsomes.

The viability of the microsomal preparation was evaluated by measuring formaldehyde as a metabolite of aminopyrine (5mM) by means of the Nash assay (65). This method is based upon the Hantzch reaction for the synthesis of pyrimidines (147), in which 2 moles of

acetylacetone condense with 1 mole of ammonia and 1 mole of formaldehyde to yield 3,5-diacetyl-1,4-dihydrolutidine plus 3 moles of water. In this case the determination was performed as described by Werringloer (148) and a calibration curve was prepared using paraformaldehyde as a standard (25 to 250$\mu g ml^{-1}$).

3.8.2 Determination of covalent binding

The covalent binding of ^{14}C-NMF metabolites to microsomal protein was determined following incubation of 7mM O^{14}C-NMF or $^{14}CH_3$-NMF (specific activity 71$\mu Ci\ mmol^{-1}$) with microsomes for 2 hours as described in 3.8.1. The incubation was terminated by precipitation of the microsomal protein with 4ml of acetone. The microsomal protein was transferred quantitatively to a centrifuge tube and washed extensively with water and water methanol (1:1) until no further radioactivity could be removed (see Figure 3.6). The tissue pellet was solubilized in 2ml of 1N NaOH, and two 50µl aliquots were taken for protein determination (141) and two 1ml aliquots were transferred to scintillation vials and the residual radioactivity was determined as described in Section 3.1 (Quench curve 3). The results were expressed as ng NMF equivalents bound mg^{-1} of microsomal protein.

3.8.3 Attempts to modify covalent binding of NMF to microsomes

Incubations were also performed with microsomes isolated from mice pretreated with phenobarbitone as described in Section 3.6.4.1 or in the presence of SKF525A (0.1mM).

The potential of GSH to abolish the covalent binding of NMF metabolites to microsomal protein was evaluated by addition of GSH in a final concentration of 10mM to the incubations.

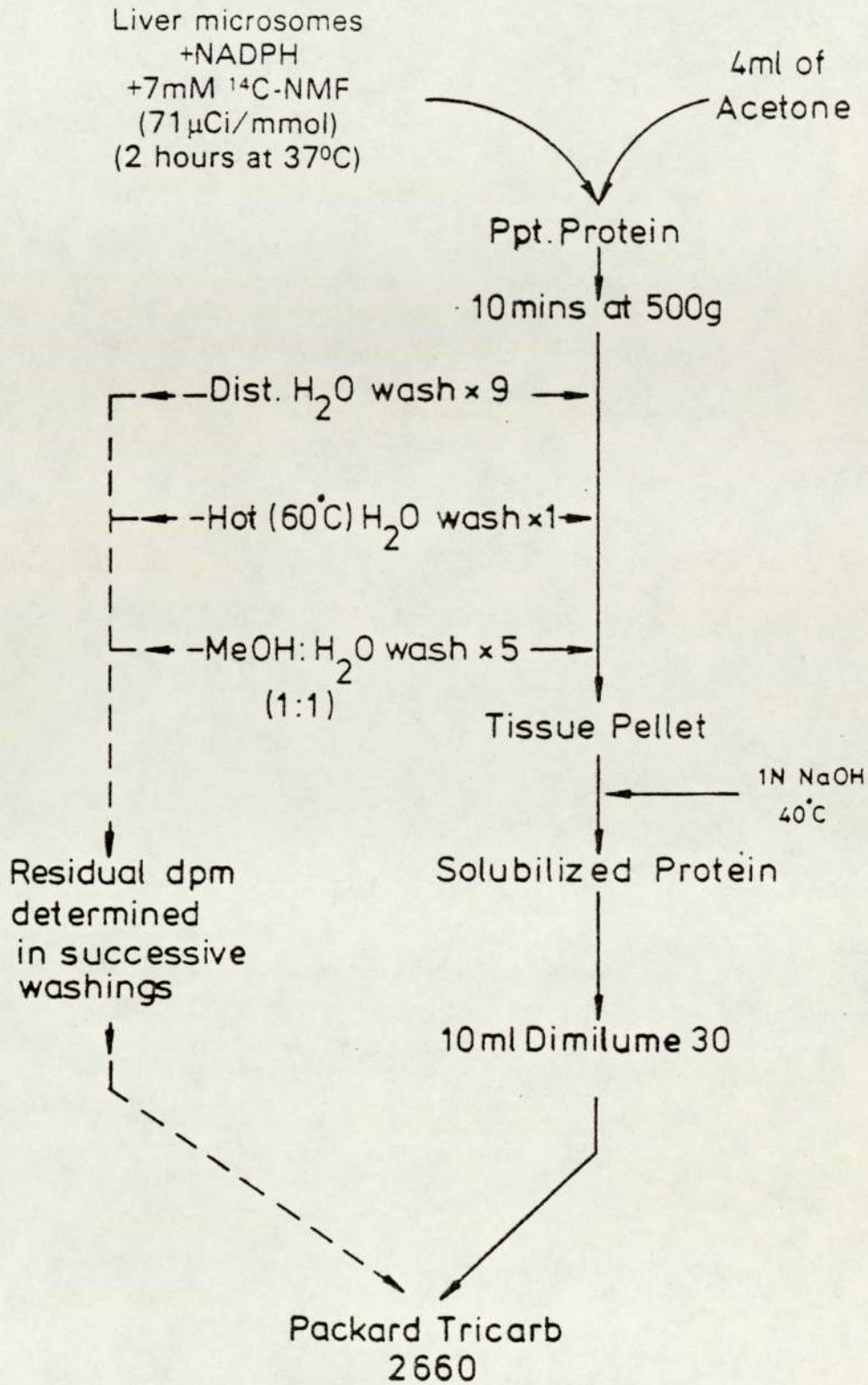


Figure 3.6 Procedure for determining covalent binding of ^{14}C-NMF metabolites to microsomal protein *in vitro*

3.9 Characterization and modification of NMF hepatotoxicity

3.9.1 Determination of the activity of liver enzymes in the plasma of mice

3.9.1.1 Plasma Samples

Mice were anaesthetised in a sealed dessicator containing cotton wool balls impregnated with `Ether for anaesthesia´ (Hills Pharmaceuticals, Burnley). Blood samples (1ml) were collected, by cardiac puncture, into 1ml syringes containing 50µl of Heparin injection BP 2,500 units in 1ml (Evans Medical, Speke). Plasma was obtained by centrifugation in a Beckman microfuge for 1 minute, and was stored at $4^{o}C$ prior to measurement of enzyme activity. All plasma samples were assayed within 6 hours of collection.

3.9.1.2 Sorbitol dehydrogenase (SDH)

The activity of SDH in plasma was assayed according to a method described by Rose and Henderson (144).

700µl NADH (disodium salt, 355µM) in Tris HCl (100mM pH 6.6, $37^{o}C$) was placed in a reduced volume 1cm pathlength cuvette. Plasma (100µl) was added, and the endogenous reaction allowed to proceed to completion (3-5 minutes; monitored by ΔA_{340}). 200µl β-D-fructose (2.5M in 100mM Tris HCl, pH 6.6, $37^{o}C$) was added to initiate the SDH reaction and the absorbance at 340nm was monitored (5 minutes). The activity of SDH in this system is given by

$$\text{SDH activity } (Ul^{-1}) = 1608.\ \Delta A_{340}\ min^{-1}$$

(where one unit (U) of SDH activity is equal to the reduction of 1µmol of fructose per litre per minute)

3.9.1.3 Aspartate aminotransferase (AST)

The activity of AST in plasma was assayed according to a method described by Kachamar and Moss (145).

The following reagents (See Section 2.8.3.2) were added to a reduced volume 1cm pathlength cuvette; L-aspartate (767µl; 228mM), NADH (33µl; 6.5mM), pyridoxal-5´-phosphate (33µl, 4.5mM), malate dehydrogenase (16µl, 36,000Ul^{-1}) lactate dehydrogenase (16µl; 72,000 UL^{-1}) and plasma (67µl). The cuvette and contents were then preincubated in the thermostated (37^{o}C) cuvette holder of a Beckman DU 7 spectrophotometer for 5 min to permit the endogenous side reactions to proceed to completion. The AST reaction was then initiated by adding α-Ketoglutaric acid solution (67µl; 225mM) to the cuvette and measuring the absorbance change at 340nm for 5 min. The activity of AST in this system is given by

$$\text{AST activity (UL}^{-1}) = 2410.\ \Delta A_{340}\ \text{min}^{-1}$$

Where one unit (U) of AST activity is equal to the oxidation of 1µmol of NADH per litre per minute.

3.9.1.4 Alanine aminotransferase (ALT)

This assay is identical to the one for AST except that L-alanine replaces L-aspartate as the amino group donor and lactate dehydrogenase replaces malate dehydrogenase as the indicator enzyme. Thus the mixture is as above with L-alanine (767µl; 525mM) being added, lactate dehydrogenase being present at a higher concentration (33µl; 72,000 UL^{-1}) and L-aspartate and malate dehydrogenase not present

$$\text{ALT activity (UL}^{-1}) = 2410.\ \Delta A_{340}\ \text{min}^{-1}.$$

Where one unit (U) of ALT activity is equal to the

oxidation of 1μmol of NADH per litre per minute

3.9.2 Histopathological examination of livers

Livers were excised immediately after the blood sample was obtained, and fixed in 10% Formol Saline for 7 days. Slices (4mm) were taken from the three major lobes of each liver and left in 10% Formol Saline for a further day. The liver slices were processed in a (Shandon Southern Products Ltd) 2 litre tissue processor: they were dehydrated through graded alcohols (70%, 90%, 90%, 100%, 100%), and were cleared in Xylene (50: 50; Xylene: alcohol, Xylene, Xylene), before being placed in a molten Paramat wax (3 changes). The liver slices were then embedded into wax blocks using Paramat wax in a (Shandon Southern Products Ltd) Tissue Embedding System.

The wax blocks were then transverse sectioned at 4μM on an Anglia Scientific Rotary Microtome, and the wax sections mounted on glass slides.

The sections were then stained with `Harris´ haematoxylin stain and counterstained with Navy Eosin (149). All the slides were then randomized prior to a `blind´ pathological evaluation by Dr Iona Pratt (University College Dublin, Eire).

3.9.3 Dose related nature of NMF hepatotoxicity in Balb and CBA/CA mice

Male Balb/C mice received saline, 100, 200 or 400mgkg^{-1} NMF. After 24h the livers were removed for histopathological examination (Section 3.9.2) and plasma samples were obtained (Section 3.9.1.1) for analysis of liver enzyme activities (Section 3.9.1.2 to 3.9.1.4). Male CBA/CA mice received saline, 200 or 400mgkg^{-1} NMF, and were treated in the same way as the Balb/C mice.

3.9.4 Pretreatments to influence the severity of NMF hepatotoxicity

In an attempt to establish the mechanism of NMF induced hepatotoxicity, Balb/C mice were pretreated with a number of agents prior to administration of various doses of NMF as described in Section 3.9.3.

The role of drug metabolism in the hepatotoxicity of NMF was evaluated by pretreating mice with phenobarbitone (Section 3.6.4.1) prior to administration of 100mgkg^{-1} NMF or with SKF 525A (Section 3.6.4.1) prior to administration of 400mgkg^{-1} NMF. In addition mice were pretreated with Methimazole (200mgkg^{-1}) 15 min before administration of 200mgkg^{-1} NMF.

The role of GSH in NMF hepatotoxicity was evaluated by depletion of hepatic GSH with BSO (1600mgkg^{-1}) 4h prior to administration of 100mgkg^{-1} NMF, and also be pretreating mice with thiol containing compounds. N-Acetylcysteine was administered as either a dose of 100mgkg^{-1} 1h before and 8h after NMF (200 or 400mgkg^{-1}) or orally (1200mgkg^{-1}) 20 min before NMF (200 or 400mgkg^{-1}). In addition cysteine (300mgkg^{-1}) was administered 10 min before and 20 min after 200mgkg^{-1} NMF.

The influence of free radical scavengers was evaluated by pretreating mice with vitamin E (100mgkg^{-1}) 15h prior to 200 or 400mgkg^{-1} NMF. In addition mice were pretreated with promethazine (24mgkg^{-1}) 10 min before 200mgkg^{-1} NMF.

For all of these pretreatments the severity of NMF hepatotoxicity was evaluated as described in Section 3.9.1 and 3.9.2.

SECTION 4

RESULTS

4.1 In vivo metabolism of NMF by Balb/C and CBA/CA mice

4.1.1 Introduction

Many, otherwise inert, xenobiotics undergo phase I oxidative metabolism to a reactive species. The reactive intermediate may subsequently be detoxified by either sulphation, glucuronidation or conjugation with GSH. The resulting phase II metabolite may then be excreted in the faeces or in the urine as a sulphate, glucuronide or mercapturate (76). Alternatively, the reactive species can react with potentially critical cellular macromolecules to initiate a series of events leading to cell death.

Similarities between the pattern of hepatotoxicity of NMF and hepatotoxins which are metabolically activated to necrogenic species suggested that metabolism is a prerequisite for NMF toxicity (Section 1.2).

The metabolism of NMF (see Section 1.3) has previously been evaluated in CBA/CA mice in this laboratory (4, 18, 41). But, the nature of the hepatotoxicity has been characterized in the Balb/C mouse (26), in which NMF is more potently hepatotoxic than in other strains (21). It is conceivable that the marked strain difference in the hepatotoxicity of NMF may be related to differences in the ability of the strains to metabolize NMF to a reactive species. In this thesis evidence is presented (Section 4.9) which supports the hypothesis that the hepatotoxicity of NMF is associated with the metabolic generation of a reactive species. To investigate the potential strain difference in metabolism of NMF, a quantitative study of the fate of NMF has been undertaken in CBA/CA and Balb/C mice.

Following the administration of 400mgkg^{-1} OH^{14}C-NMF or $^{14}CH_3$-NMF the excretion of radioactivity in expired air (as $^{14}CO_2$) and in

the urine of Balb/C and CBA/CA mice has been evaluated for 72 hours (see Section 3.4). In addition, the amount of unchanged NMF and the metabolites CH_3NH_2 and F excreted in the urine were determined by G.L.C or H.P.L.C respectively.

The hepatotoxic dose threshold of NMF has been established at $150mgkg^{-1}$ in Balb/C mice (21). Here, the metabolism of NMF was studied using doses of $400mgkg^{-1}$ and $100mgkg^{-1}$.

The aims of this study were twofold:

1) To investigate the dose related nature of NMF metabolism in Balb/C mice using a hepatotoxic and a non-hepatotoxic dose.
2) To evaluate quantitative differences in the metabolism of NMF between Balb/C and CBA/CA mice.

4.1.2 Results

Unchanged NMF and F (or HMF) were determined in urine by G.L.C. (see Section 3.4.3), a typical chromatogram is shown in Figure 4.1. Methylamine, the principle metabolite in urine was determined by H.P.L.C. following derivatization with DNBS (see Section 3.4.4), a typical chromatogram is shown in Figure 4.2.

In a preliminary study $400mgkg^{-1}$ NMF was administered to CBA/CA and Balb/C mice and levels of NMF in the urine were determined at 2 hour intervals for the first 8 hours, and then at 12 hour and 24 hour post dose (Figure 4.3). Over the 24 hour period the urinary excretion profile of unchanged NMF was qualitatively similar in both Balb/C and CBA/CA mice. But, CBA/CA mice excreted twice as much unchanged NMF (25.4%) compared to Balb/C (13.0%).

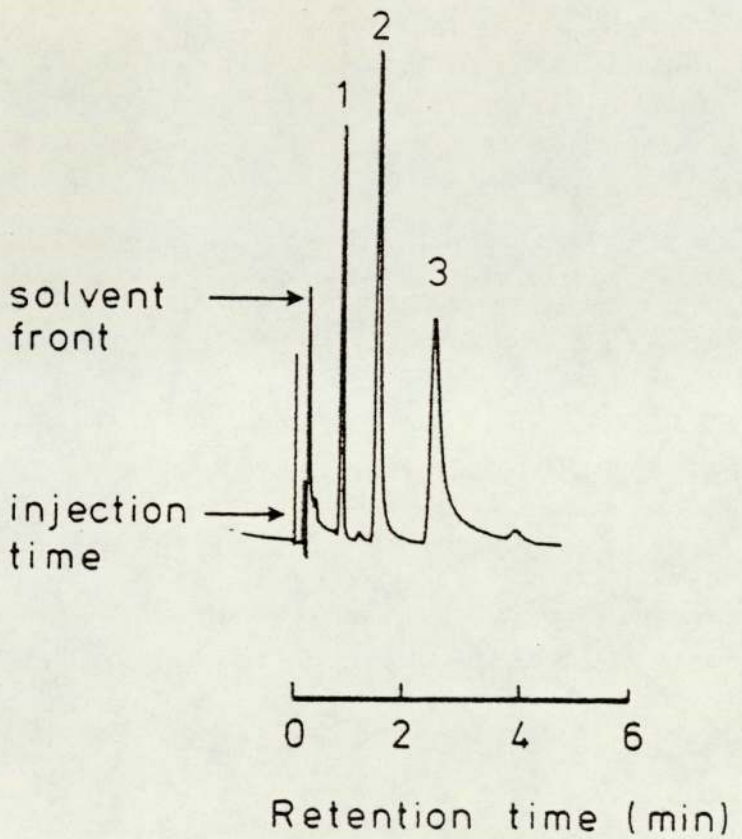


Figure 4.1 A typical G.L.C. chromatogram of urine from Balb/C mice administered N-methylformamide.

1) Tetramethylurea (Internal standard)

2) N-Methylformamide

3) Formamide

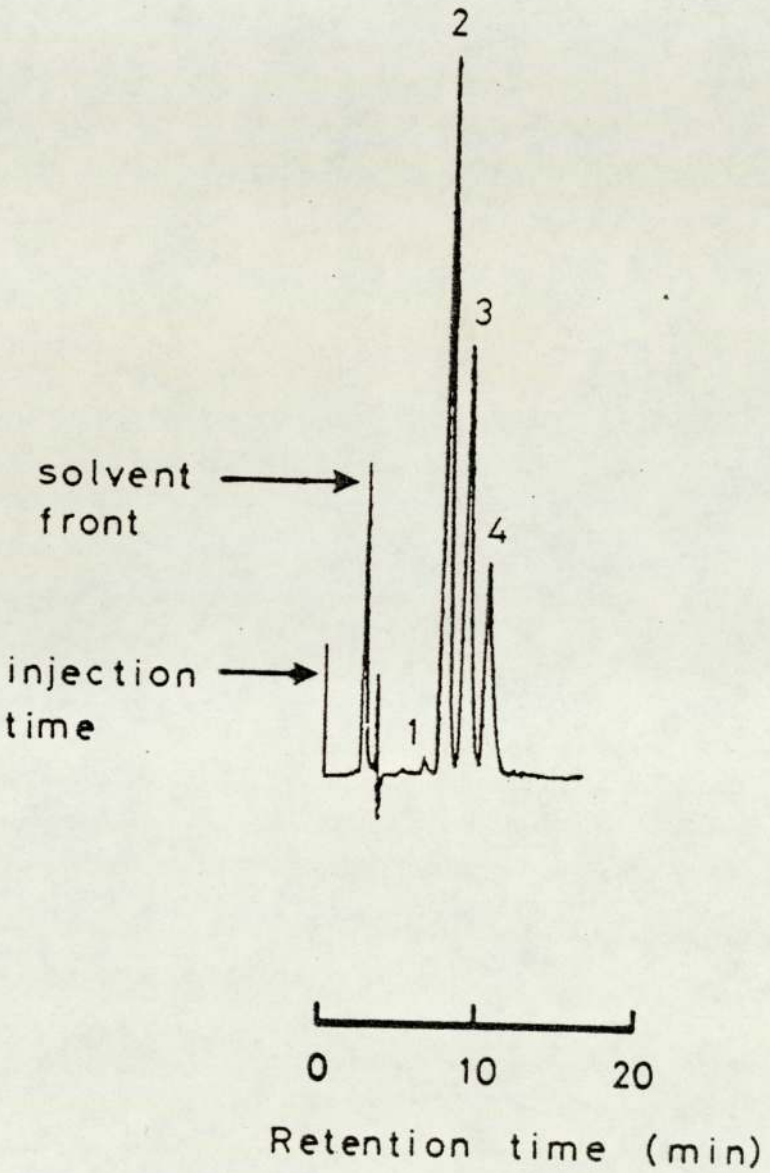


Figure 4.2 A typical H.P.L.C. chromatogram of urine from Balb/C mice administered N-methylformamide, after derivatization of the urine with DNBS.

1) DNP-ethanolamine

2) DNP-ammonia

3) DNP-butylamine (Internal standard)

4) DNP-methylamine

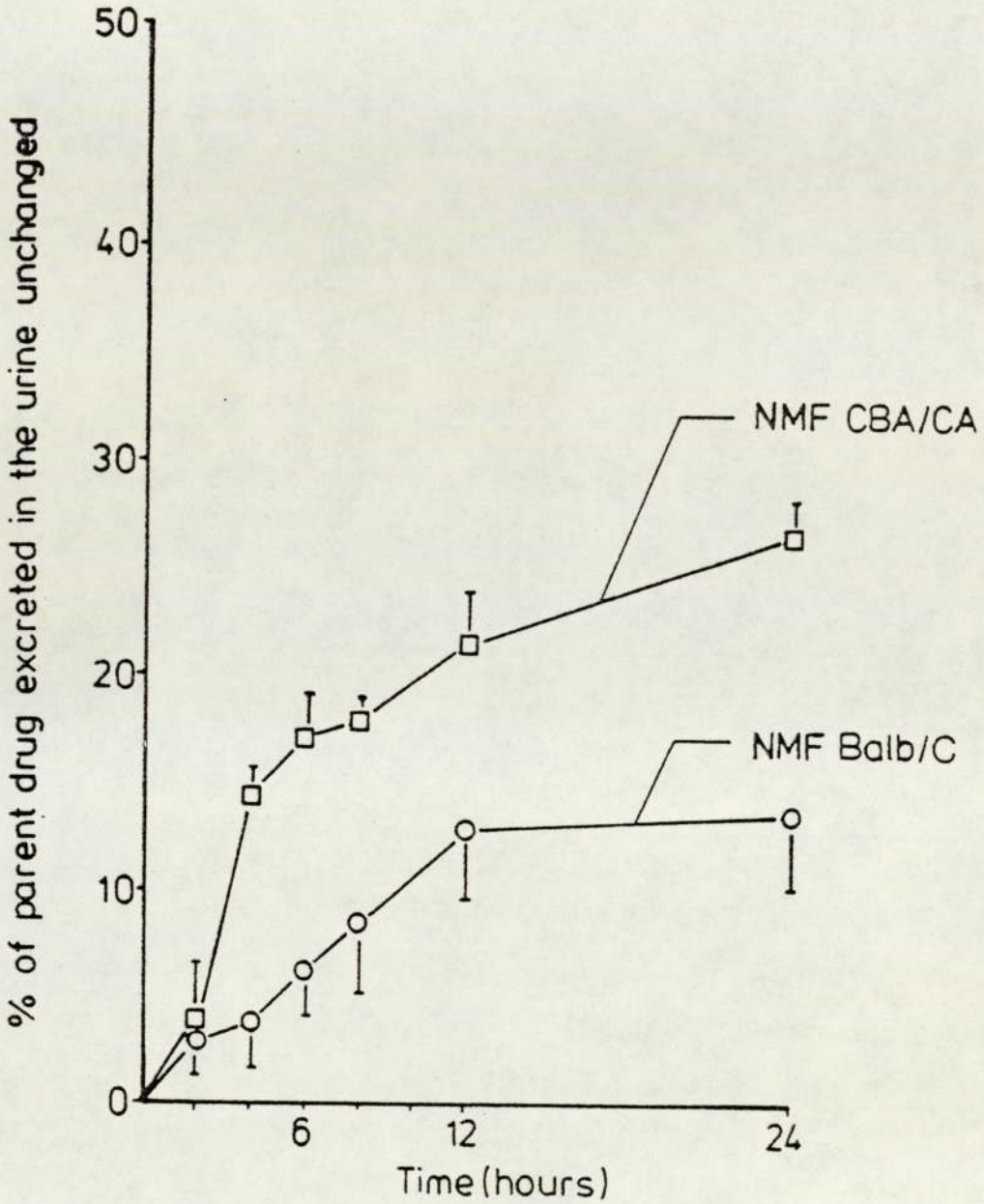


Figure 4.3 Excretion of NMF in the urine of Balb/C and CBA/CA mice, for a 24 hour period following a single i.p. injection of 400mgkg^{-1} NMF (mean ± s.e. mean; n = 4)

Following the preliminary study, the mass balance for the excretion of $OH^{14}C$-NMF and $^{14}CH_3$-NMF in both Balb/C and CBA/CA mice was evaluated. The results of this study are presented in Tables 4.1 to 4.3 and graphically in Figures 4.4 to 4.17. Also, the results are tabulated in Appendix I.

The profiles of excreted radioactivity derived from either $OH^{14}C$-NMF or $^{14}CH_3$-NMF in the breath and urine of Balb/C mice are shown in Figure 4.4. Following administration of $OH^{14}C$-NMF, the radioactivity was cleared principally via the lungs as $^{14}CO_2$ (51.3% in 24 hours) with 31% excreted in the urine over the same period. Conversley $^{14}CH_3$-NMF was cleared principally via the urine (55.6% in 24 hours) compared with 16% as $^{14}CO_2$ in the breath. Faecal excretion accounted for only 3.2% and 6.1% in case of $OH^{14}C$-NMF and $^{14}CH_3$-NMF respectively (Appendix I). Total recovery of radioactivity (urine + breath + faeces) was significantly greater for $OH^{14}C$-NMF ($P < 0.05$) than for $^{14}CH_3$-NMF (Figure 4.6) at 24 hour. Residual radioactivity remaining in the mouse was calculated as the original dose less the total recovery of radioactivity (urine + breath + faeces) for time points up to 60 hours post dose. The residual radioactivity in the mouse at 72 hour post dose was determined experimentally (Section 3.4.2).

G.L.C. analysis of the urine (see Figure 4.5) indicated that within 24 hours, 10% of the dose of NMF was excreted unchanged, and 5.8% was excreted as F. Beyond 48 hours after drug administration no further excretion of NMF or F could be detected in the urine of Balb/C mice.

H.P.L.C. analysis of the urine revealed that 27.4% of the dose was excreted as methylamine within 24 hours. By 72 hours, 40% of

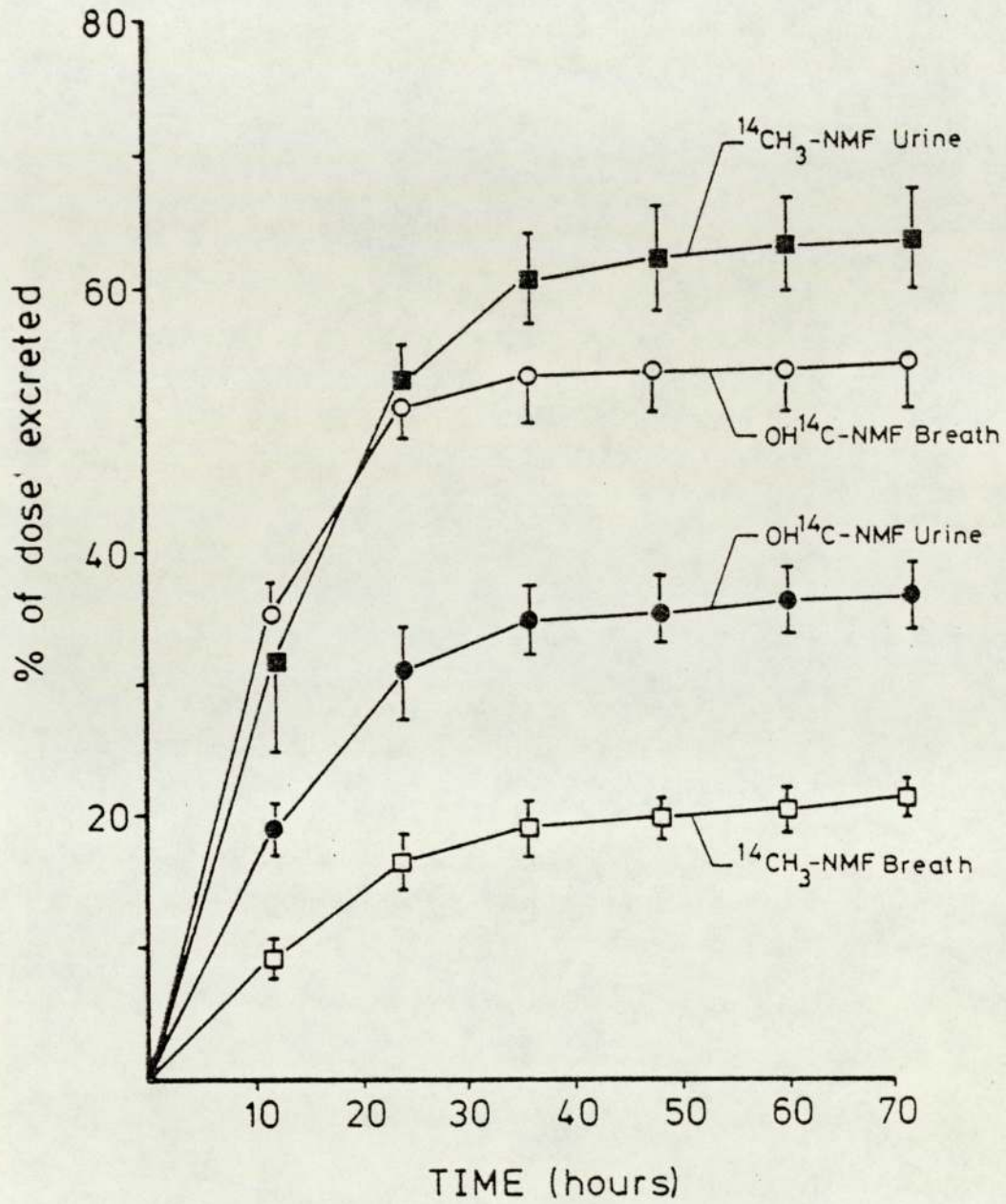


Figure 4.4 Excretion of ^{14}C-NMF metabolite(s) in the urine and breath of Balb/C mice following a dose of 400mgkg^{-1} $^{14}CH_3$-NMF or OH^{14}C-NMF (mean ± s.e. mean; n = 3)

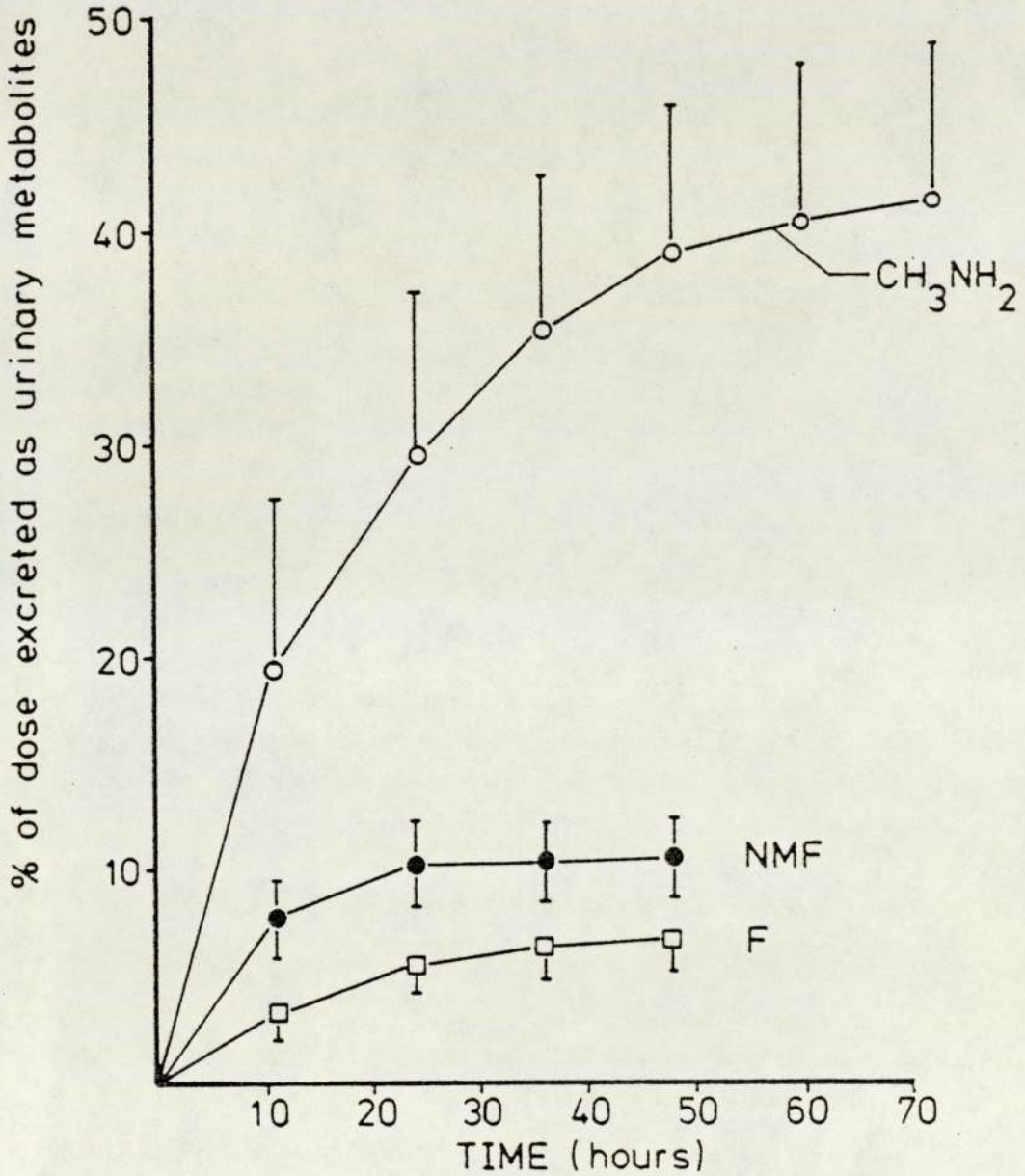


Figure 4.5 Excretion of NMF, F and methylamine in the urine of Balb/C mice following a single i.p. injection of 400mgkg^{-1} NMF (mean ± s.e. mean; $n \geq 4$)

Figure 4.6

Total recovery of a single dose of 400 mg/kg^{-1} OH^{14}C-NMF (●——●) and $^{14}CH_3$-NMF (○——○) administered to Balb/C mice (mean ±s.e. mean; n = 3).

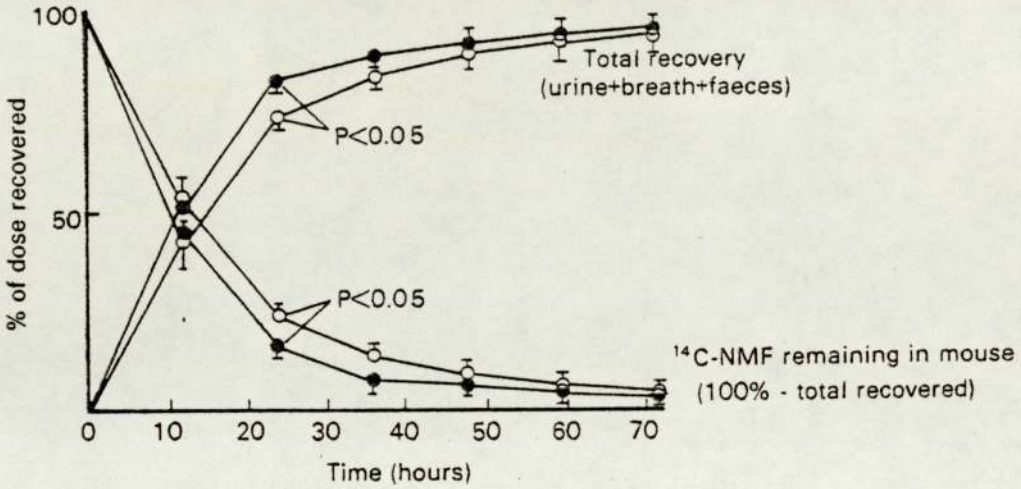
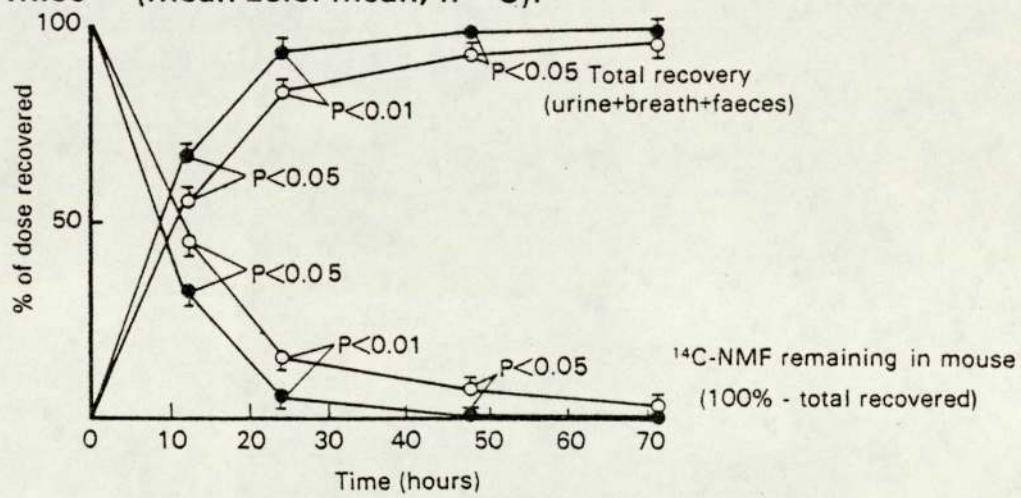


Figure 4.7

Total recovery of a single dose of 400 mg/kg^{-1} $^{14}CH_3$-NMF (○——○) and OH^{14}C-NMF (●——●) administered to CBA/CA mice (mean ±s.e. mean; n = 3).



the radioactivity injected with the dose had appeared in the urine (Figure 4.5) as methylamine. The methylamine excretion following NMF has been corrected for excretion of endogenous methylamine (43.6μg in 24 hours) (see Table 4.1).

Table 4.1 Endogenous excretion of methylamine in the urine of Balb/C mice (24 hour; mean ± s.e.m; n = 4)

Mouse	Methylamine excreted in 24h (μg)
1	43.0
2	48.9
3	66.7
4	15.8
mean ± s.e. mean	46.3 ± 12.3

Total urinary metabolites, determined as total radioactivity less the amount of unchanged NMF, comprised 45.5% and 20% of the dose for $^{14}CH_3$-NMF and $OH^{14}C$-NMF respectively at 24 hours. The 24 hour excretion figures for Balb/C mice are summarized in Table 4.2 to

enable a comparison to be made with CBA/CA mice.

The excretion of radioactivity derived from $^{14}CH_3$-NMF in the breath and urine of Balb/C and CBA/CA mice are compared in Figure 4.8. Balb/C mice exhaled more $^{14}CO_2$ derived from $^{14}CH_3$-NMF (21.1%) than did CBA/CA mice (14.2%; $p < 0.01$) by 72 hours, however this difference is not significant when the values obtained within 24 hours are compared. Balb/C mice also exhaled significantly more $^{14}CO_2$ derived from OH^{14}C-NMF (51.3%) than did CBA/CA mice (40%) measured within 24 hours ($P < 0.05$) (Figure 4.9 and Table 4.2).

In both strains the total recovery of radioactivity which stemmed from OH^{14}C-NMF was greater than that derived from $^{14}CH_3$-NMF at 24h (Figure 4.6 and 4.7). Total urinary metabolites (determined as total radioactivity in urine less the amount of unchanged NMF) in both strains was similar for each of the labels.

The differences in the quantitative metabolism of NMF by Balb/C and CBA/CA mice 24 hours after a single dose of 400mgkg^{-1} are summarized in Table 4.2.

The metabolism of NMF has also been investigated at a lower dose of NMF, 100mgkg^{-1}, which is not hepatotoxic in any of the strains investigated. Balb/C mice were used in this part of the study, and the results compared with the quantitative metabolism of a dose of 400mgkg^{-1} are summarized in Table 4.3. The excretion profile of radioactivity derived from 100mgkg^{-1} OH^{14}C-NMF or $^{14}CH_3$-NMF in the breath and urine of Balb/C mice are shown in Figure 4.12. In the case of OH^{14}C-NMF the pulmonary clearance of $^{14}CO_2$ at 24 hour post dose was

Table 4.2 Excretion of ^{14}C-NMF in the urine and expired air of Balb/C and CBA/CA mice 24 hours after a single i.p. dose of 400mgkg^{-1} (% of dose administered; mean ± s.e. mean; n ⩾ 3)

Metabolite	Balb/C	CBA/CA
EXPIRED AIR		
$^{14}CO_2$ derived from OH^{14}C-NMF	51.3±2.8	40.0±2.6 *(P<0.05)
$^{14}CO_2$ derived from $^{14}CH_3$-NMF	16.0±1.5	12.3±0.76
URINE		
NMF	10.1±1.5	26.4±1.9 (P<0.001)
Methylamine	27.4±9.1	33.3±8.9
Formamide	5.8±1.4	<2%
Total metabolites from OH^{14}C-NMF$^+$	20%	20.8%
Total metabolites from $^{14}CH_3$-NMF$^+$	45.5%	40.5%

* Students t-test

+(Total metabolites determined as total radioactivity in urine less unchanged NMF)

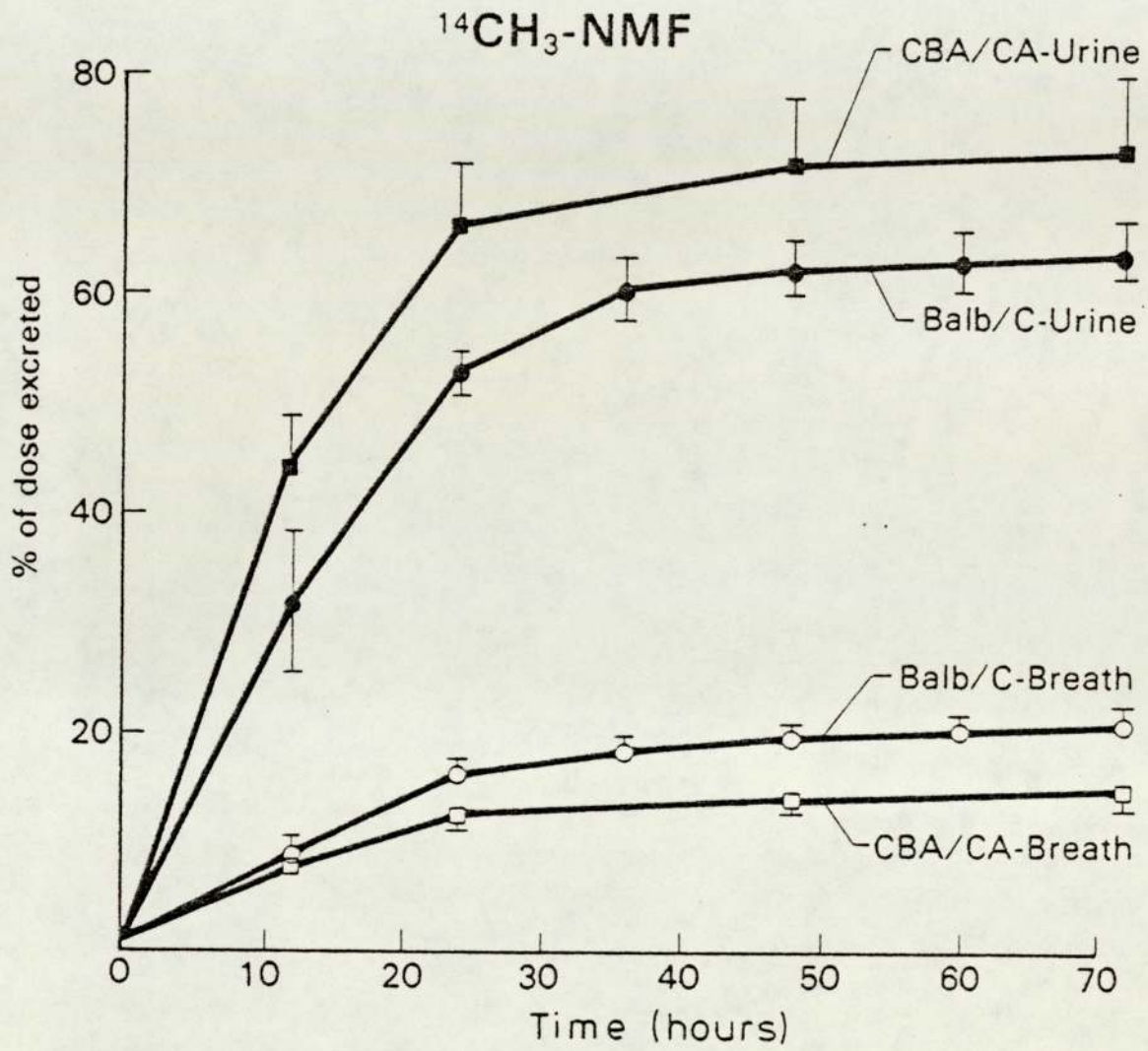


Figure 4.8 Excretion of ^{14}C-NMF in the urine and expired air of Balb/C and CBA/CA mice after a single dose of 400mgkg^{-1} $^{14}CH_3$-NMF (Mean ± s.e. mean; n = 3)

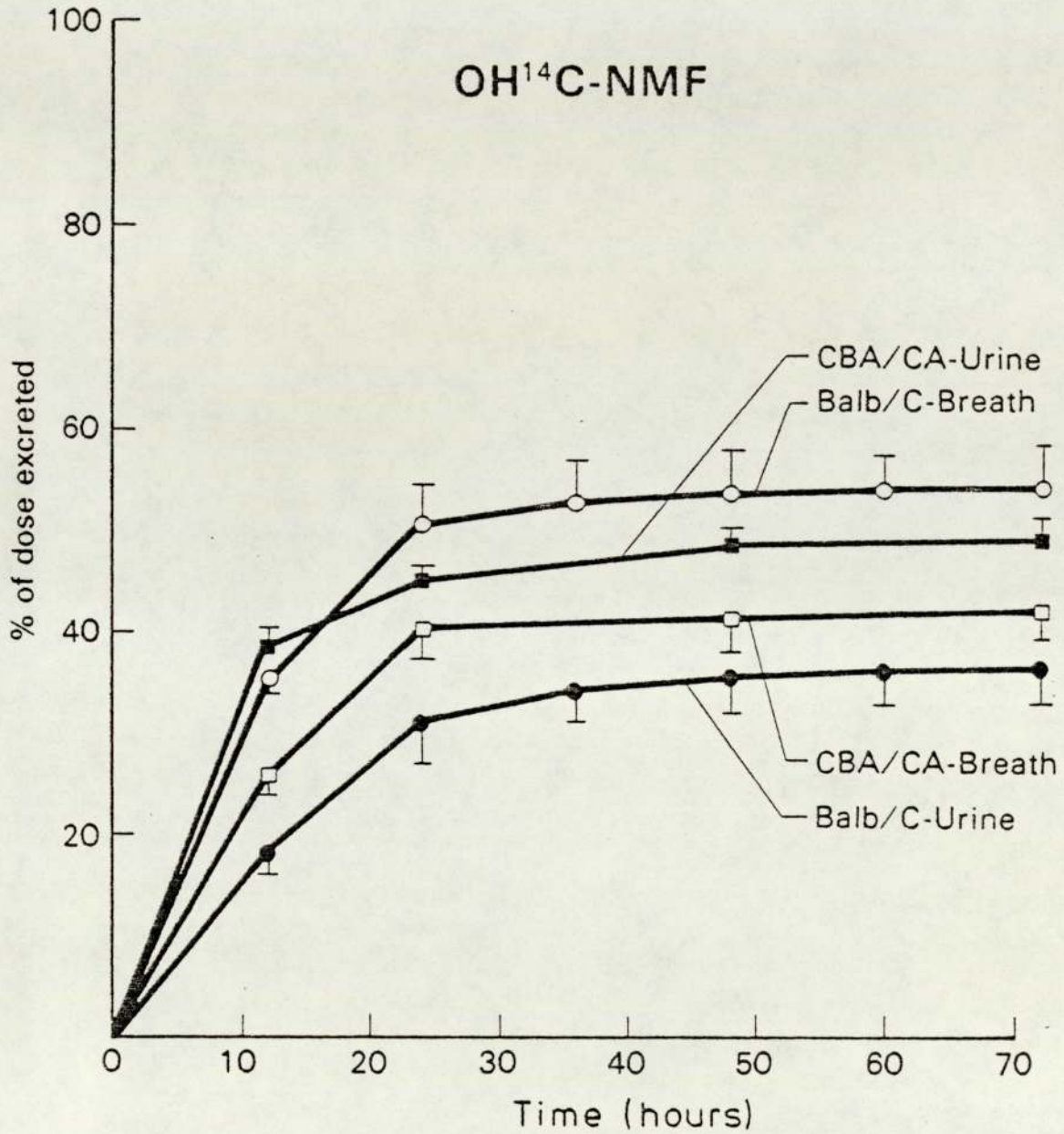


Figure 4.9 Excretion of ^{14}C-NMF in the urine and expired air of Balb/C and CBA/CA mice after a single dose of 400mgkg^{-1} OH^{14}C-NMF (mean ± s.e. mean; n = 3)

Figure 4.10

Total recovery of a single dose of 400 mg/kg OH^{14}C-NMF administered to Balb/C (o—o) and CBA/CA mice (•—•) (mean ±s.e. mean; n = 3).

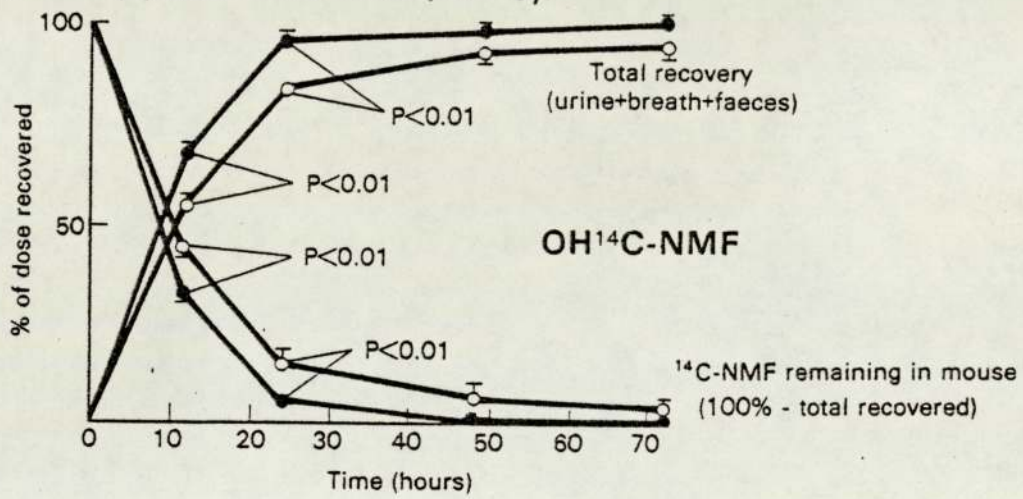


Figure 4.11

Total recovery of a single dose of 400 mg/kg $^{14}CH_3$-NMF administered to Balb/C (o—o) and CBA/CA mice (•—•) (mean ±s.e. mean; n = 3).

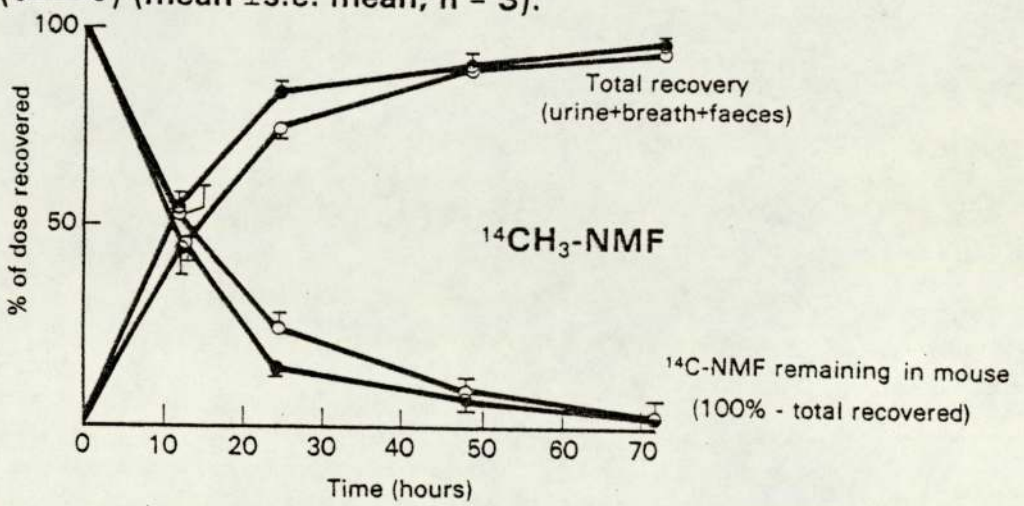


Table 4.3 Excretion of ^{14}C-NMF in the urine and expired air of Balb/C mice 24 h after a single i.p. dose of 100mgkg^{-1} and 400mgkg^{-1} (% of dose administered, mean ± s.e.; n ⩾ 3)

Metabolite	400mgkg^{-1}	100mgkg^{-1}
EXPIRED AIR		
$^{14}CO_2$ derived from OH^{14}C-NMF	51.3±2.8	56.4±7.1
$^{14}CO_2$ derived from $^{14}CH_3$-NMF	16.0±1.5	23.6±1.0 *(P<0.01)
URINE		
NMF	10.1±1.5	4.5±2.1 *(P<0.05)
Methylamine	27.4±9.1	37.6±6.25
Formamide	5.8±1.4	ND
Total metabolites from OH^{14}C-NMF^{+}	20%	19.8
Total metabolites from $^{14}CH_3$-NMF^{+}	45.5%	49.1

*Students t-test

ND = Not determined

$^{+}$Total metabolites determined as total radioactivity in urine less unchanged NMF.

similar for both doses (Table 4.3). However, significantly more ($P < 0.01$) $^{14}CO_2$ derived from $OH^{14}C$-NMF was exhaled within 12 hours after a dose of $100mgkg^{-1}$ (57.9%; Figure 4.12) than after $400mgkg^{-1}$ (35.1%; Figure 4.4). A smaller percentage of radioactivity derived from $OH^{14}C$-NMF was excreted in the urine following a dose of $100mgkg^{-1}$ NMF (Figure 4.12) than after $400mgkg^{-1}$ NMF (Figure 4.4 and Table 4.3). The difference is due to differences in the relative amounts of unchanged NMF in the urine, as the total metabolites (total radioactivity in the urine less the amount of NMF) in the urine was similar at both doses. Total clearance of $OH^{14}C$-NMF (urine + breath + faeces) (Figure 4.14) determined within 12 hours after administration was greater at the lower dose ($P < 0.001$).

The excretion profile of $100mgkg^{-1}$ $^{14}CH_3$-NMF in the breath and urine of Balb/C mice is shown in Figure 4.12. The pulmonary clearance of $^{14}CO_2$ derived from $^{14}CH_3$-NMF was significantly greater at the lower dose (Table 4.3). The urinary excretion of radioactivity derived from $^{14}CH_3$-NMF was also greater at the lower dose, but this was not significant ($P = 0.058$). The percentage of total urinary metabolites derived from $^{14}CH_3$-NMF, was greater at the lower dose. Likewise the urinary excretion of methylamine was greater at the lower dose, but this was not significant (Table 4.3; Figure 4.13). Concomitantly less NMF was excreted in the urine unchanged at the lower dose ($P < 0.05$).

The excretion of radioactivity derived from ^{14}C-NMF in the faeces has also been determined. Following a dose of $400mgkg^{-1}$ ^{14}C-NMF, 5.1% and 11.0% of the radioactivity derived from $OH^{14}C$-NMF and $^{14}CH_3$-NMF, respectively, were excreted in the faeces. After a dose of $100mgkg^{-1}$ only 2.8% and 5.0% of the total radioactivity derived from

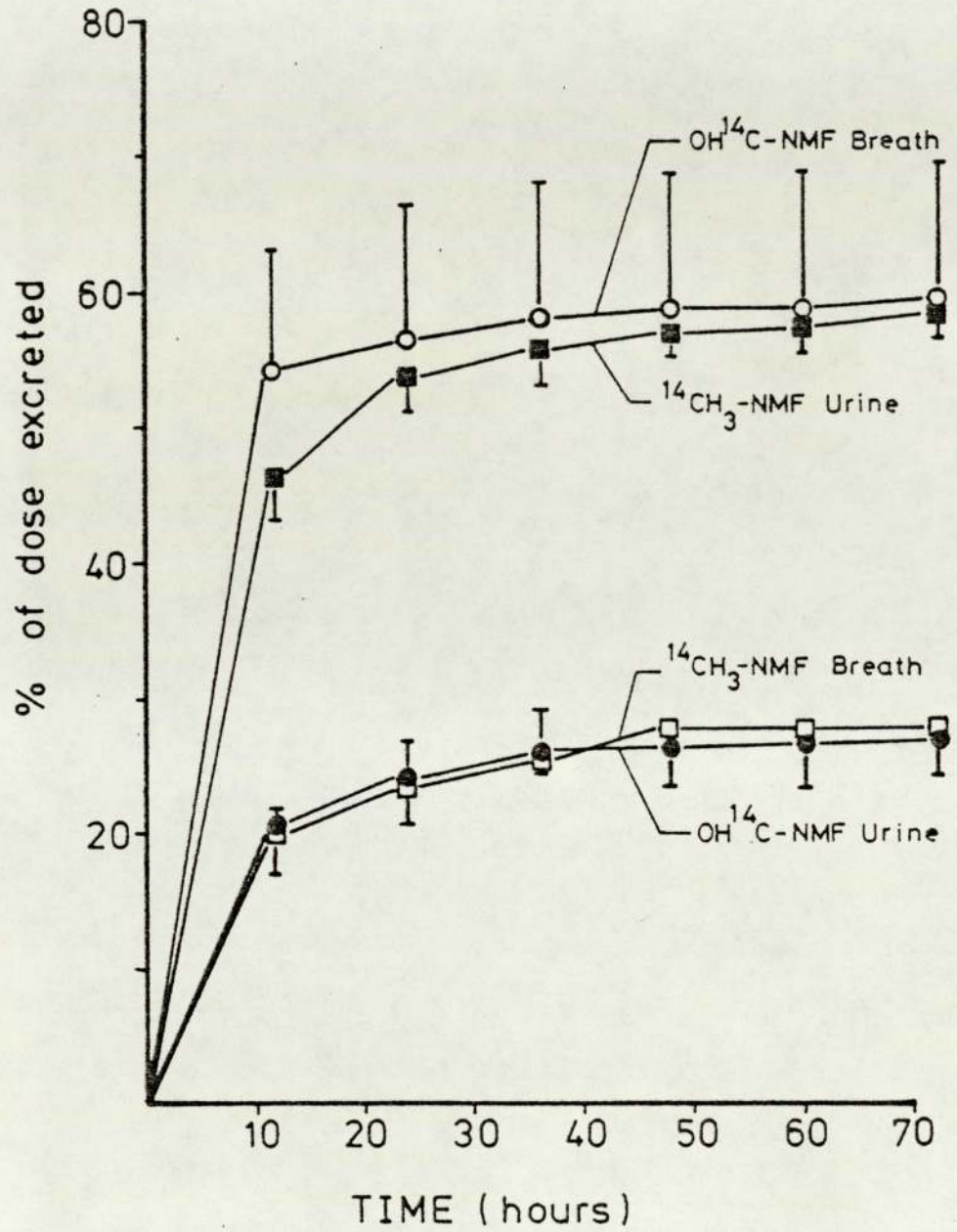


Figure 4.12 Excretion of ^{14}C-NMF derived radioactivity in the urine and breath of Balb/C mice following a dose of 100mgkg^{-1} $^{14}CH_3$-NMF or OH^{14}C-NMF (mean ± s.e.m; n = 3)

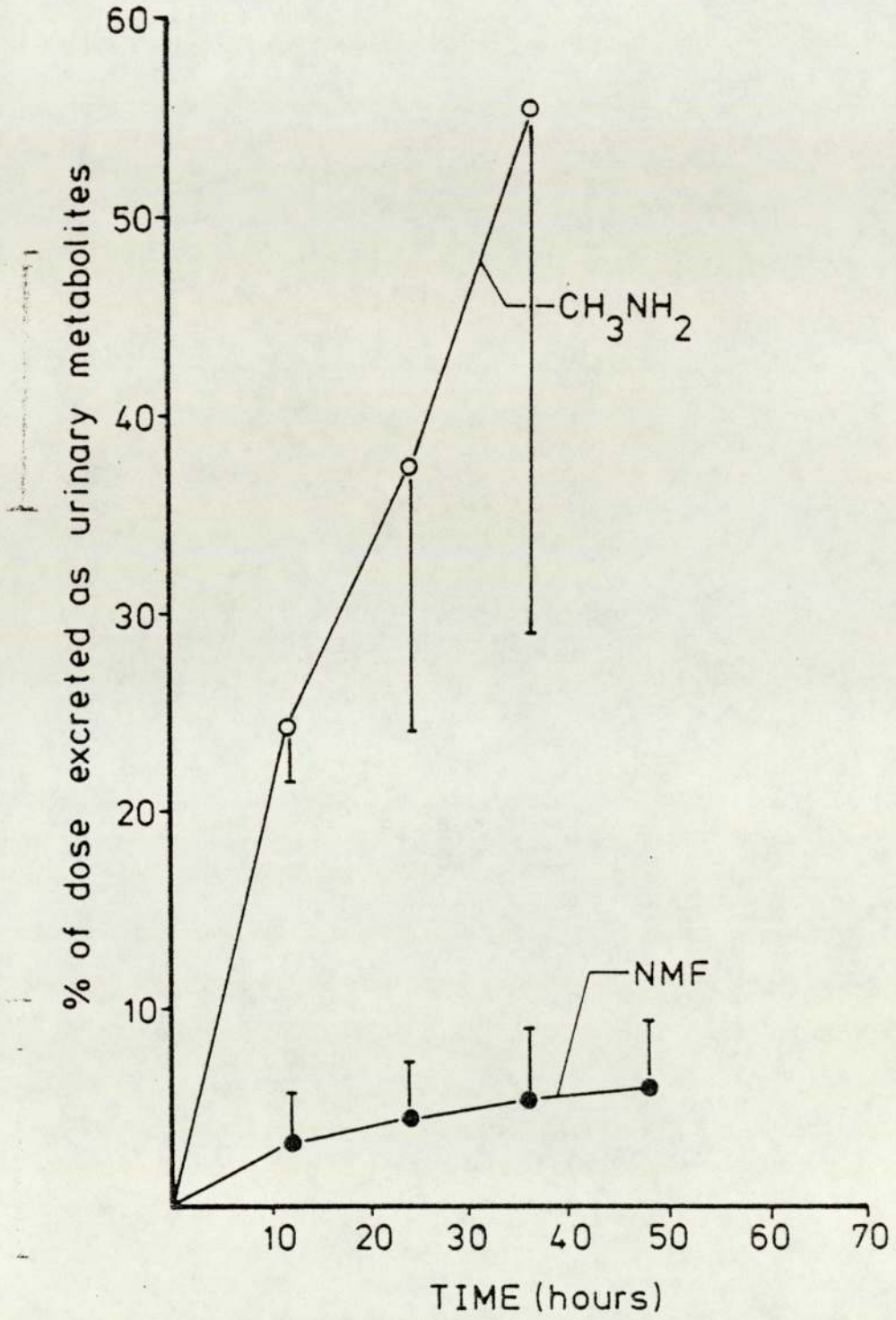


Figure 4.13 Excretion of NMF, F and methylamine in the urine of Balb/C mice following a single i.p. injection of 100mgkg^{-1} NMF (mean ± s.e. mean; n ≥ 3)

Figure 4.14

Total recovery of a single dose of 100 mg/kg (●——●) and 400 mg/kg OH^{14}C-NMF (○——○) administered to Balb / C mice (mean ±s.e. mean; n = 3).

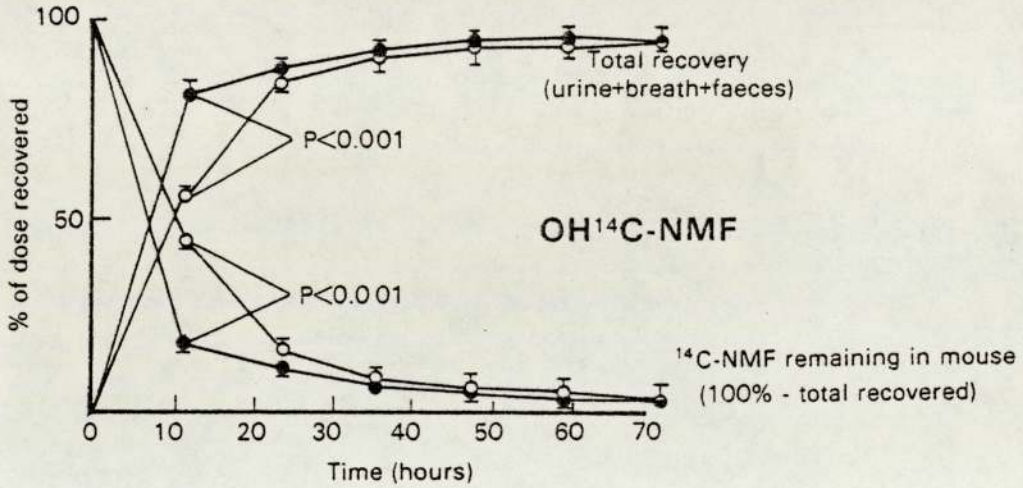
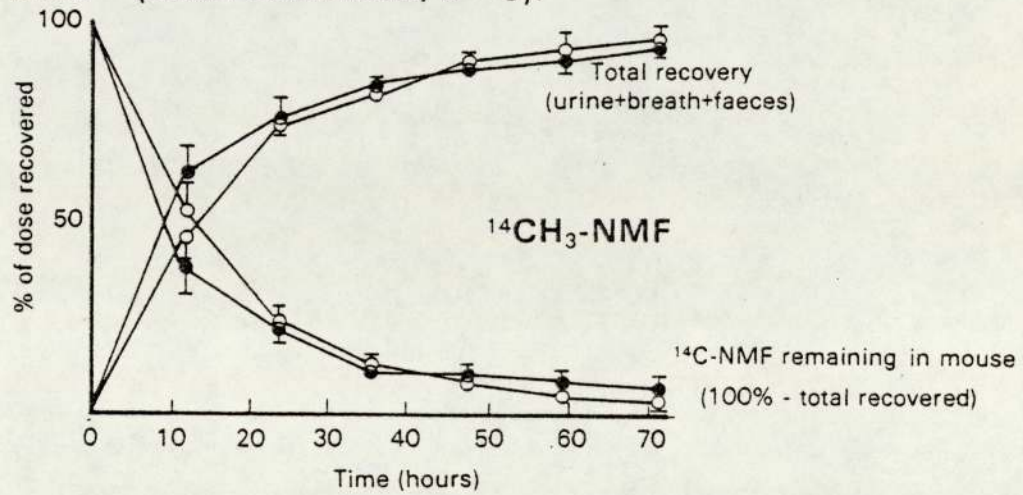


Figure 4.15

Total recovery of a single dose of 100 mg/kg (●——●) and 400 mg/kg $^{14}CH_3$-NMF (○——○) administered to Balb / C mice (mean ±s.e. mean; n = 3).



$OH^{14}C$-NMF and $^{14}CH_3$-NMF, respectively were excreted in the faeces. At the higher dose the faecal excretion of $^{14}CH_3$-NMF metabolites was greater than at 100mgkg^{-1} NMF.

4.1.3 Discussion

The results indicate that there is a strain difference in the metabolism of NMF between Balb/C and CBA/CA mice. NMF is more extensively metabolised in the Balb/C mouse, since the urinary excretion of unchanged NMF was significantly lower (Table 4.2). In addition Balb/C mice excreted more F in urine within 24 hours than did CBA/CA mice. There was however no significant difference in the urinary excretion of the major metabolite methylamine, or the total amounts of urinary metabolites.

Balb/C mice exhaled more $^{14}CO_2$ derived from $OH^{14}C$-NMF than did CBA/CA mice, a finding consistent with more extensive metabolism having occurred in the Balb/C mouse. Also Balb/C mice exhaled more $^{14}CO_2$ derived from $^{14}CH_3$-NMF over 72 hours ($P < 0.01$). Total recovery of $OH^{14}C$-NMF (urine + breath + faeces) was greater for CBA/CA mice than for Balb/C mice. Also total recovery of $^{14}CH_3$-NMF was greater in CBA/CA mice, however, this was not significantly different at 24 hours.

The apparent differences in the metabolism and disposition of NMF are important in view of the suggestion that metabolism is a prerequisite for hepatotoxicity (26). The mechanism of NMF hepatotoxicity may be mediated via the metabolic generation of a reactive species. Therefore the more extensive metabolism of NMF by Balb/C mice is consistent with the greater susceptibility of the Balb/C mouse to NMF hepatotoxicity (21) (see Section 4.10). The

strain difference in metabolism of NMF is summarized schematically in Figures 4.16 and 4.17.

The difference in the elimination of metabolites of NMF between the two strains is not profound. However, the amount of ^{14}C-NMF metabolites remaining in the mouse has greater toxicological implications, as the amount of covalently bound metabolites may determine the severity of toxicity of a foreign compound (see Section 1.5). In the CBA/CA mouse, the total clearance of ^{14}C-NMF is greater than in Balb/C mice, as a consequence the amount of ^{14}C-NMF remaining in the CBA/CA mouse is smaller than that in Balb/C mice (Figure 4.10 and 4.11). Again, the difference between the strains is small, but it may gain significance when compared with the differences between strains in the amount of ^{14}C-NMF irreversibly associated with hepatic macromolecules in vivo (Figure 4.47). This comparison reveals that the greater amounts of ^{14}C-NMF remaining in Balb/C mice compared with CBA/CA (Figures 4.10 and 4.11) reflects a difference in the extent of association of ^{14}C-NMF metabolites with hepatic macromolecules in these strains (see Section 4.8). Furthermore, the greater clearance of OH^{14}C-NMF compared with $^{14}CH_3$-NMF in both strains (Figures 4.6 and 4.7) reflects the more extentive association of radioactivity derived from $^{14}CH_3$-NMF than that of OH^{14}C-NMF with hepatic macromolecules (Section 4.8). At the lower dose of 100mgkg^{-1} NMF in Balb/C mice, the amount of ^{14}C-NMF associated with hepatic macromolecules is much less, (Section 4.6) and the total recovery of NMF is greater, than at the higher hepatotoxic dose (Figures 4.14 and 4.15).

At the dose of 100mgkg^{-1}, NMF is more extensively biotransformed in the Balb/C mouse as significantly less NMF is

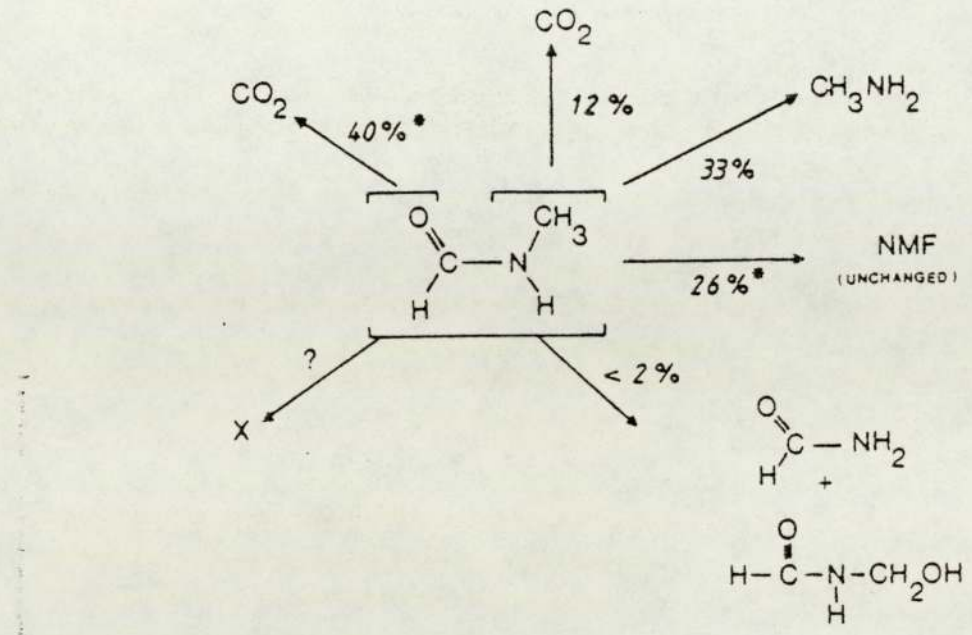
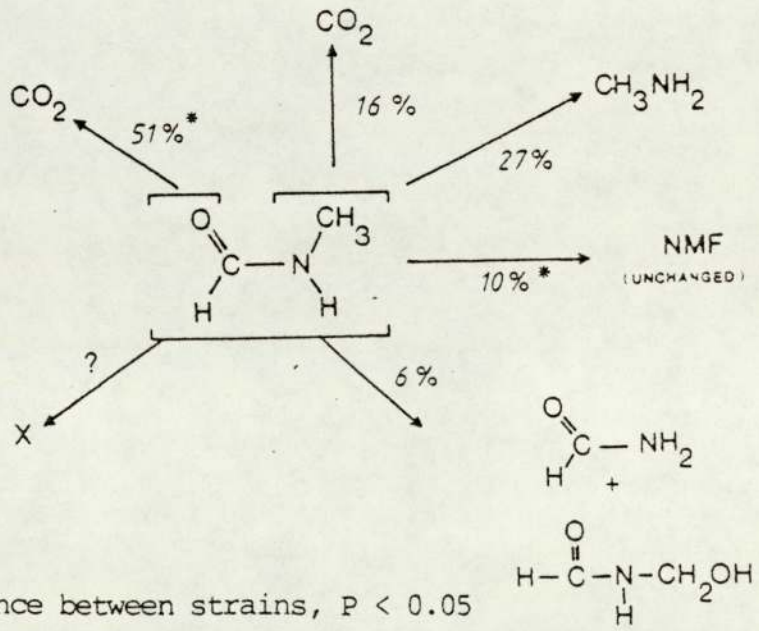


Figure 4.16 Summary of NMF biotransformation by CBA/CA mice (24 hours after administration of 400mgkg^{-1} NMF)



* significant difference between strains, $P < 0.05$

Figure 4.17 Summary of NMF biotransformation by Balb/C mice (24 hours after administration of 400mgkg^{-1} NMF)

excreted in the urine unchanged (Table 4.3). In addition more of the major metabolite, methylamine, was excreted in the urine. However at the lower dose the methylamine excretion was variable and, as a consequence on statistical analysis was not greater than that observed at 400mgkg^{-1} (Table 4.3). The expiration of $^{14}CO_2$ as a metabolite of ^{14}C-NMF also reflected the differences in metabolism between the doses.

The observation that NMF is less extensively metabolised at the higher dose of 400mgkg^{-1} than at the lower dose is interesting in view of the suggestion that DMF inhibits its own metabolism at high doses (42). Upon administration of increasing doses of ^{14}C-DMF to rats, the relative amount of radioactivity excreted in the urine remained unaltered while increasing amounts of unchanged DMF were excreted. Also the urinary excretion of HNMF was reduced indicating that DMF is metabolized to a lesser extent at high doses (42).

In the Balb/C mouse, after a dose of 100mgkg^{-1} NMF, less unchanged NMF was excreted in the urine compared with a dose of 400mgkg^{-1} NMF (Table 4.3). This suggests that NMF may exhibit non-linear pharmacokinetics over the dose range of 100mgkg^{-1} to 400mgkg^{-1} in the Balb/C mouse. In a previous study, the plasma levels of NMF were measured after administering doses of 80mgkg^{-1} and 400mgkg^{-1} to CBA/CA mice (18). For both doses the plasma concentration - time course for the disposition of NMF did not obey linear kinetics. In addition, the plot of NMF plasma concentration versus time after administration exhibited a linear relationship which suggested zero order kinetics for the elimination of NMF from the plasma of CBA/CA mice. However, when the plasma levels obtained after each dose were divided by the dose, the curves obtained were almost superimposable.

Hence it was not possible in that study to conclude whether or not the plasma disposition of NMF was governed by non-linear kinetics (18).

The results from the present study in Balb/C mice support the suggestion that the metabolism and disposition of NMF is dose related, and that NMF is more rapidly metabolized at a lower dose. In addition, the total recovery of OH^{14}C-NMF was greater at 24 hours after 100mgkg^{-1} than after 400mgkg^{-1} NMF. The lower total recovery of ^{14}C-NMF at the higher dose may reflect the greater amounts of covalently bound metabolites in the liver than at the lower non-hepatotoxic dose (Figure 4.14 and 4.15).

4.2 Thioether excretion in the urine of mice administered N-alkylformamides

4.2.1 Introduction

N-Methylformamide has been shown previously (4) and in this work (see Section 4.4) to deplete hepatic GSH in a dose related manner. This depletion is specific to NMF and is not caused by other non-hepatotoxic formamides (4), and thus is probably the consequence of a reaction between NMF metabolites and GSH.

Metabolically generated reactive species are known to be detoxified in the liver by conjugation with GSH, and they are subsequently excreted in the urine as mercapturic acids or cysteine conjugates (Section 1.4). Alkaline hydrolysis, of urine from mice which had received NMF, liberated increased amounts of thiol compounds, suggesting the presence of metabolites with a thioether structure such as N-acetylcysteine derivatives (18). Thus determination of urinary thioethers can provide preliminary evidence for the formation of a reactive electrophilic species.

The measurement of thiols after alkaline hydrolysis of urine from chemical workers has provided an index of exposure to alkylating agents (128). Also the excretion of thioethers in the urine of cigarette smokers, correlated well with the extent of exposure to cigarette smoke, and with the mutagenicity of the urine in a bacterial mutation reversion assay (129).

The urine of mice treated with NMF has been examined for thioether compounds following alkaline hydrolysis according to a method modified from Van Doorn (129). Whilst it was not possible to

gain definitive information about the structure of the mercapturate, this study has been undertaken to establish the following:-

1. The structural requirements within a series of N-alkylformamides, for the excretion of thioethers in the urine.
2. The dose dependent nature of the urinary thioether excretion induced by NMF.
3. The time course following a single i.p. injection of NMF for the appearance of thioethers in the urine.

4.2.2 Results

The estimation of urinary thioethers was performed essentially by alkaline hydrolysis followed by colorimetric estimation of the liberated thiols. Alkaline treatment leads to hydrolysis of thioethers (S-R) and also disulphides (S-S) in the urine. This method has more recently been modified by the addition of a preliminary reduction with sodium borohydride to reduce disulphides to thiols (129). Thus by measuring thiol content colorimetrically with DTNB after reduction and after hydrolysis, it is possible to determine hydrolyzable thioethers only.

It was found that the high pH of the reduction conditions (approximately pH 11) caused an increase in urinary thiols similar to that obtained on alkaline hydrolysis. Presumably, the thioether formed as a consequence of the interaction of NMF metabolite(s) and GSH is insufficiently stable to survive the reduction. As a consequence the reduction stage was not performed in the present investigation.

Structural requirements for urinary thioether production was

established by administering equimolar doses of a series of formamide analogues (Figure 4.18). Of the formamides administered, 400mgkg^{-1} NMF caused an approximately four fold elevation of urinary thioethers above controls within 24h ($P<0.01$). The other analogues tested, F, HMF and DMF caused no significant elevation of urinary thioethers.

The dose related excretion of urinary thioethers by Balb/C mice 24h after a single i.p. dose of NMF is shown in Figure 4.19. At a dose of 100mgkg^{-1} the 24h urinary thioether excretion did not differ significantly from control. However at 200mgkg^{-1} and 400mgkg^{-1} the thioether excretion was approximately three and four times that of control respectively. This clearly indicates a dose-related excretion of urinary thioethers following NMF administration.

The time course for the appearance of thioethers in the urine of Balb/C mice following a single i.p. dose of 400mgkg^{-1} is illustrated in Figure 4.20. Total urinary thioethers were determined at 6, 12, 24 and 36 hours post NMF administration. During the first 24h the rate of excretion appeared to be linear, and by 36h the thioether excretion returned to control values. Control values for total 24h thioether excretion are shown in Table 4.4. During the first 6h after a dose of 400mgkg^{-1}, urinary thioether excretion approached 24h control values and by 12h had exceeded the 24h control values (Table 4.4). The total thioether excretion in 24h, determined with a single urine collection, did not significantly differ from the value obtained after collection at time intervals during the 24 hours (Table 4.4).

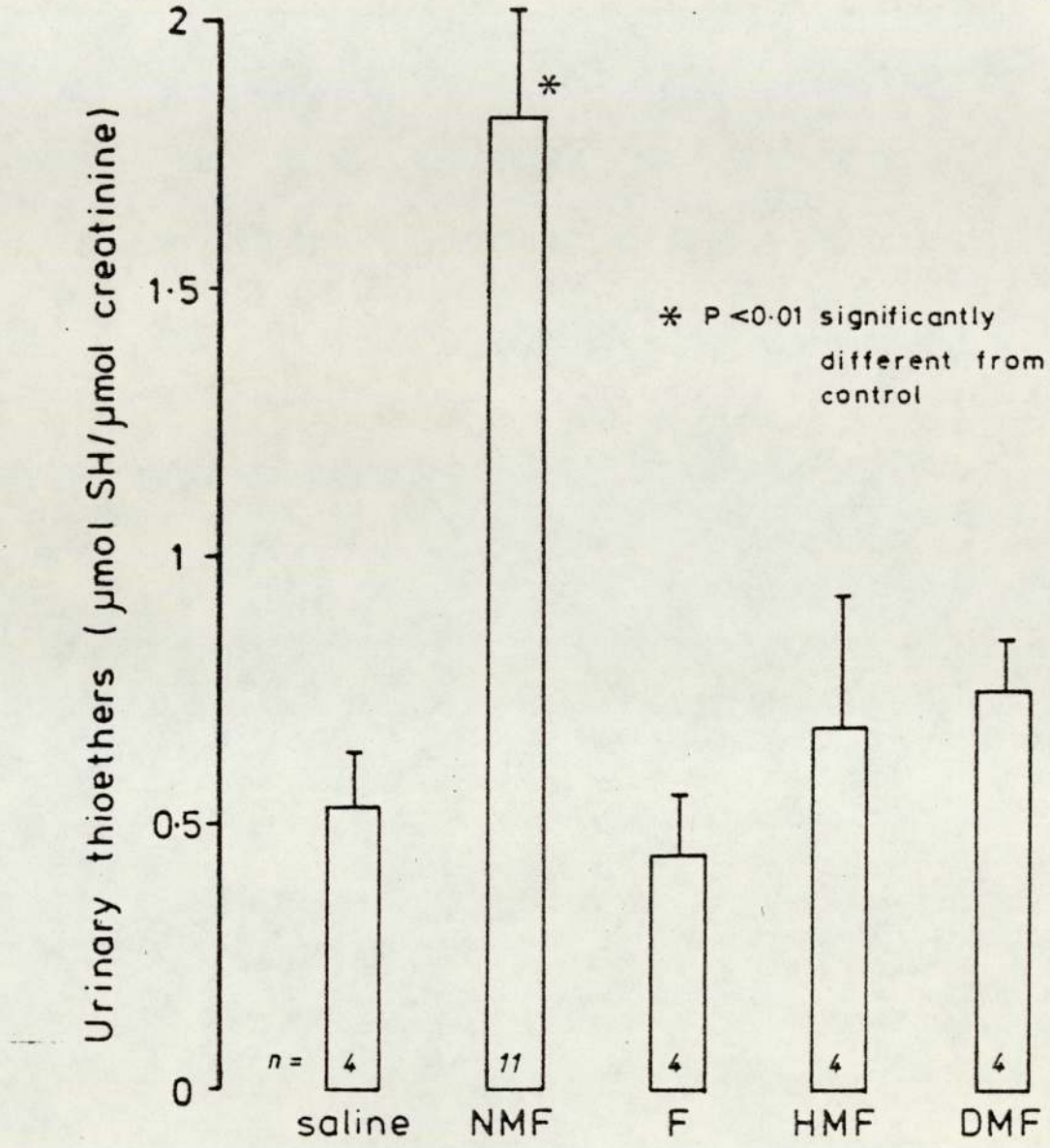


Figure 4.18 The appearance of thioethers in the urine of Balb/C mice during a 24h interval after administration of 6.8mmol kg^{-1} of various formamide analogues (mean ± s.e. mean)

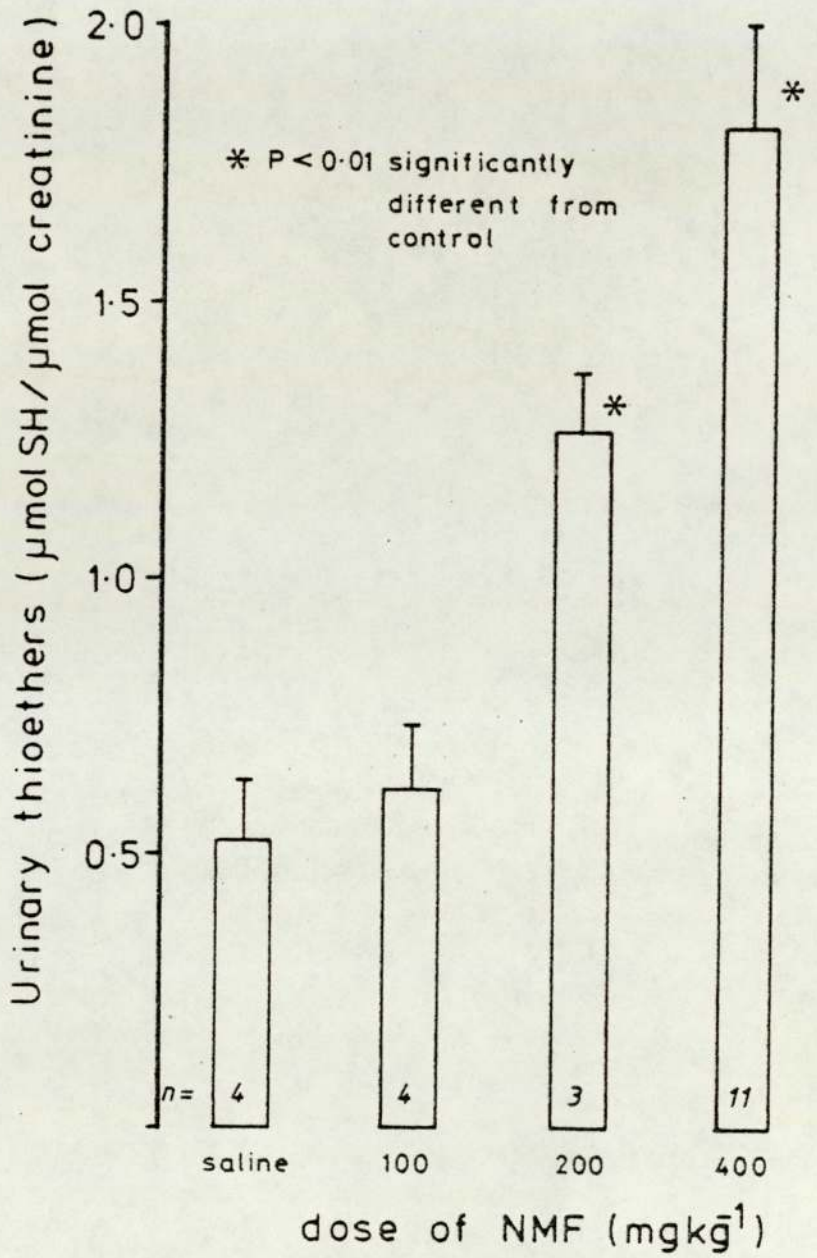


Figure 4.19 Dose related excretion of urinary thioethers by Balb/C mice during a 24h interval after a single i.p. dose of NMF (mean ± s.e. mean)

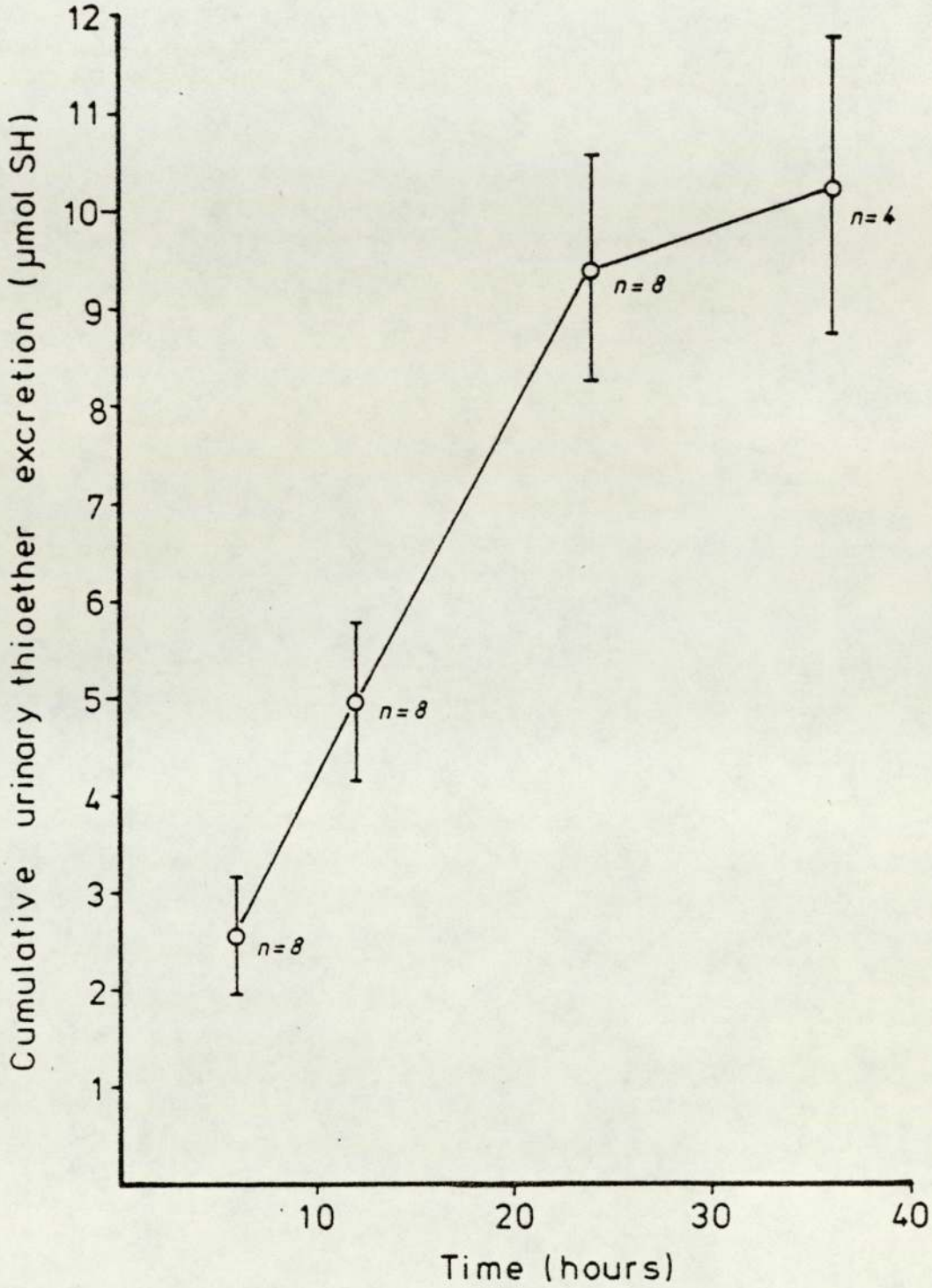


Figure 4.20 Time course for urinary thioether excretion by Balb/C mice following a single i.p. dose of 400mgkg^{-1} NMF (mean ± s.e. mean)

Table 4.4 Time course for thioether excretion by Balb/C mice at various intervals during the 24h after a single i.p. dose of 400mgkg^{-1} NMF ($n \geq 4$)

COLLECTION	Total urinary thioether (μmol) mean ± s.e. mean
CONTROL 24h	3.22 ± 0.43
0-6h	2.58 ± 0.59
0-12h	4.98 ± 0.83
0-24h	9.40 ± 1.17
SINGLE 24h COLLECTION	11.95 ± 1.24

4.2.3 Discussion

The results indicate that NMF caused a marked increase in urinary thioether excretion over control. In the series of formamides evaluated, F and HMF did not elevate urinary thioethers. This is consistent with the finding that neither F or HMF depleted hepatic NPT (4, 15). DMF did not increase urinary thioether excretion at a dose equimolar to that of NMF which induced elevation. This may suggest that the mechanism of hepatotoxicity of DMF and NMF are different ie. that DMF does not form a reactive metabolite that reacts with GSH, alternatively this may reflect the greater potency of NMF as a hepatotoxin (Section 1.2).

The structural requirements for thioether excretion also parallel the features for in vivo antitumour activity (4, 15).

It is clear that NMF induced urinary thioether excretion occurs in a dose related manner (Figure 4.19), and this reflects the dose related nature of hepatic GSH depletion (4) (Section 4.4). The threshold for NMF induced elevation of plasma SDH activity in the Balb/C mouse is $150mgkg^{-1}$ (21). Below this threshold urinary thioether excretion does not differ from control. Above $150mgkg^{-1}$ NMF, urinary thioethers are significantly elevated ($P<0.01$).

Examination of the time course of NMF induced thioether excretion indicated that the rate of production was constant over the first 24h. In addition control urinary total thioether levels were exceeded between 6 and 12 hours after a single i.p. dose of $400mgkg^{-1}$. This is interesting as the onset for the elevation of plasma SDH, following the same dose of NMF, occurred between 10 and 24 hours post dose (26). Therefore the urinary thioether elevation occurs prior to the onset of hepatotoxicity.

In conclusion, NMF produced a specific dose related elevation of urinary thioethers,which closely paralleled the pattern of hepatic NPT depletion. In addition the thioether excretion preceeds changes in indices of hepatotoxicity. These observations suggest that a reactive species generated from NMF may be detoxified by conjugation with GSH prior to excretion as a urinary thioether.

4.3 Depletion of hepatic GSH by chemical means, and by inhibition of biosynthesis

4.3.1 Introduction

The deliberate alteration of tissue GSH levels has proven to be a useful tool in detoxification and drug metabolism studies. Depletion of hepatic GSH to less than 30% of control values can result in altered drug metabolism and enhanced toxicity of electrophilic metabolites. Depletion of hepatic GSH with DEM has enhanced the toxicity of paracetamol (109) and bromobenzene (71).

The observation that NMF depleted hepatic GSH in a dose related manner (4) and also depleted GSH in isolated hepatocytes (26) implies a role for GSH in the hepatotoxicity of NMF. It was considered that by depleting hepatic GSH prior to NMF administration it may be possible to correlate GSH depletion with increased covalent binding (Section 4.7) and a lowered threshold for NMF hepatotoxicity (Section 4.10).

Hepatic GSH may be depleted by substrates of glutathione transferases eg. methyliodide and DEM (85, 150) or by inhibition of GSH biosynthesis with BSO (87). The aim of this section is to establish suitable dosing protocols for DEM and BSO which may be employed as tools to establish a role for GSH in the hepatotoxicity of NMF (Section 4.10).

4.3.2 Results

DEM was administered as a single i.p. dose of 0.3, 0.5 or $0.7 ml kg^{-1}$ and hepatic GSH determined 1 hour later (Figure 4.21). A dose of $0.7 ml kg^{-1}$ reduced total hepatic GSH to 17.1% of control values compared with injection vehicle alone. The lower doses of 0.3 and

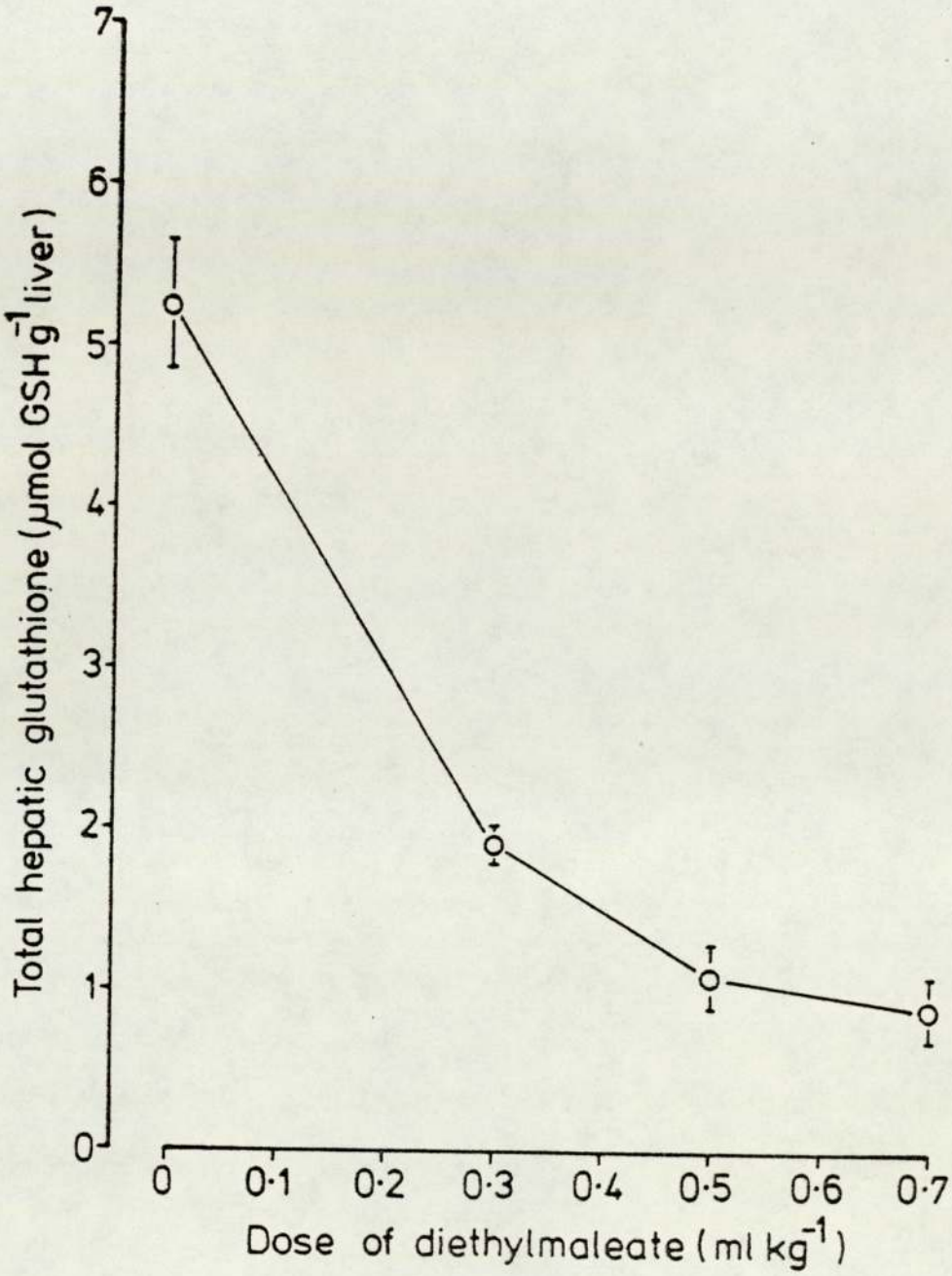


Figure 4.21 Dose-related depletion of hepatic GSH 1 hour after DEM administration (mean ± s.e. mean; n = 4)

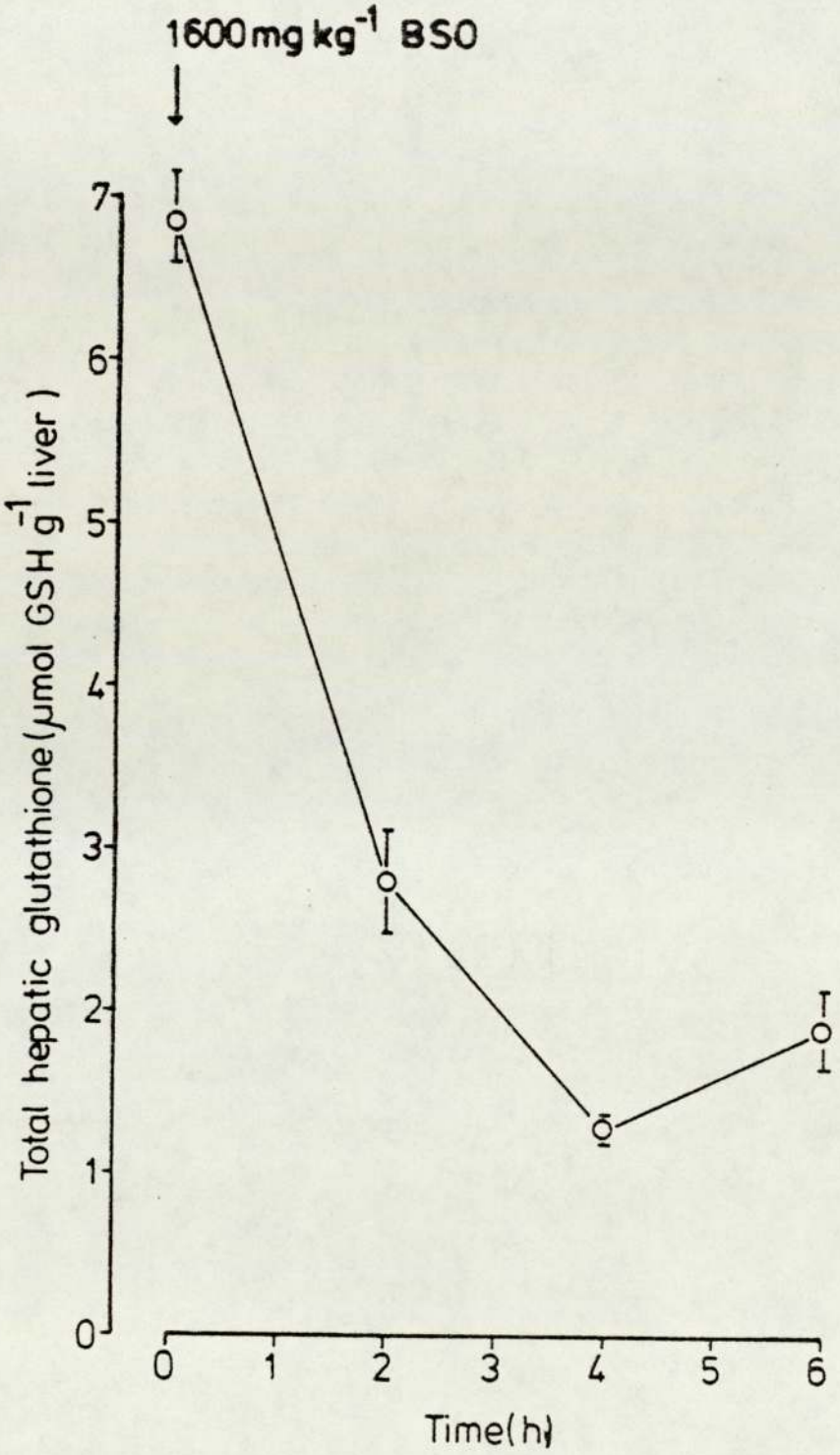


Figure 4.22 Time course of the depletion of hepatic GSH by buthionine sulphoximine (BSO) (mean ± s.e. mean; n = 4)

0.5mlkg^{-1} reduced hepatic GSH to 36.5% and 20.9% of control respectively.

BSO depleted GSH by inhibition of γ- glutamylcysteine synthetase (γ - GCS), thus the fall in GSH is indicative of hepatic GSH turnover. As a consequence it is important to establish the time course for hepatic GSH depletion by BSO.

A dose of 1600mgkg^{-1} BSO produced a gradual decline in hepatic GSH to 18.6% of control values at 4h post dose (Figure 4.22). Six hours after BSO administration the hepatic GSH levels had risen to 29.8% of control indicating that the nadir occurs between 4 and 6 hours post dose.

4.3.3 Discussion

The results clearly support the findings in many laboratories that hepatic GSH levels can be effectively lowered by DEM and BSO. Doses of 0.3 to 0.7mlkg^{-1} DEM rapidly lowered hepatic GSH in Balb/C mice to below 30% of control values within one hour. This compares favourably with reports of doses of 0.6 to 1.0mlkg^{-1} reducing hepatic GSH in rats to 6-20% of control values in 30 minutes (85, 150). During the depleted state the rate of GSH biosynthesis is increased (151), and GSH levels rise to approximately twice control values within 24 hours before returning to normal (85).

A dose of 1600mgkg^{-1} BSO has previously been shown to deplete hepatic GSH in C3H mice to 35% of control values within 4 to 6 hours (88). This observation has been confirmed in the present study in Balb/C mice. The nadir for GSH depletion occured at 18.6% of control values 4h after a dose of 1600mgkg^{-1} BSO. Also, from as early as 2h after BSO administration hepatic GSH levels are considerably depressed

in Balb/C mice. The GSH depletion profile following BSO administration mirrors that previously reported and is superimposable upon the profile for γ-GCS inhibition in the livers of C3H mice (88).

In conclusion, both DEM and BSO produce a significant depletion of GSH at the dose protocols evaluated. The GSH depletion induced by DEM and BSO lasted for 2-4h (85, 88) thus permitting the role of GSH in the hepatotoxicity of NMF to be evaluated (Section 4.10).

4.4 Depletion of GSH by NMF in Balb/C BDF_1 and CBA/CA mice

4.4.1 Introduction

The ability of NMF to deplete hepatic NPT in a dose-related manner has clearly been established (4). A dose of $400mgkg^{-1}$ caused a 59.8% depletion of hepatic NPT within 1 hour of administration. This effect is specific to NMF as other formamides did not deplete hepatic NPT (4, 15). The NMF induced disturbance of hepatic GSH is short lived. Upon repeated daily administrations of $400mgkg^{-1}$ NMF, for five days, to Balb/C, CBA/CA and BDF_1 mice, hepatic GSH + GSSG levels determined 24h after the last dose did not differ from control (26).

The extent of depletion of NPT (59.8%) 1 hour after a dose of $400mgkg^{-1}$ NMF is insufficient to account for the extent of centrilobular necrosis reported (4, 26). Paracetamol administered to mice at a dose of $225mgkg^{-1}$ produced a hepatic GSH depletion of 54% without any histopathological evidence of necrosis. At a larger dose of $325mgkg^{-1}$, hepatic GSH was depleted by 75% and extensive centrilobular necrosis was apparent (109). Depletion of hepatic GSH to 25% of control values has been suggested to represent an almost total depletion of the cytosolic pool of GSH (90). This extent of depletion of hepatic GSH following paracetamol administration has been confirmed recently in young rats (153).

It is conceivable that NMF causes centrilobular necrosis, following an extensive localized depletion of hepatic GSH which would not be reflected by drastic changes in total hepatic GSH. Indeed, the existence of a GSH gradient in the liver lobule has been established, and partially accounts for the particular sensitivity of the centrilobular region to oxidative stress (48).

Examination of the time-course of ^{14}C-NMF levels in the liver following a dose of 400mgkg^{-1} administered to CBA/CA mice indicated that peak levels of radioactivity in the liver occured 8 hours post dose (139). In view of the delay in achieving peak levels of ^{14}C-NMF in the liver, it is reasonable to evaluate the extent of GSH depletion for several hours after NMF administration.

Studies on the toxicology of NMF has revealed a marked difference between strains of mice in their susceptibility to NMF hepatotoxicity (21), which may be a consequence of a strain difference in metabolism (Section 4.1) of NMF to a reactive species, and subsequent depletion of hepatic GSH to differing degrees.

The aims of this section are threefold:-

1) To confirm the dose-related nature of hepatic GSH depletion, previously determined as NPT (4).
2) To establish precisely the time and extent of the nadir for GSH depletion in Balb/C mice.
3) To evaluate strain differences in NMF induced GSH depletion.

4.4.2 Results

Various doses of NMF were administered to Balb/C mice and total hepatic GSH was determined 1 hour later according to the method of Tietze (133) (Figure 4.23). It is apparent that maximal depletion of hepatic GSH at 52% of control values, occurs 1h after a dose of 200mgkg^{-1} NMF. At the higher dose of 400mgkg^{-1} the extent of depletion (59% of control) did not differ significantly from a dose of 200mgkg^{-1}.

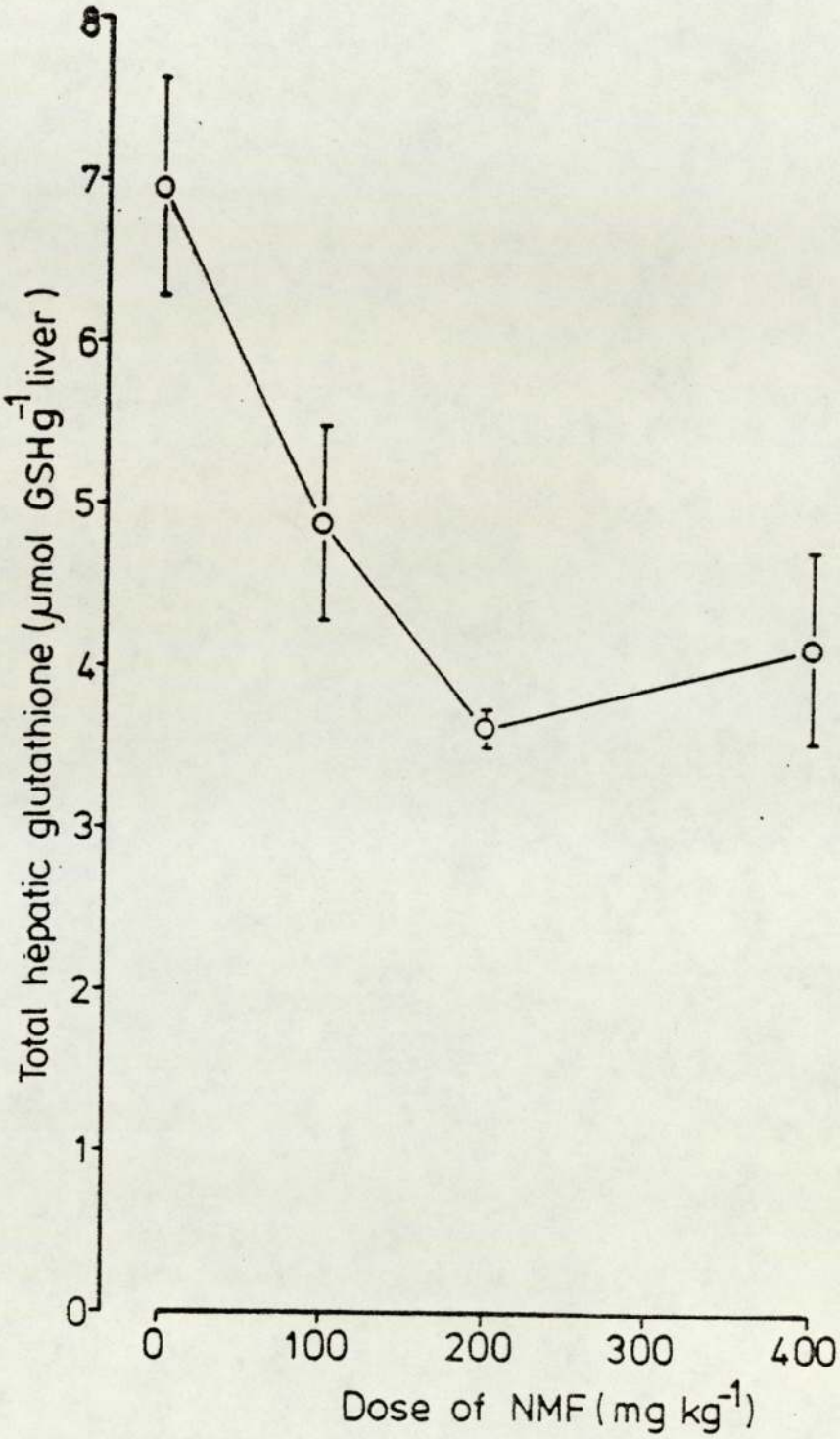


Figure 4.23 Dose related depletion of hepatic GSH in Balb/C mice 1h after a single i.p. injection of NMF (mean ± s.e. mean; n = 4)

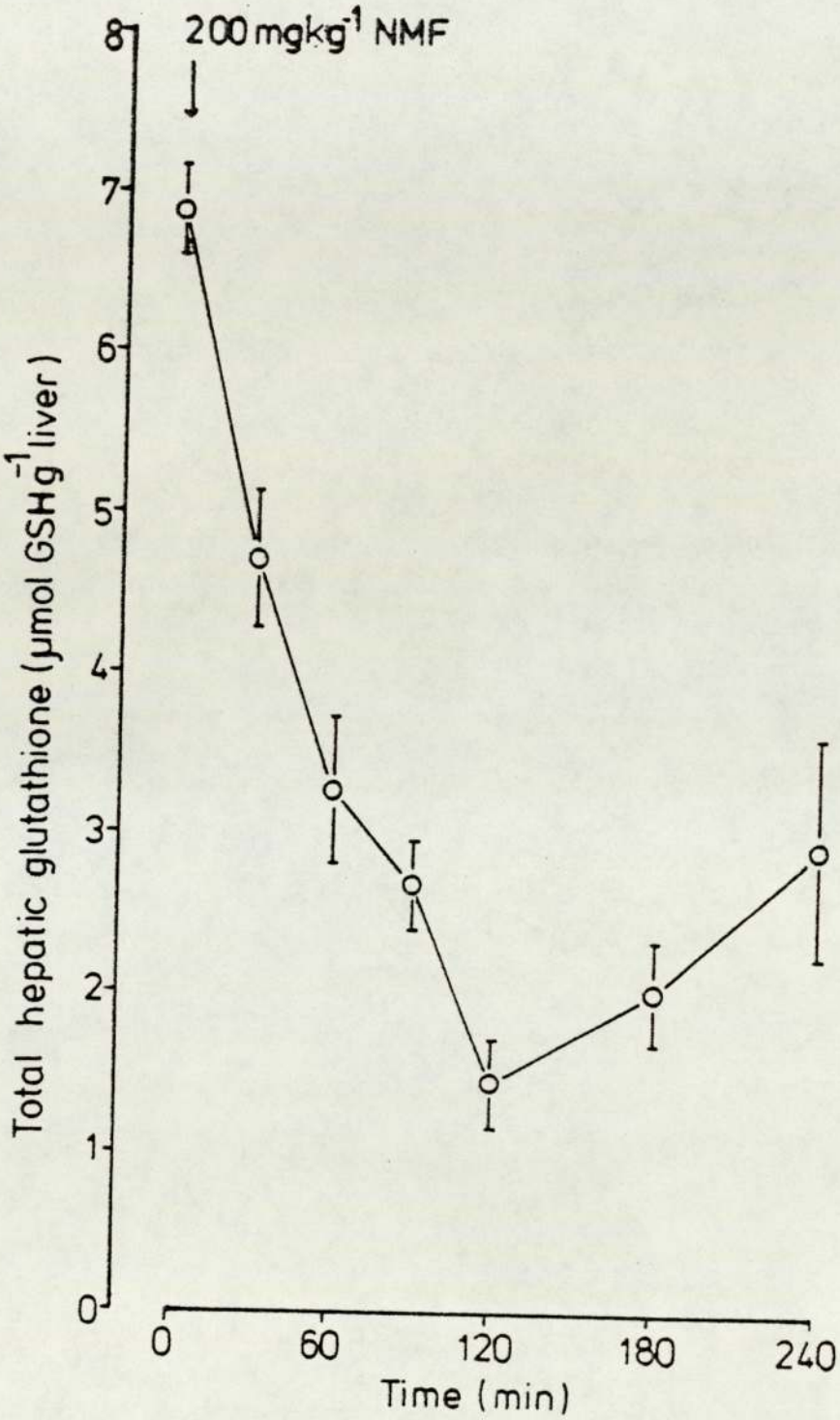


Figure 4.24 Time course for the depletion of hepatic GSH in Balb/C mice after a single i.p. injection of 200mgkg^{-1} NMF (mean ± s.e. mean; n = 4)

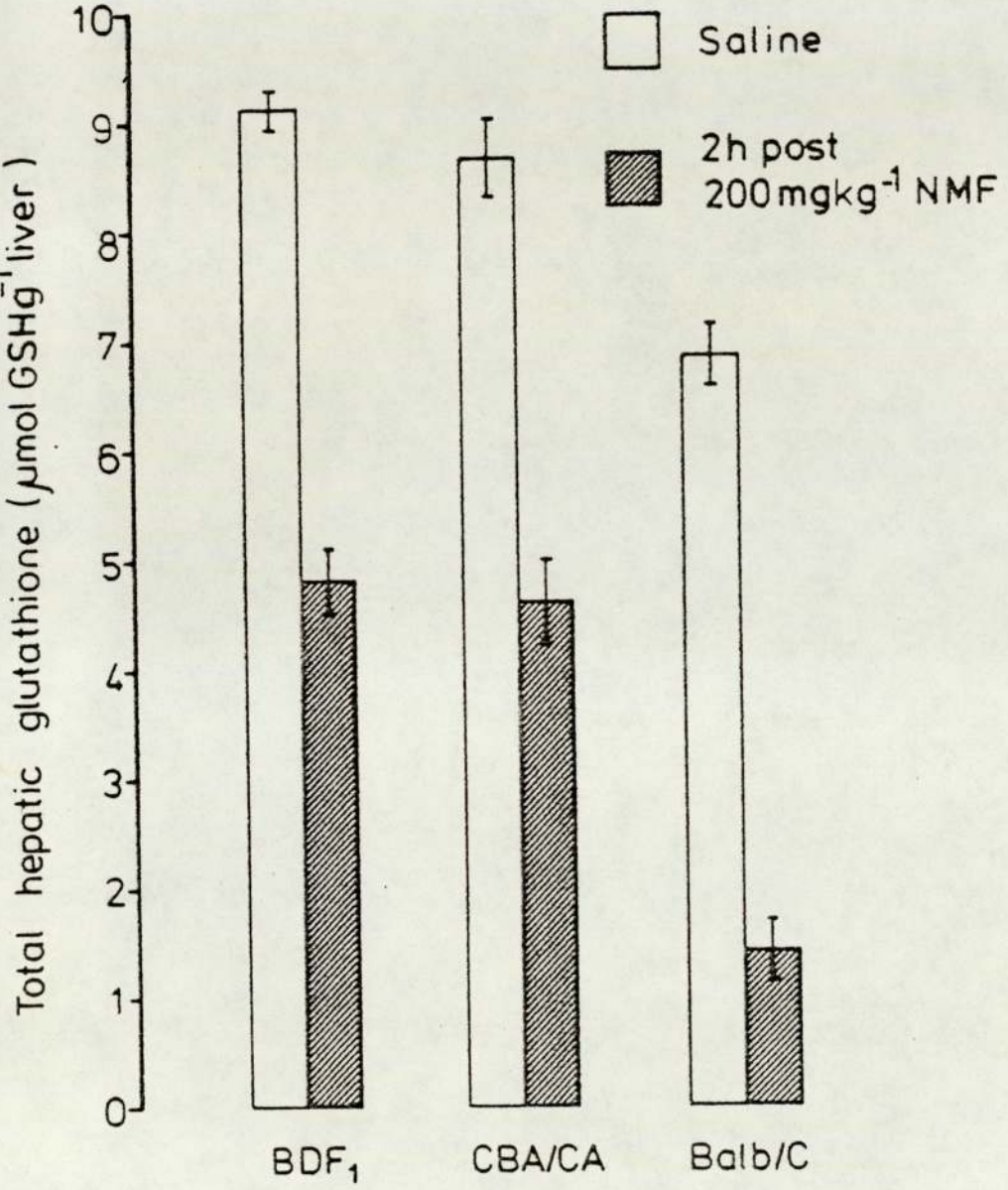


Figure 4.25 Strain differences in depletion of hepatic GSH in mice 2h after a single i.p. injection of 200mgkg^{-1} NMF (mean ± s.e. mean; n = 4)

The time course for depletion was evaluated after a dose of 200mgkg^{-1} NMF at periods up to 4 hours post dose (Figure 4.24). The results indicate maximal hepatic GSH depletion occured after 2h at 20.9% of control values. When a dose of 200mgkg^{-1} NMF was administered to BDF_1 and CBA/CA mice, the extent of GSH depletion (52.9% and 53.2% of control respectively) was considerably less than observed with Balb/C mice. In addition control GSH levels in BDF_1 and CBA/CA mice were significantly greater than in Balb/C mice ($P<0.05$) (Figure 4.25).

4.4.3 Discussion

The results confirm the dose related nature of NMF induced depletion of hepatic GSH (4). However, the extent of depletion at 1h after a dose of 400mgkg^{-1} (to 59% of control GSH) was less than previously reported (to 41% of control GSH). Also, the maximal depletion of hepatic GSH was obtained with a dose of 200mgkg^{-1} NMF. In the previous study hepatic NPT were maximally depleted by a dose of 400mgkg^{-1}, and no further depletion was observed at a dose of 1000mgkg^{-1}. In the current study, a dose of 400mgkg^{-1} failed to deplete GSH to a greater extent than 200mgkg^{-1} NMF.

Examination of the time course of hepatic GSH levels indicated that maximal GSH depletion occured after 2h at 20.9% of control values. Within 4h hepatic GSH had returned to 42% of control values, indicating a rapid recovery consistent with previous observations on the duration of NMF induced GSH depletion in mice (26). The NMF induced depletion of hepatic GSH to 20.9% of control values is considerably greater than previously observed (4) and is consistent with the extent of depletion seen with other necrogenic

xenobiotics (71, 109, 153).

The extent of depletion in BDF_1 and CBA/CA mice was considerably less than that observed in Balb/C mice. This strain difference is in accordance with the observation that the hepatotoxic dose threshold for NMF in the Balb/C mouse ($150 mg kg^{-1}$) was lower than that in CBA/CA (Section 4.10) or BDF_1 mice (21). Therefore the extent of GSH depletion in the different strains appears to be related to their susceptibility to NMF hepatotoxicity. The higher control GSH levels in CBA/CA and BDF_1, compared to Balb/C mice, may confer resistance to NMF hepatotoxicity.

In conclusion, the extent of NMF induced hepatic GSH depletion in Balb/C mice is consistent with the severity of centrilobular necrosis previously reported (27). Furthermore the particular susceptibility of the Balb/C mouse to the hepatotoxicity of NMF, appears to be related to the extent of GSH depletion. The strain of mice which were more resistant to the hepatotoxicity of NMF exhibited less extensive GSH depletion. This in turn is probably a consequence of either less extensive biotransformation of NMF in these resistant strains (Section 4.1), or alternatively due to the higher basal GSH levels in these mice.

4.5 Attempts to modify NMF induced depletion of GSH

4.5.1 Introduction

NMF causes a marked depletion in hepatic GSH when administered to mice at hepatotoxic doses. The extent of GSH depletion within strains of mice paralleled the extent of metabolism, and the severity of hepatotoxicity of NMF (see Section 4.10). A previous study reported that SKF525A, an inhibitor of hepatic mixed function oxidases (156, 157), partially prevented NMF induced hepatic GSH depletion (4). These observations suggest that a metabolically generated reactive species is involved in the hepatotoxicity of NMF.

NMF depletes GSH in isolated hepatocytes and subsequently leads to the production of breakdown products of lipid peroxides at levels significantly above control (26). A relationship has been established between GSH depletion and lipid peroxidation during pro-oxidative stress in the rat liver (158). Increased levels of breakdown products of lipid peroxides were found in the liver mitochondria of Balb/C mice 24h after administration of $400 mgkg^{-1}$ NMF. However, it is not clear if this is a cause or a consequence of the toxicity as necrosis is apparent at this time (26). In order to elucidate the relationship between GSH depletion, extent of necrosis and lipid peroxidation caused by NMF, it may be worthwhile to consider briefly the well-studied hepatotoxin carbontetrachloride (CCl_4).

Carbontetrachloride exerts its toxicity through cytochrome P450 mediated formation of a trichloromethyl radical ($CCl_3^{\cdot}$) or trichloromethylperoxy radical ($CCl_3O\dot{O}$) (159) which may subsequently decompose to phosgene ($COCl_2$) and an electrophilic chlorine species (160). The free radicals formed from CCl_4 injure the hepatocyte by

lipid peroxidation, and covalent binding to cellular structures. GSH can protect the liver microsomal membrane against lipid peroxidation and covalent binding under aerobic conditions (159). In addition the antioxidants, vitamin E and promethazine prevent CCl_4 induced free radical attack of PUFA and protect against CCl_4 hepatotoxicity (138, 161).

In analogy to the approach outlined for CCl_4 it was considered that the role of GSH in NMF hepatotoxicity may be more clearly defined by pretreating mice with antioxidants and GSH precursors prior to NMF administration. By using this approach it may be possible to establish a relationship between GSH depletion and hepatotoxicity. In addition the influence of phenobarbitone, an inducer of drug metabolism (136), and also SKF525A, an inhibitor of cytochrome P-450 will be evaluated.

Therefore the aim of this section is to elucidate factors that influence the NMF induced depletion of GSH in Balb/C mice, following pretreatment of mice in the following ways:-

1) With an inhibitor or inducer of drug metabolism ie. SKF525A and phenobarbitone (PB).
2) With the antioxidants promethazine and vitamin E.
3) With the GSH precursors N-acetylcysteine or cysteine.

The influence of these pretreatments upon the severity of the hepatotoxicity of NMF is described in Section 4.10.

4.5.2 Results

The influence of a variety of pretreatments upon NMF induced GSH depletion was determined either 1h or 2h after a dose of $200 mgkg^{-1}$.

A dose of 200mgkg^{-1} NMF depleted hepatic GSH in Balb/C mice to 61% of control values within 1 hour (Figure 4.26). Pretreatment with PB (50mgkg^{-1} for 4 days) or SKF525A (60mgkg^{-1} 1h prior to NMF), did not change the NMF-induced GSH depletion, which were 61% and 52% of control values respectively (Figure 4.26). The extent of GSH depletion following concomitant administration of SKF525A and NMF appeared to be greater than that observed after NMF alone, but this difference was not statistically significant.

After administration of vitamin E (100mgkg^{-1}) 15h prior to NMF, NMF depleted hepatic GSH to 55% of control values (Figure 4.27). Promethazine, 78μmol kg^{-1} (24mgkg^{-1}), prevented the NMF induced depletion of GSH when administered 15min beforehand. Neither vitamin E nor promethazine alone had an effect upon hepatic GSH levels (Figure 4.27).

The GSH precursors cysteine and NAC were administered using a range of dosing protocols that have been reported to protect against the toxicity of a range of foreign compounds (73, 150, 154, 155).

Cysteine HCl (300mgkg^{-1}) administered 10min before and 20min after NMF, completely prevented the GSH depletion, and elevated hepatic GSH when given alone (Figure 4.28). NAC (100mgkg^{-1}) administered 1 hour before NMF failed to protect against the GSH depletion (Figure 4.28). However, administration of a larger dose of NAC (1200mgkg^{-1}, p.o.) 20 min prior to 200mgkg^{-1} NMF substantially protected against the GSH depletion measured 2h after NMF injection (Figure 4.29). It is pertinent to note in this experiment that whereas NMF markedly depleted hepatic GSH to 20.9% of control values, NAC (1200mgkg^{-1}) significantly elevated hepatic to GSH to 125% of

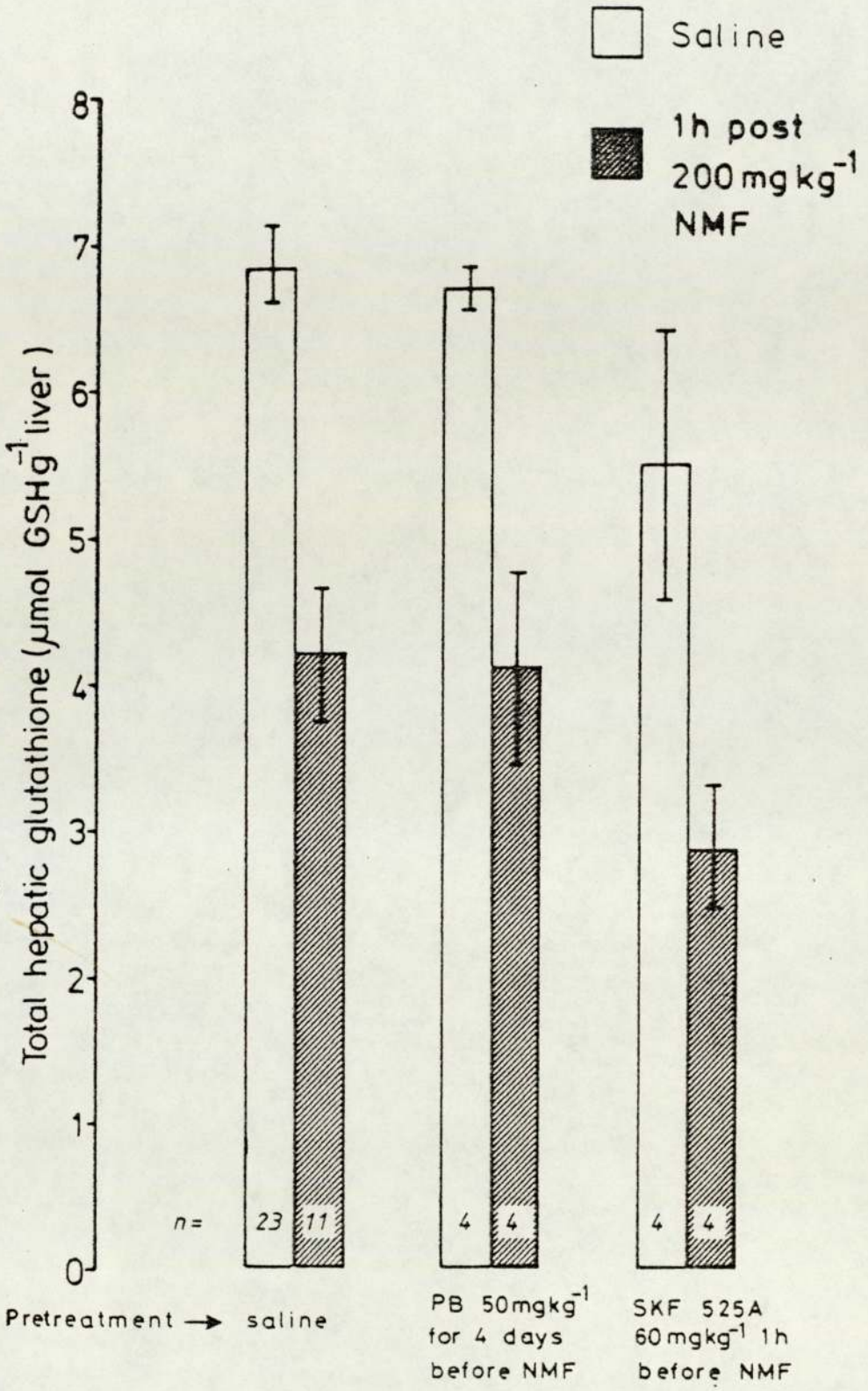


Figure 4.26 Influence of phenobarbitone and SKF525A pretreatment upon NMF induced depletion of hepatic GSH in Balb/C mice (mean ± s.e.m)

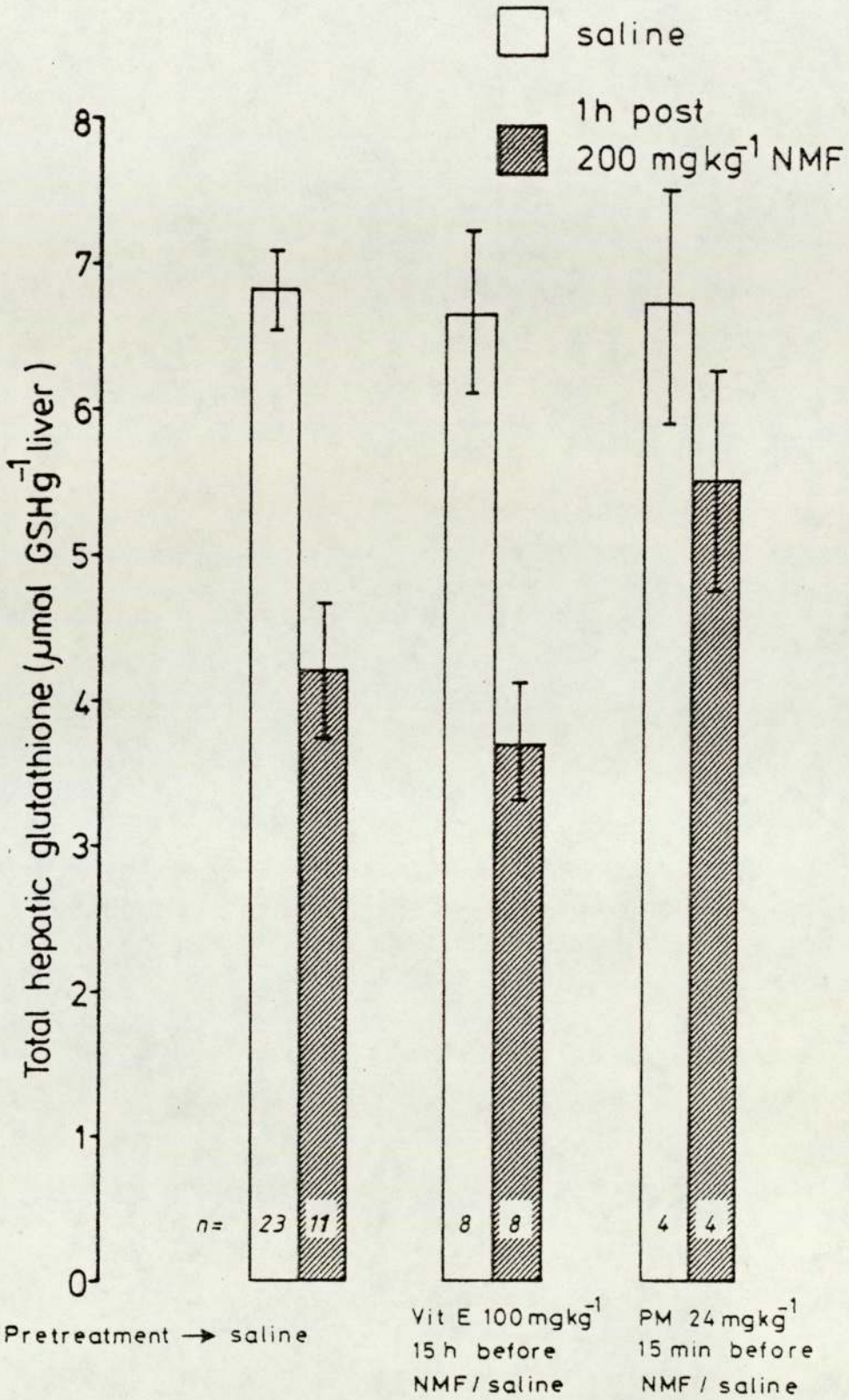


Figure 4.27 Influence of Vitamin E and promethazine pretreatment upon NMF induced depletion of hepatic GSH in Balb/C mice (mean ± s.e.m.)

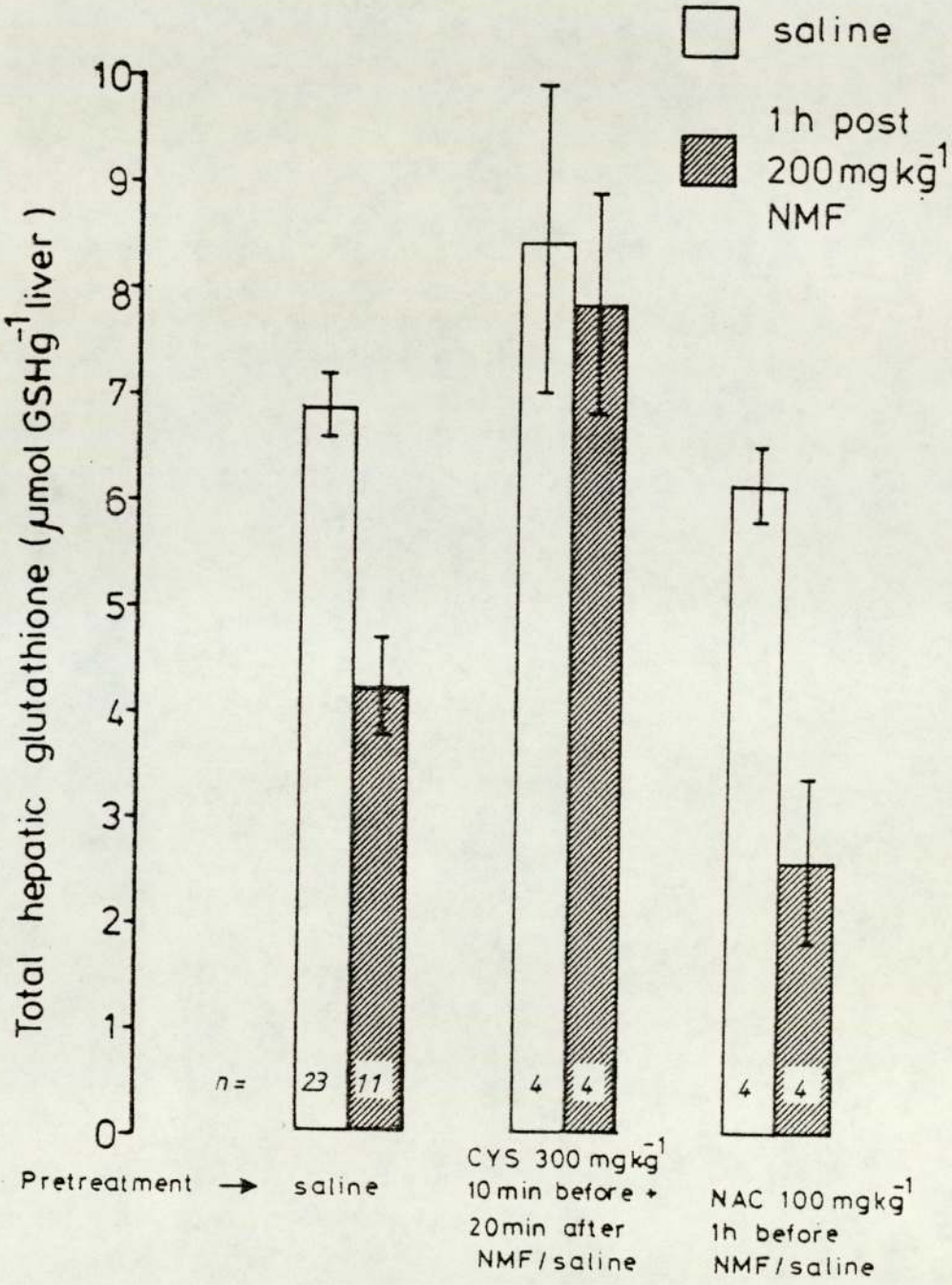


Figure 4.28 Influence of cysteine and N-acetylcysteine pretreatment upon NMF induced depletion of hepatic GSH in Balb/C mice 1h after administration of 200mgkg^{-1} NMF (mean ± s.e.m.)

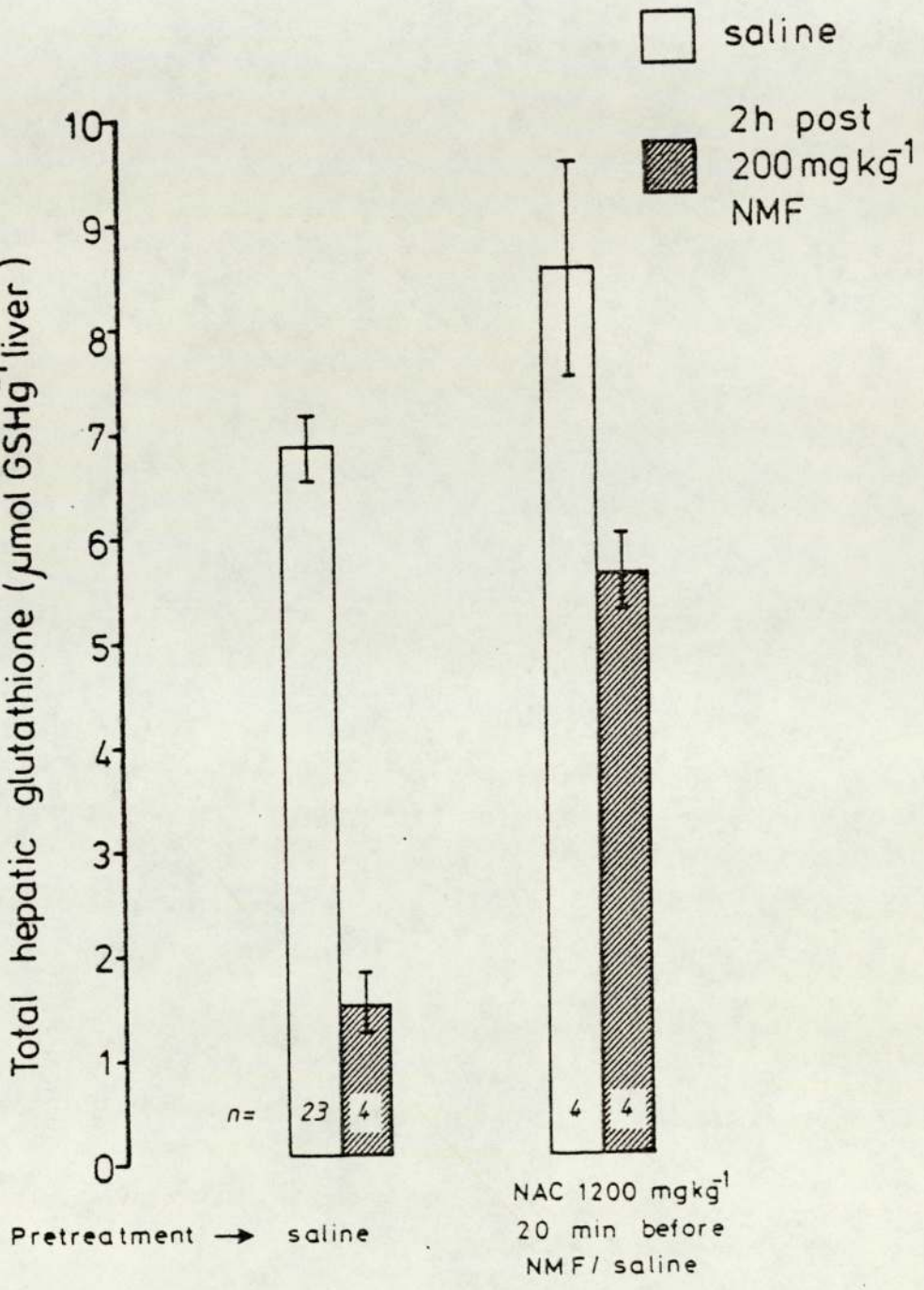


Figure 4.29 Influence of N-acetylcysteine pretreatment upon NMF induced depletion of hepatic GSH in Balb/C mice 2h after administration of 200mgkg^{-1} NMF (Mean ± s.e. mean)

control values obtained after injection of saline alone ($P<0.05$).

4.5.3 Discussion

The observation that SKF525A failed to prevent the NMF induced GSH depletion conflicts with a previous report (4) that implicated cytochrome P-450 in the GSH depletion. PB also had no effect upon NMF induced depletion of liver GSH stores (Figure 4.26). Paracetamol toxicity has been postulated to arise from cytochrome P-450 mediated formation of the reactive species NAPQI. Pretreatment with PB increased both the extent of hepatic GSH depletion (109) and the severity of necrosis (106) caused by paracetamol. Conversely inhibition of drug metabolism reduced the extent of GSH depletion and severity of toxicity. The observations presented here and in Section 4.9 suggest that NMF is probably activated by a system independent of cytochrome P-450. One has to consider that whereas the pretreatments employed cannot yield a definitive answer, the use of inhibitors and inducers of cytochrome P-450 can give hints as to the involvement of P-450 in the production of reactive NMF metabolites (162). This concept will be expanded upon when considering the metabolic activation of NMF to a covalently bound species by mouse liver microsomes (Section 4.9).

Vitamin E did not protect against the NMF induced GSH depletion when administered to mice 15h before NMF. This is particularly interesting as pretreatment of rats with vitamin E 15h prior to isolation of hepatocytes protected against the elevation in indices of lipid peroxidation which resulted when the hepatocytes were incubated with 4-hydroxynonenal. However, vitamin E did not protect against 4-hydroxynonenal induced depletion of GSH in hepatocytes,

which was attributed to the formation of a conjugate between GSH and 4-hydroxynonenal (137). The inability of vitamin E to protect against NMF induced GSH depletion indicates that the lipid peroxidation induced by NMF in the livers of mice (26) is probably unrelated to the GSH depletion.

In contrast the radical scavenger promethazine completely prevented GSH depletion 1h after 200mgkg^{-1} NMF. Promethazine is also reported to protect against paracetamol induced depletion of GSH in isolated rat hepatocytes (163).

The GSH precursor cysteine, completely protected against the NMF related depletion of GSH, a similar dosing schedule also completely abolished DEM induced depletion of hepatic GSH in the rat (150). Cysteine administered alone caused a slight, but not significant increase in liver GSH.

NAC only protected against the GSH depletion at a very high dose. A dose of 100mgkg^{-1} NAC administered to mice 1h before and 7h after adriamycin prevented cardiac GSH depletion and microscopic myocardial lesions (154, 155). Administration of 100mgkg^{-1} NAC to Balb/C mice failed to prevent the GSH depletion induced by NMF. However, 1200mgkg^{-1} NAC dramatically prevented the NMF induced depletion of GSH when determined 2h after NMF. This dose of NAC has recently been shown to protect efficiently against the covalent binding and hepatotoxicity of paracetamol in mice (73). In addition NAC reversed paracetamol-induced depletion of glutathione in rats by increasing glutathione synthesis from 0.54 to 2.69 µmol/g/hr (164). In the experiment described in Figure 4.29 NAC (1200mgkg^{-1}) alone significantly elevated hepatic GSH above control values ($P<0.05$).

In conclusion, the NMF induced GSH depletion could be prevented by pretreatment with the thiol compounds cysteine and N-acetylcysteine, and by the radical scavenger promethazine. The influence of these pretreatments upon the severity of NMF hepatotoxicity is outlined in Section 4.10.

4.6 The covalent binding of ^{14}C-NMF to hepatic macromolecules in mice

4.6.1 Introduction

NMF is extensively metabolised in mice (Section 4.1) depletes hepatic GSH (Section 4.4), causes centrilobular necrosis in the liver and is hepatotoxic (6, 21, 26). As outlined in Section 1.5, a number of hepatotoxic xenobiotics have been shown to form reactive metabolites which are bound to macromolecules in the liver and this process has been linked with toxicity.

It is reasonable to speculate that NMF may also be metabolised to a reactive species which might covalently bind to endogenous macromolecules and this hypothesis has now been tested in this work.

The potential of NMF to covalently interact with hepatic macromolecules has been investigated in the Balb/C mouse using NMF labelled with ^{14}C either in the methyl moiety ($^{14}CH_3$-NMF) or the formyl moiety (OH^{14}C-NMF). It is clear from metabolism studies (Section 4.1) that both OH^{14}C-NMF and $^{14}CH_3$-NMF are metabolized to $^{14}CO_2$, and display the potential for incorporation of NMF metabolites into hepatic macromolecules. As a consequence what is described in sections 4.6 to 4.8, as covalent binding of NMF to hepatic macromolecules *in vivo*, may represent either covalent binding, incorporation or a combination of both processes.

The results are expressed as ng NMF equivalents bound to or incorporated into 1mg of protein (referred to as binding).

The aim of this section is to establish:-

1) The extent of covalent binding of $^{14}CH_3$-NMF and OH^{14}C-NMF to hepatic macromolecules.

2) The time course of covalent binding of ^{14}C-NMF to hepatic macromolecules as the rate of formation of reactive species will determine when the peak of binding occurs (104, 105).

3) The dose-related nature of the binding at hepatotoxic (400mgkg^{-1}) and non-hepatotoxic doses (20mgkg^{-1} and 100mgkg^{-1}).

4.6.2 Results

Following a single dose of 400mgkg^{-1} $^{14}CH_3$-NMF, binding increased with time and plateaud between 4 and 8h, remaining constant up to 16h (Figure 4.30). The binding increased in an approximately linear fashion within the dose range 20, 100 and 400mgkg^{-1} NMF. The binding profile obtained following a single dose of 400mgkg^{-1} OH^{14}C-NMF was qualitatively similar to that obtained after 400mgkg^{-1} $^{14}CH_3$-NMF, but the extent of binding was only approximately 50% of that obtained for $^{14}CH_3$NMF (Figure 4.31). At the lower doses of 20mgkg^{-1} and 100mgkg^{-1}, the pattern of binding of OH^{14}C-NMF was not dissimilar to that of $^{14}CH_3$-NMF (Figure 4.32). However, at 400mgkg^{-1} OH^{14}C-NMF, the binding was not substantially greater than that observed after a dose of 100mgkg^{-1} OH^{14}C-NMF. This indicates a clear difference between the fate of the formyl and the methyl moities of NMF at a hepatotoxic dose of 400mgkg^{-1}.

By expressing the results as total drug bound in the liver as a % of that administered, it is apparent that 2.3% of $^{14}CH_3$-NMF was bound 8h after a dose of 400mgkg^{-1} NMF (Figure 4.33). As previously observed (Figure 4.31) the extent of binding of $^{14}CH_3$-NMF was approximately twice that of OH^{14}C-NMF.

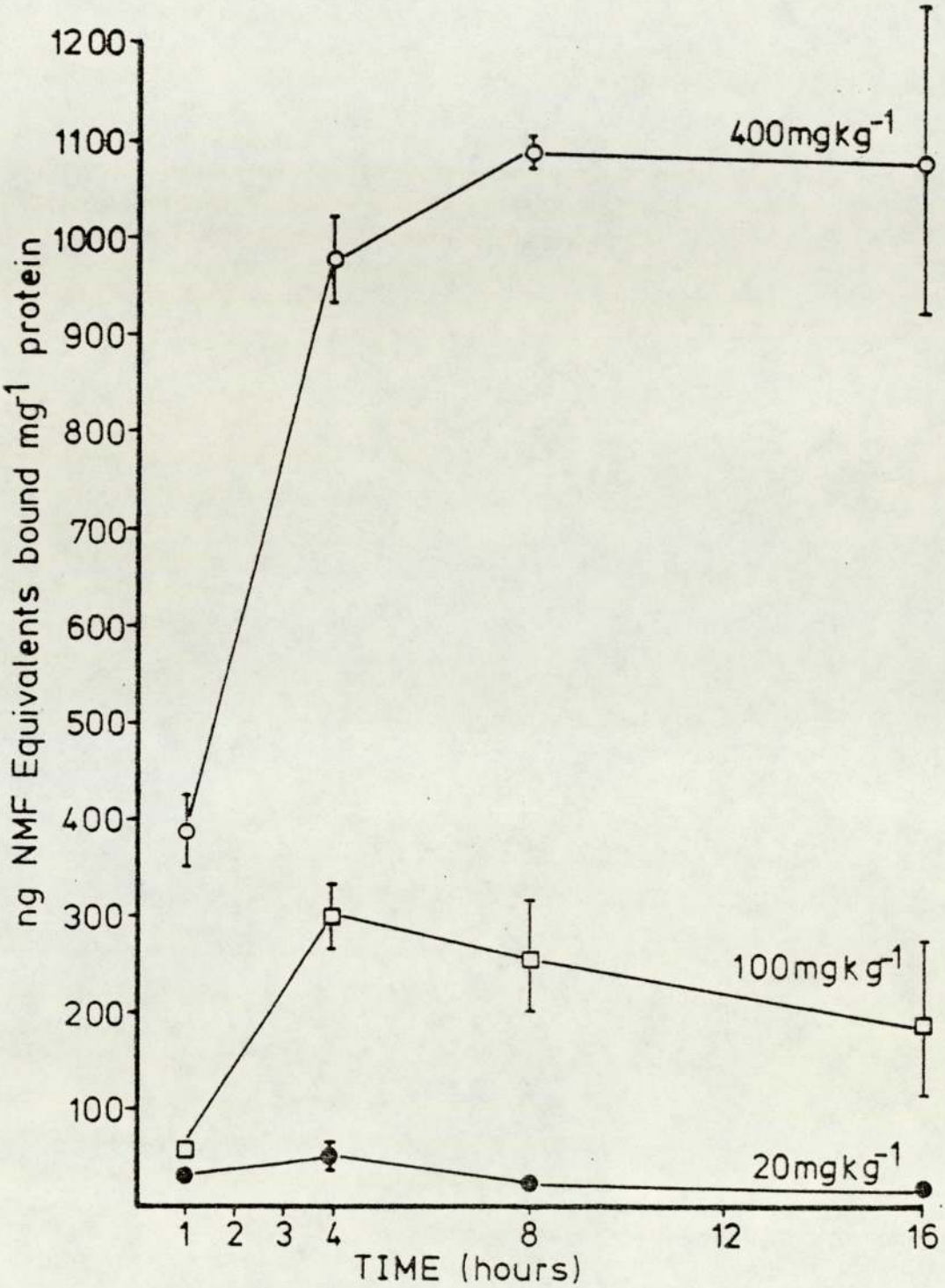


Figure 4.30 Dose related binding of $^{14}CH_3$-NMF to hepatic macromolecules of Balb/C mice (mean ± s.d.; n = 3)

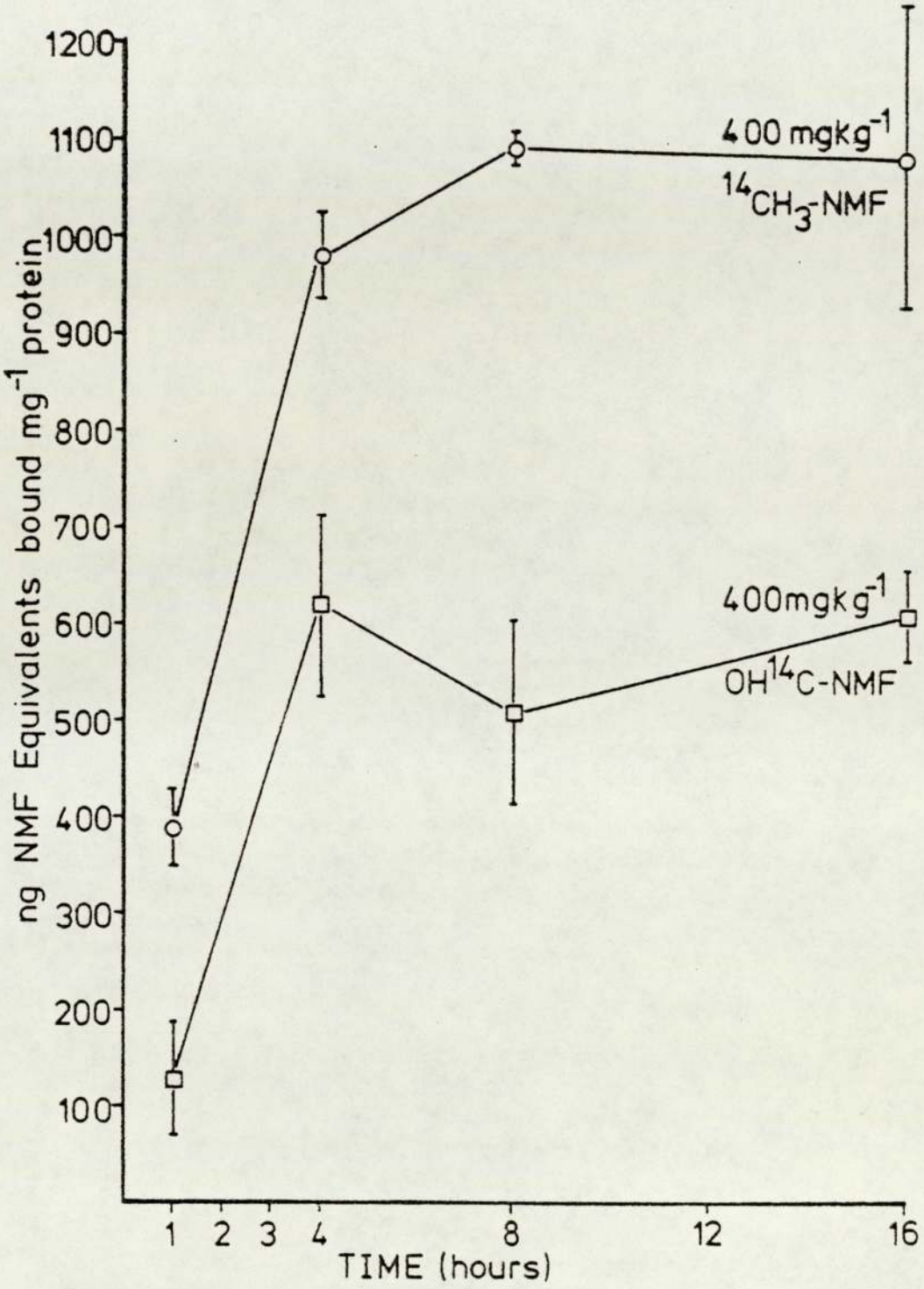


Figure 4.31 Binding of $^{14}CH_3$-NMF and $OH^{14}C$-NMF to hepatic macromolecules in Balb/C mice (mean ± s.d; n = 3)

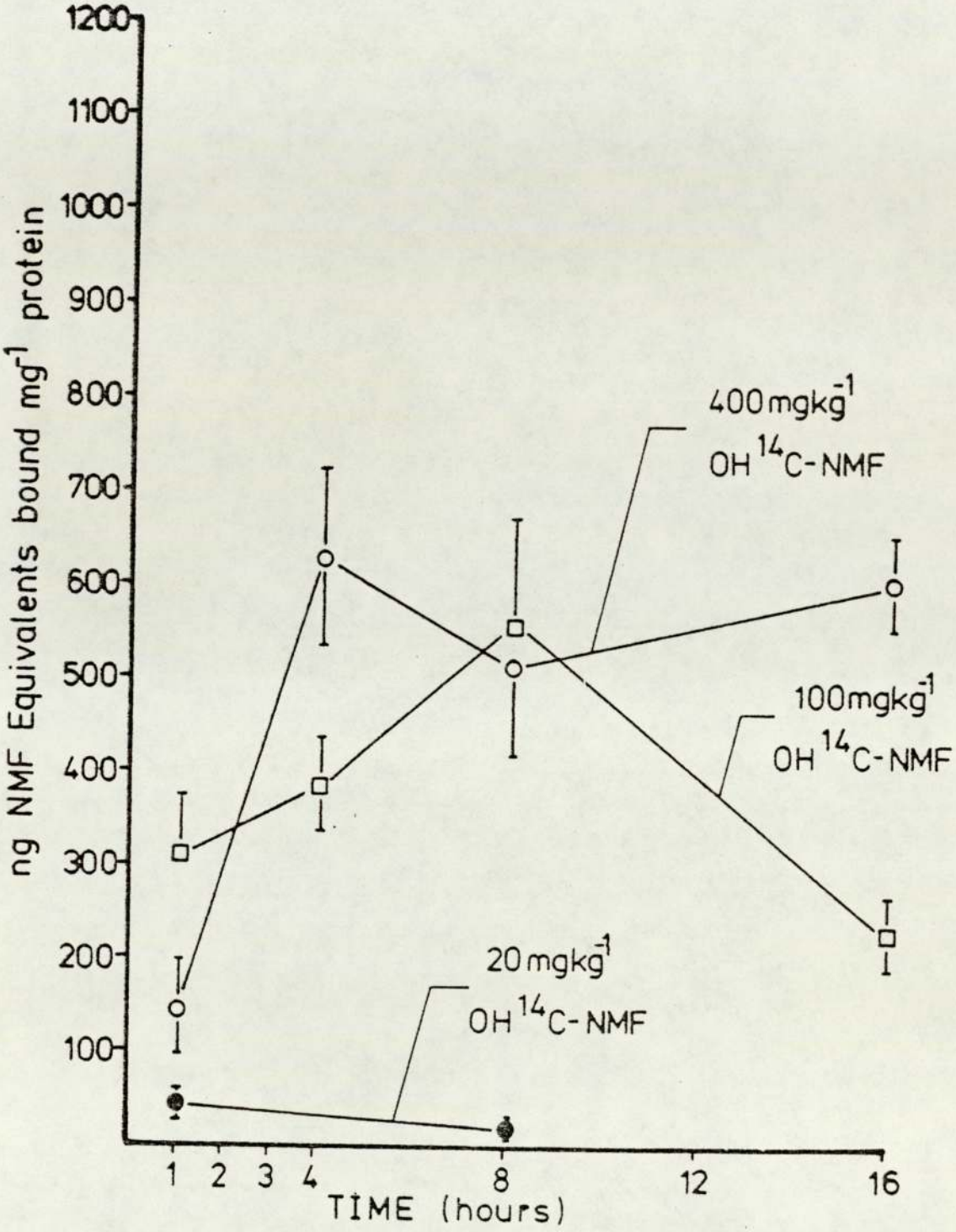


Figure 4.32 Dose related binding of $OH^{14}C$-NMF to hepatic macromolecules in Balb/C mice (mean ± s.d; n = 3)

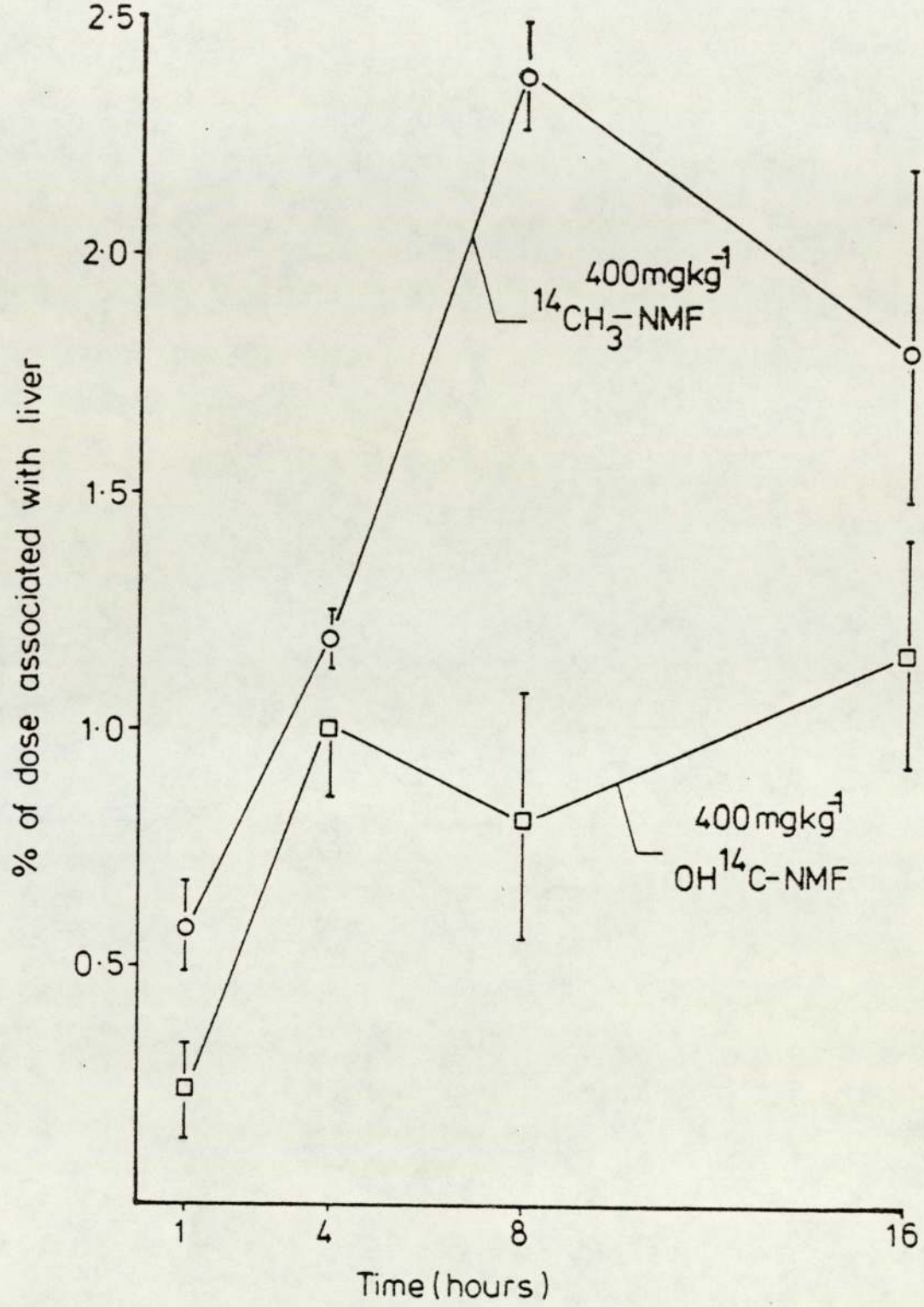


Figure 4.33 Time course of binding of NMF to hepatic macromolecules, expressed as a % of the dose, following administration of a single i.p. dose of 400mgkg^{-1} OH^{14}C-NMF or $^{14}CH_3$-NMF to Balb/C mice (mean ± s.d; n = 3)

4.6.3 Discussion

NMF undergoes dose related covalent binding to hepatic macromolecules following a dose of $^{14}CH_3$-NMF within the dose range 20-400mgkg^{-1}. This finding is correlated with the dose-related depletion of hepatic GSH induced by NMF (Section 4.4). The depletion of hepatic GSH to a nadir 2h after a dose of 200mgkg^{-1} appears to preceed the rise in covalent binding to a plateau between 4 and 8 h post dose. Administration of 400mgkg^{-1} NMF to Balb/C mice produced a rise in plasma SDH activity between 12 and 24h post dose (26), indicating that the peak of covalent binding preceeded manifestations of hepatotoxicity by at least 4h.

Following a non-hepatotoxic dose of 20mgkg^{-1} NMF the extent of binding of OH^{14}C-NMF and $^{14}CH_3$-NMF was minimal, indicating that large doses of NMF are required to produce significant binding. At a dose of 100mgkg^{-1}, the extent of binding of both labels rose to a peak at 4-8h and then declined over the 16h period with a half-life of approximately 20h. This is considerably faster than the reported half lifes of 3 to 11 days for hepatic proteins (58) and may represent elimination of the adduct formed between protein and a metabolite of NMF.

At a hepatotoxic dose of 400mgkg^{-1} NMF, the extent of binding of $^{14}CH_3$-NMF was markedly greater than that of OH^{14}C-NMF. This indicates that the molecule is cleaved, which is consistent with observations from the metabolism studies (Section 4.1). NMF is more extensively metabolised in the Balb/C mouse at a dose of 100mgkg^{-1} than at 400mgkg^{-1} NMF. The difference between the extent of binding of both labels at a hepatotoxic, but not an innocuous dose may indicate an impaired metabolism of the formyl moiety. The binding

observed probably represents a combination of covalent binding and incorporation (Section 4.7) and the ratio of the contribution of both processes may be dose related. The relative contribution of the incorporation of NMF metabolites into hepatic macromolecules is discussed more extensively in Section 4.7.

When expressed as a percentage of the dose bound it is clear that NMF avidly binds to hepatic tissue, 2.3% of a dose of $400mgkg^{-1}$ $^{14}CH_3$-NMF becomes irreversibly associated with the liver within 8h. The extent of binding of other hepatotoxin is often less than 1% of the total dose (103) eg. following the administration of a dose of $1000mgkg^{-1}$ ^{3}H-paracetamol to Swiss mice, approximately 0.5% of the dose was bound irreversibly to hepatic tissue within 6h.

4.7 Modification of the covalent binding of ^{14}C-NMF to hepatic macromolecules in mice

4.7.1 Introduction

The correlation between the dose related hepatic GSH depletion and the extent of covalent binding of NMF to hepatic macromolecules (Section 4.6) provides evidence for the notion that NMF is metabolized to a reactive species. Covalent binding of this species to critical nucleophilic sites in the liver may subsequently cause hepatocellular injury. In analogy to studies on other hepatotoxins (102-105) it was considered to be desirable to correlate changes in the magnitude of covalent binding with changes in the incidence and severity of toxicity in order to clearly establish a role for covalent binding in the hepatotoxicity of NMF.

NMF markedly depleted hepatic GSH, consequently it is not surprising that it was possible to modulate NMF induced GSH depletion by pretreatment with sources of thiols (Section 4.5). The deliberate alteration of hepatic GSH prior to a hepatotoxic challenge has yielded information about the mechanism of hepatotoxicity of both bromobenzene and paracetamol (71, 73, 165). Therefore it was thought that the modulation of GSH status in the livers of mice prior to NMF administration may modify hepatotoxicity (Section 4.10) and probably also covalent binding. Consequently, this kind of experiment may provide further clues as to the mechanism by which NMF causes hepatotoxicity.

The potential of NMF to enter denovo pathways and to become incorporated into endogenous macromolecules cannot be ignored. This problem may be approached in two ways (103). Firstly, the compound

under study can be labelled with ^{3}H and ^{14}C in different parts of the molecule and the fate of each label followed. Alternatively, incorporation may be distinguished from covalent binding by studying the effects of protein synthesis inhibitors upon the rate of covalent binding of radiolabelled drugs to protein in vivo (103). The latter approach has been used to distinguish covalent binding from incorporation in a study upon the association of inhaled [^{14}C]-methylchloride with macromolecules from various tissues in rats (142).

If covalent binding and hepatotoxicity are the consequence of metabolism, parallel changes in the extent of covalent binding and toxicity may be induced by pretreatment with inhibitors or inducers of drug metabolism. For example, piperonylbutoxide and colbaltous chloride pretreatment have been shown to reduce the extent of both covalent binding and liver necrosis induced by paracetamol (107).

The aim of this section is threefold:-

1) To delineate the relative contribution of incorporation and covalent binding to the association of NMF with hepatic macromolecules.
2) To evaluate the effects of depleting GSH, prior to NMF administration, upon the extent of NMF covalent binding.
3) To evaluate the effect of SKF525A upon the covalent binding of NMF to hepatic macromolecules.

4.7.2 Results

The extent of binding of $^{14}CH_3$-NMF following pretreatment with SKF525A ($60mgkg^{-1}$), 1h before NMF was approximately only half of that observed with $^{14}CH_3$-NMF alone (Figure 4.34). The influence of SKF525A upon binding of $OH^{14}C$-NMF ($400mgkg^{-1}$) was determined 1h and 8h

after NMF administration. One hour after $OH^{14}C$-NMF administration the binding was not significantly different from $OH^{14}C$-NMF alone, whereas 8h after administration of $OH^{14}C$-NMF to SKF525A pretreated mice the extent of binding was twice that of $OH^{14}C$-NMF alone (Figure 4.35). Thus pretreatment with SKF525A induced marked alterations in the binding pattern of both radiolabels.

In mice pretreated with cycloheximide ($1mgkg^{-1}$) 15 min before NMF, the association of both $OH^{14}C$-NMF (Figure 4.37) and $^{14}CH_3$-NMF (Figure 4.36) with hepatic macromolecules was significantly reduced. In control experiments this dose of cycloheximide inhibited [$1^{14}C$]-Leucine (2µCi) incorporation into liver macromolecules by approximately 50% when determined 1h and 8h after leucine administration (Figure 4.38). Cycloheximide inhibited the association of $400mgkg^{-1}$ $^{14}CH_3$-NMF with hepatic macromolecules by 75% and 38% 1 and 8 hours respectively after NMF administration. The extent of inhibition of $OH^{14}C$-NMF association by cycloheximide was approximately 75% and 50% at 1 and 8 hours respectively (Figure 4.37). Pretreatment of mice with methotrexate for 5 days prior to NMF administration, inhibited the association of $400mgkg^{-1}$ $^{14}CH_3$-NMF by 93% and 54% at 1 and 8h respectively after NMF administration (Figure 4.39) Folic acid antagonists like methotrexate can interfere with single carbon metabolism by binding to dihydrofolate reductase and blocking reduction of folic acid to tetrahydrofolate. The transfer of single carbon units is mediated by tetrahydrofolate (62).

The role of GSH as a protectant against association of NMF metabolites with hepatic macromolecules was investigated by depleting hepatic GSH with DEM or BSO prior to NMF administration. Doses of 0.3 or $0.7mlkg^{-1}$ DEM were used to deplete GSH to 31% and 17% of control

respectively (Section 4.3.2). DEM 0.7mlkg^{-1} administered 1h prior to 400mgkg^{-1} $^{14}CH_3$-NMF drastically reduced the association of $^{14}CH_3$-NMF derived species with hepatic macromolecules, at all time points evaluated over the 16h time course (Figure 4.40). Surprisingly, the lower dose of DEM (0.3mlkg^{-1}) did not alter the binding of 400mgkg^{-1} OH^{14}C-NMF at 1 and 8h after NMF administration (Figure 4.41).

When hepatic GSH was depleted with BSO (1600mgkg^{-1}), to 18.6% of control values, the hepatotoxicity of NMF was strikingly increased so that the animals died or had to be killed 5h after NMF administration (see Section 4.10). The binding profile over the first 4h after NMF administration in BSO pretreated mice was similar to that of NMF alone, with the exception that the association of OH^{14}C-NMF was increased after 1h (Figure 4.42 and 4.44).

In Figures 4.34 to 4.42 and 4.44, the association of NMF with hepatic macromolecules has been expressed as ng NMF equivalents bound mg^{-1} protein. However, a recent paper has shown that hepatotoxic drugs can cause hepatic necrosis associated with increased liver weight and accumulation of blood protein. Thus, if in such a situation binding is expressed as amount of drug bound per milligram of protein the increased amount of blood protein in liver may obscure an increase in binding to genuine liver proteins. As the additional protein enters the liver after hepatocellular damage has occurred it should not be included in the measurement of covalent binding (73). For the worst possible case of necrosis seen in this investigation, after BSO and NMF administration, the results have been expressed as ng NMF equivalents bound to hepatic protein per total liver (Figure 4.43 and 4.45). When expressed in this manner the binding profiles

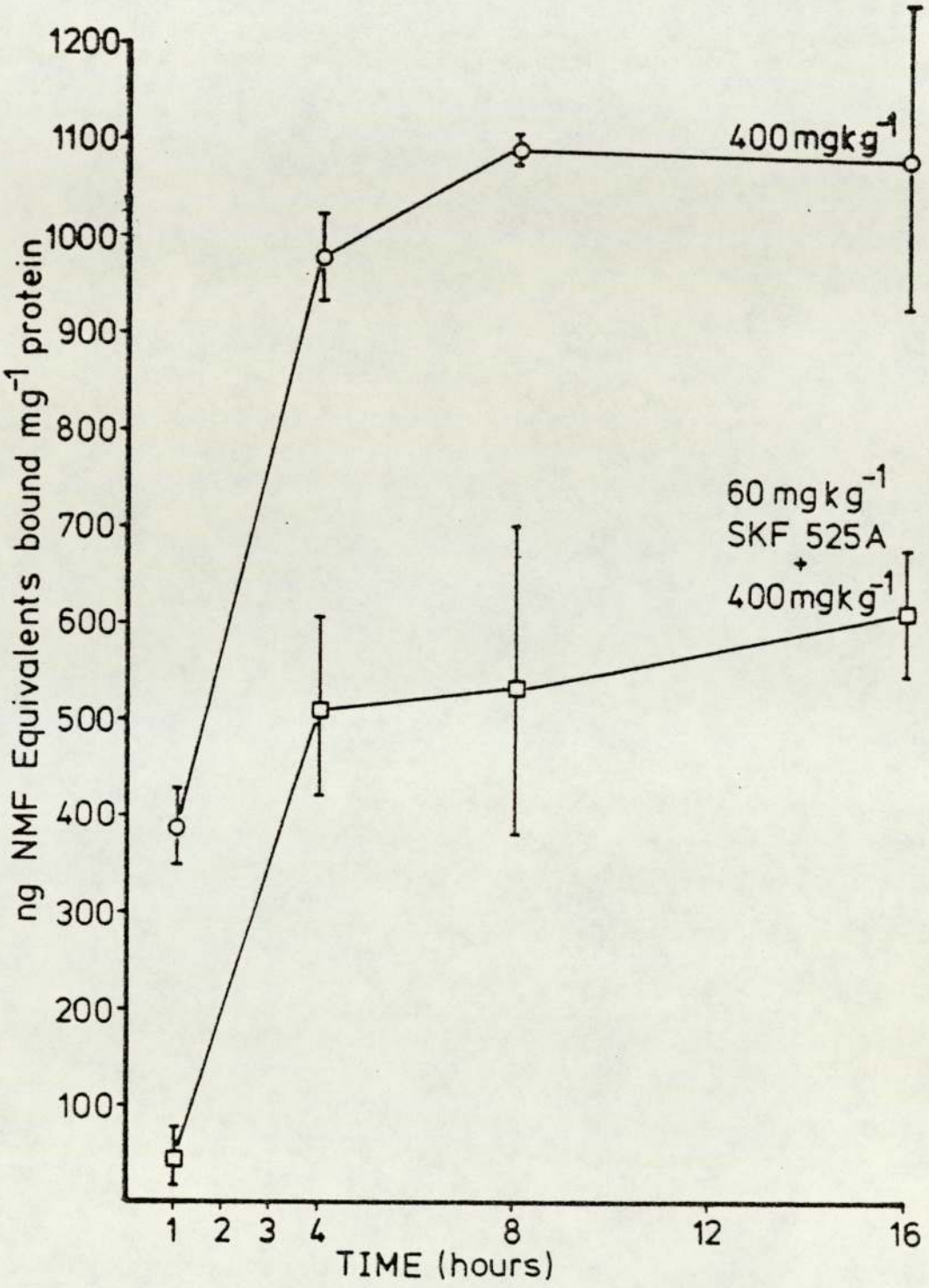


Figure 4.34 The effect of SKF525A (60mgkg^{-1}) administered 1 hour before NMF, upon the binding of $^{14}CH_3$-NMF to hepatic macromolecules in Balb/C mice (mean ± s.d; n = 3)

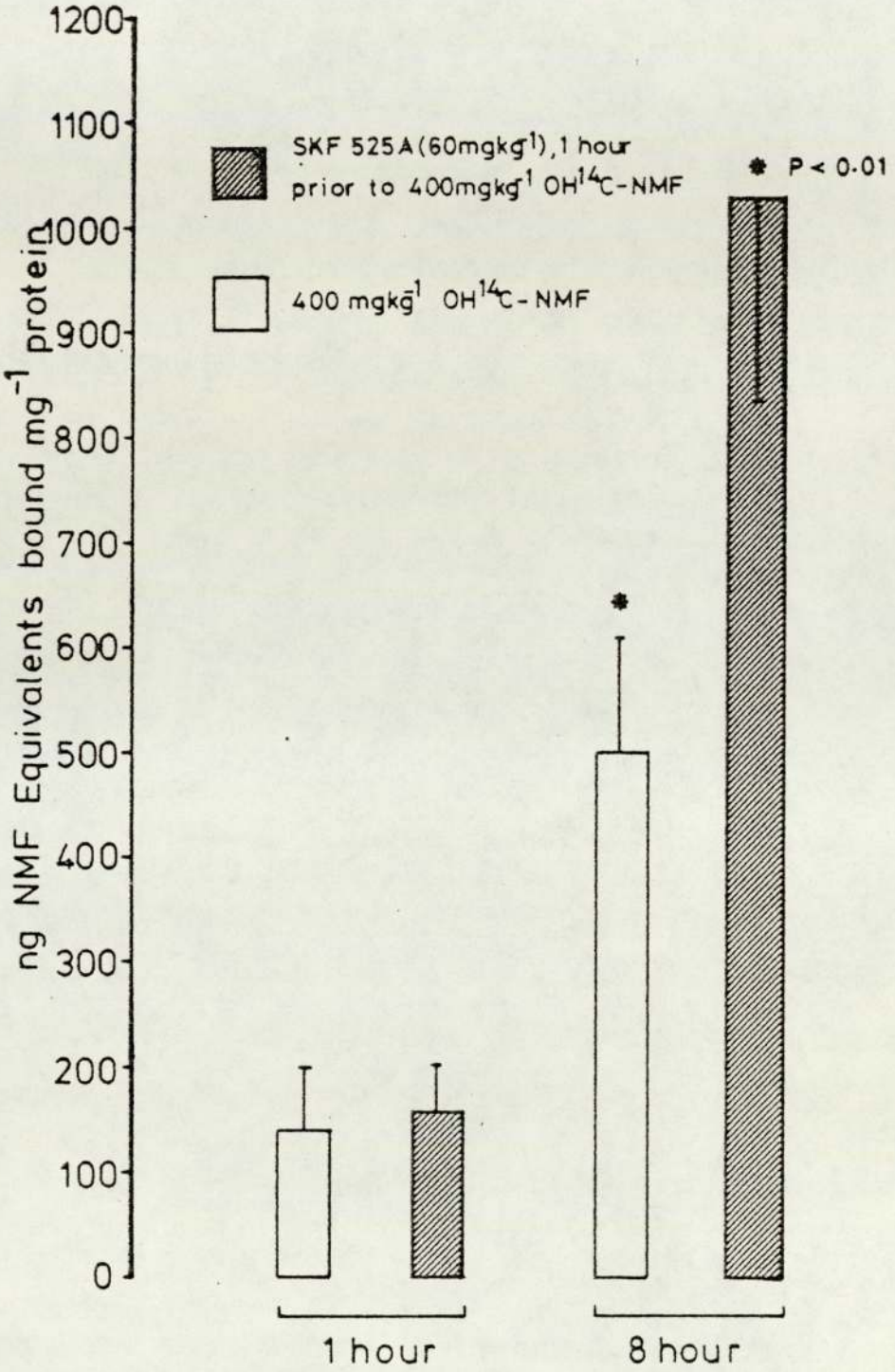


Figure 4.35 The effect of SKF525A ($60mgkg^{-1}$) administered 1 hour before NMF, upon the binding of $OH^{14}C$-NMF to hepatic macromolecules in Balb/C mice (mean ± s.d; n = 3).

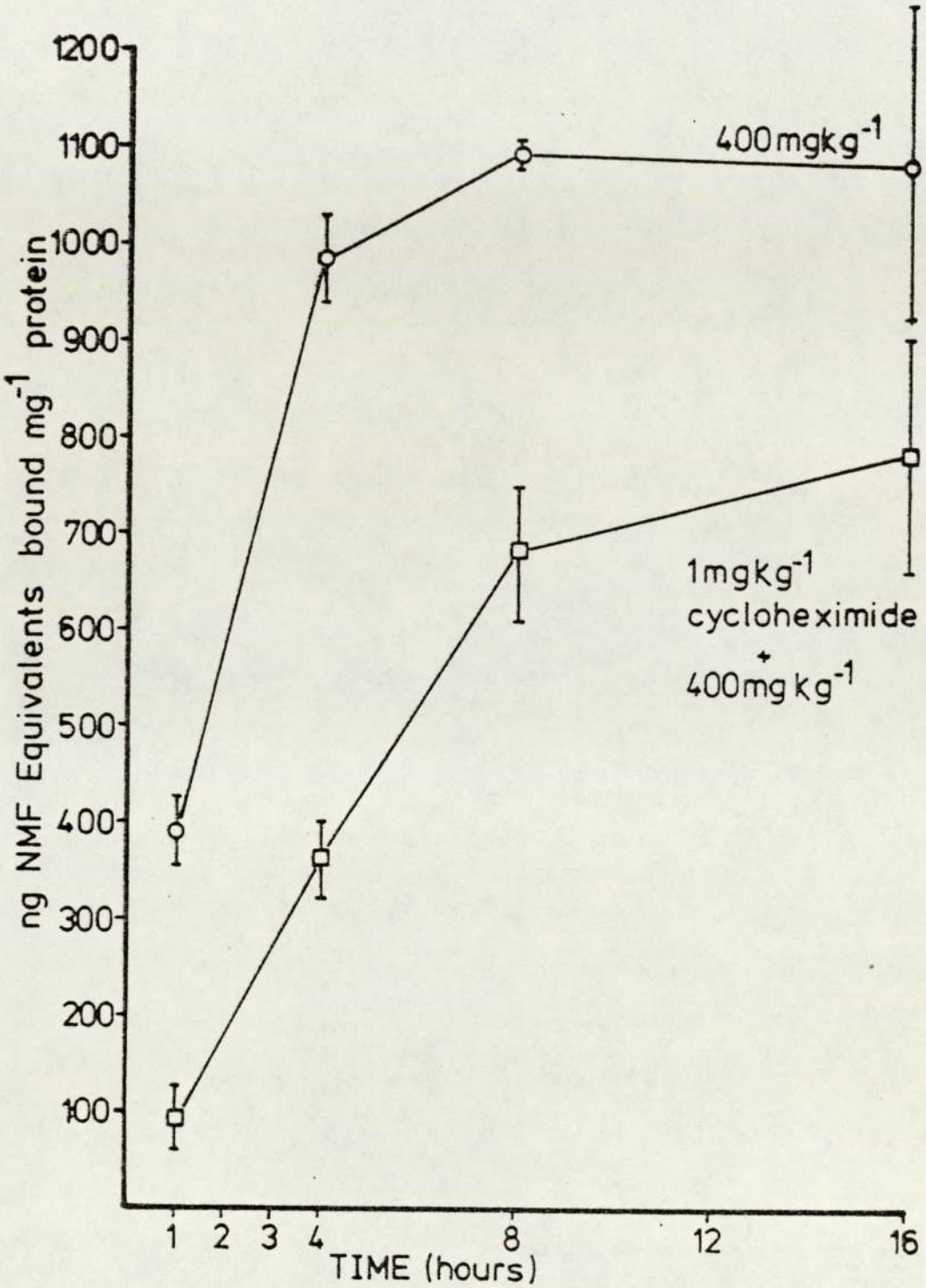


Figure 4.36 Effect of cycloheximide ($1mgkg^{-1}$), administered 15 min before NMF, upon the binding of $^{14}CH_3$-NMF to hepatic macromolecules in Balb/C mice (mean ± s.d; n = 3)

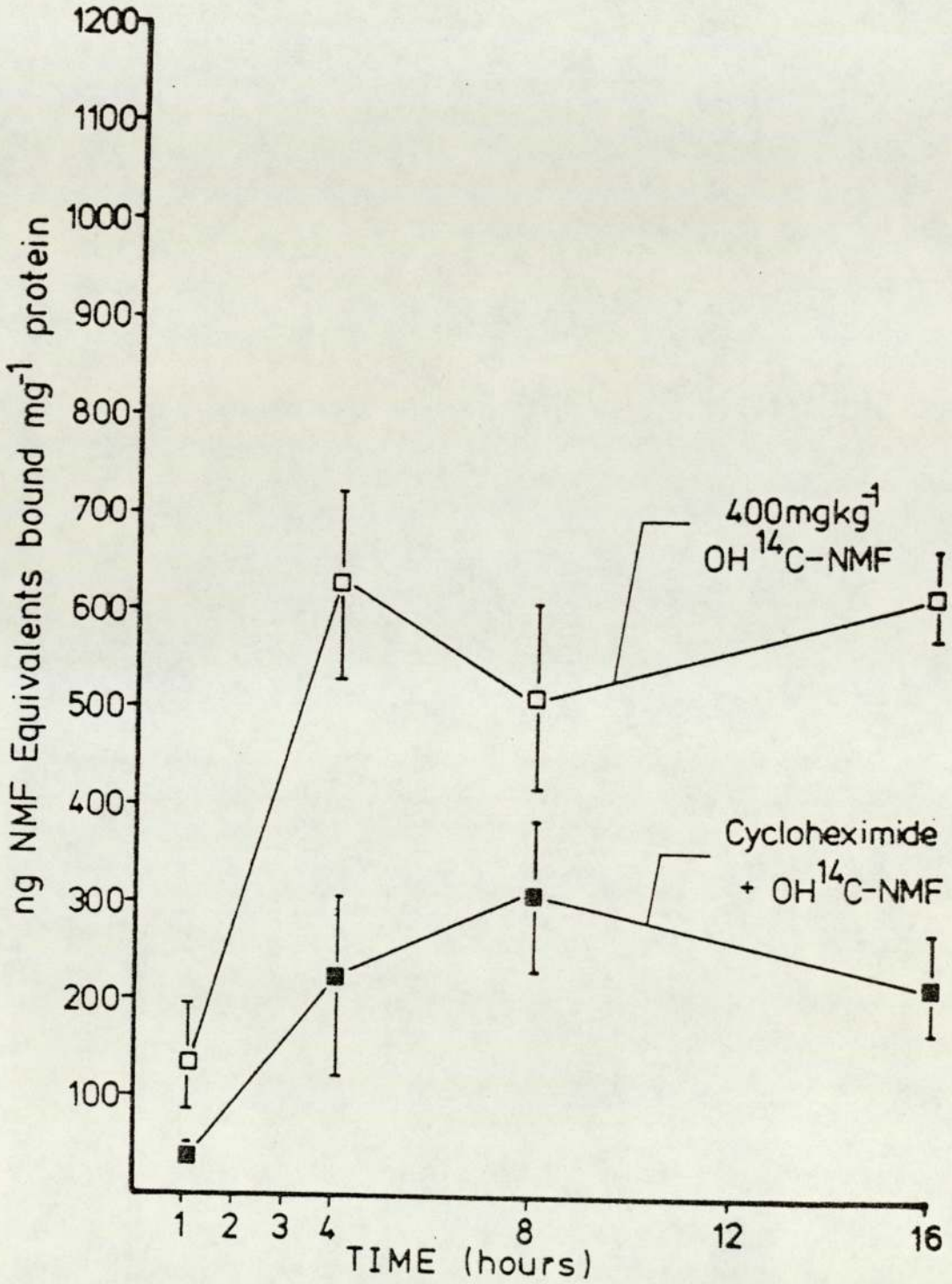


Figure 4.37 Effect of cycloheximide ($1mgkg^{-1}$), administered 15min before NMF, upon the binding of $400mgkg^{-1}$ $OH^{14}C$-NMF to hepatic macromolecules in Balb/C mice (mean ± s.d; n = 3)

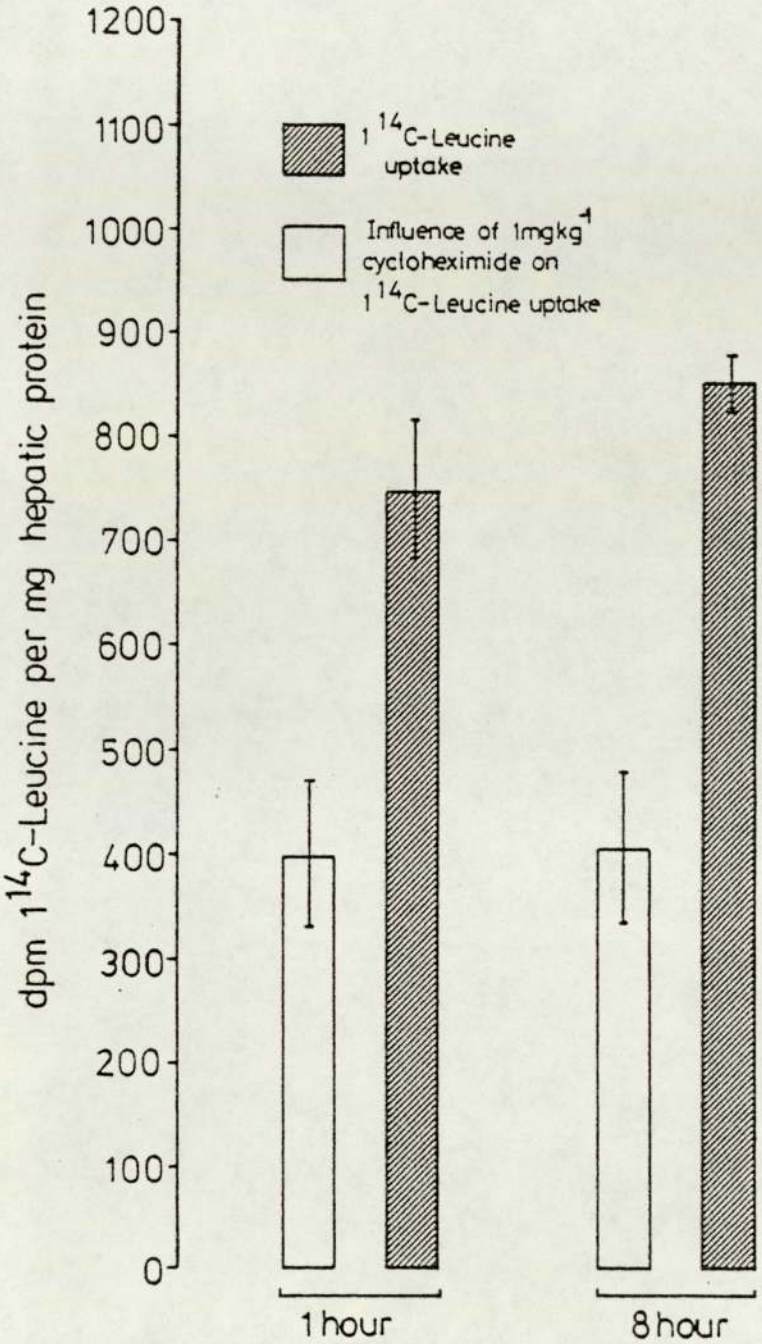


Figure 4.38 Influence of cycloheximide ($1mgkg^{-1}$) upon [$1^{14}C$]-Leucine incorporation in livers of Balb/C mice (mean ± s.d; n = 3)

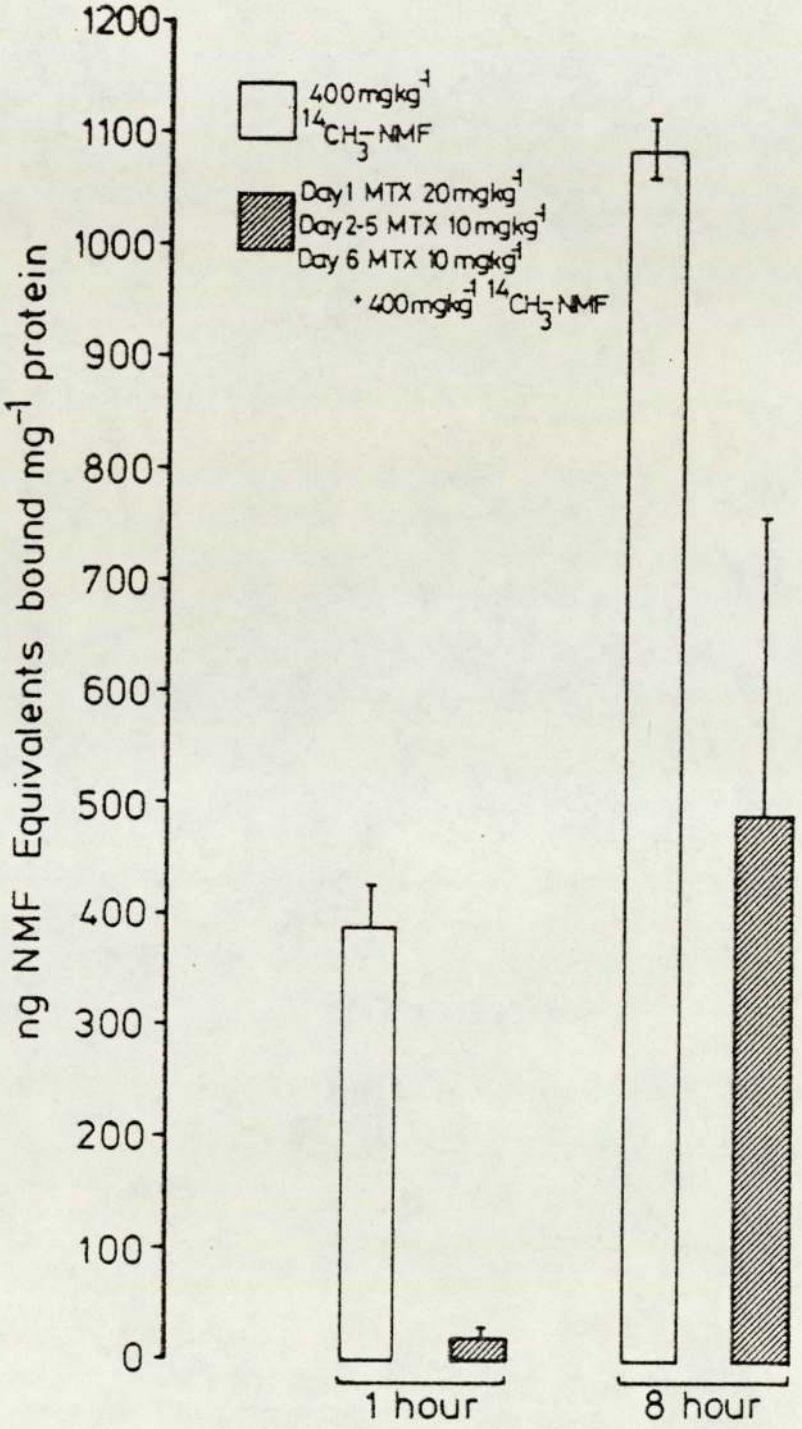


Figure 4.39 Influence of Methotrexate pretreatment upon the binding of 400mgkg^{-1} $^{14}CH_3$-NMF to hepatic macromolecules in Balb/C mice (mean ± s.d; n = 3)

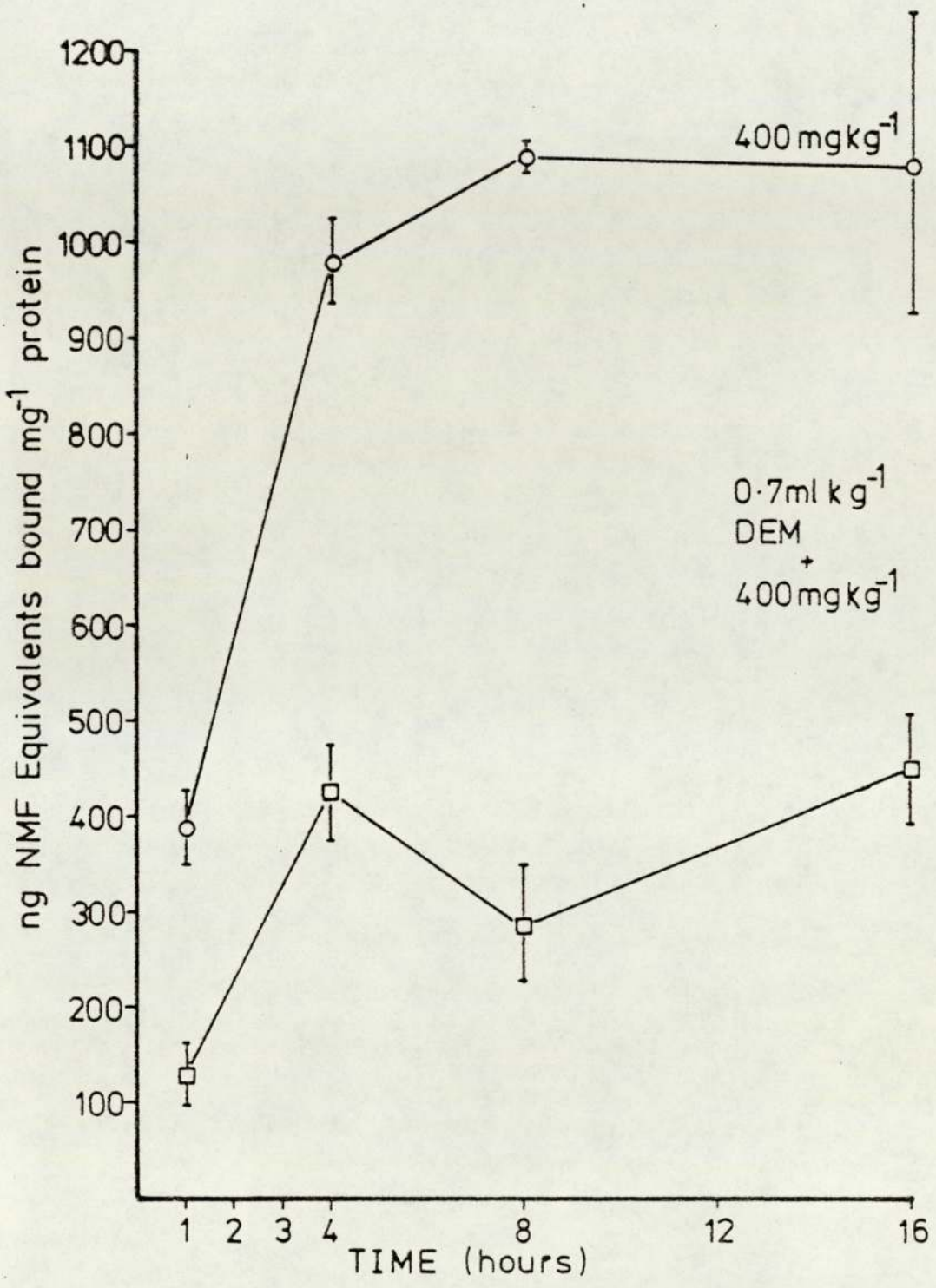


Figure 4.40 Effect of DEM ($0.7ml kg^{-1}$) administered 1 hour before NMF, upon the binding of $^{14}CH_3$-NMF to hepatic macromolecules in Balb/C mice (mean ± s.d; n = 3)

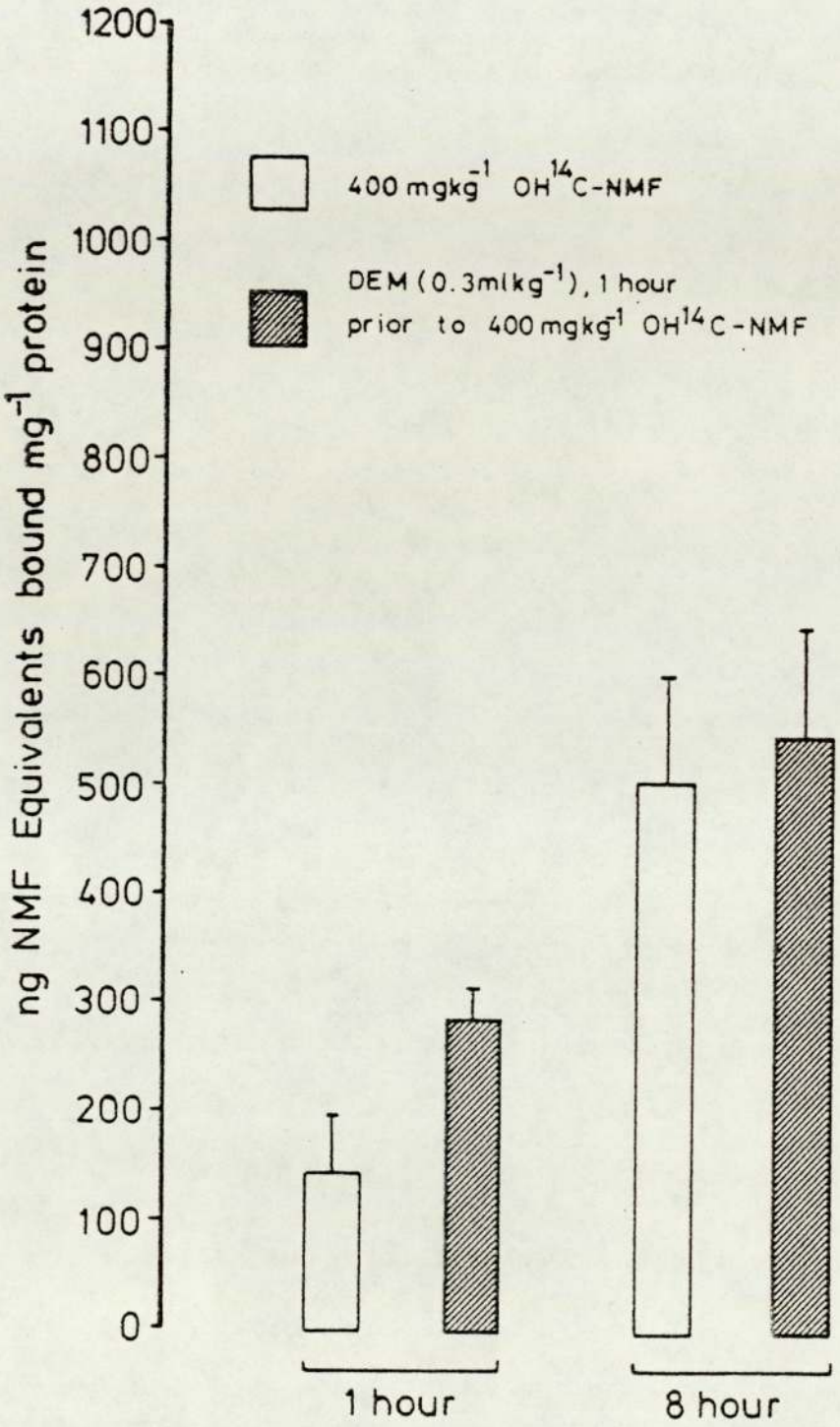


Figure 4.41 The effect of DEM ($0.3 ml kg^{-1}$) administered 1 hour before NMF, upon the binding of $OH^{14}C$-NMF to hepatic macromolecules in Balb/C mice (mean ± s.d; n = 3)

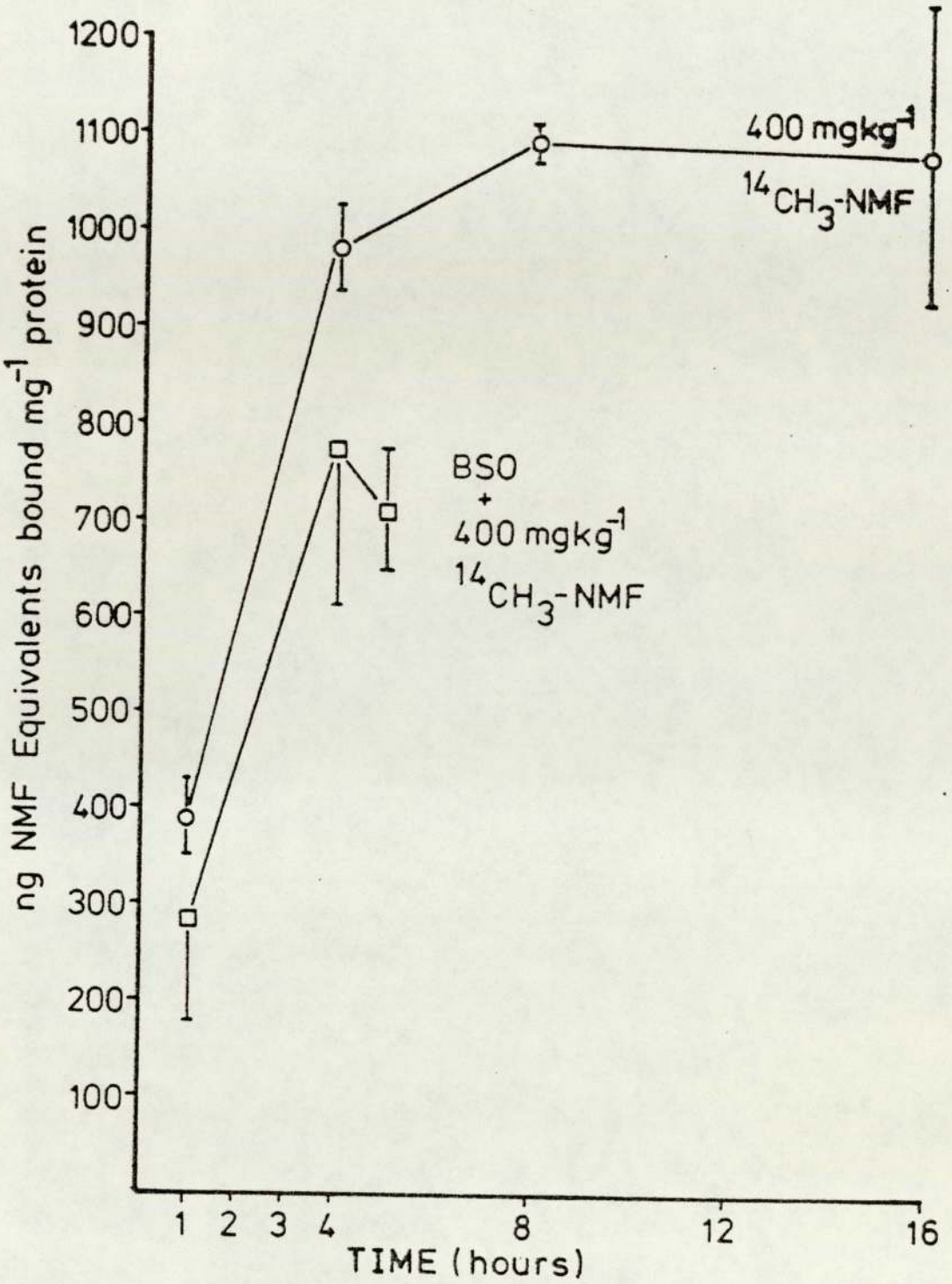


Figure 4.42 Effect of BSO (1600mgkg^{-1}), administered 4h before NMF, upon the binding of 400mgkg^{-1} $^{14}CH_3$-NMF to hepatic macromolecules in Balb/C mice (mean ± s.d; n = 3)

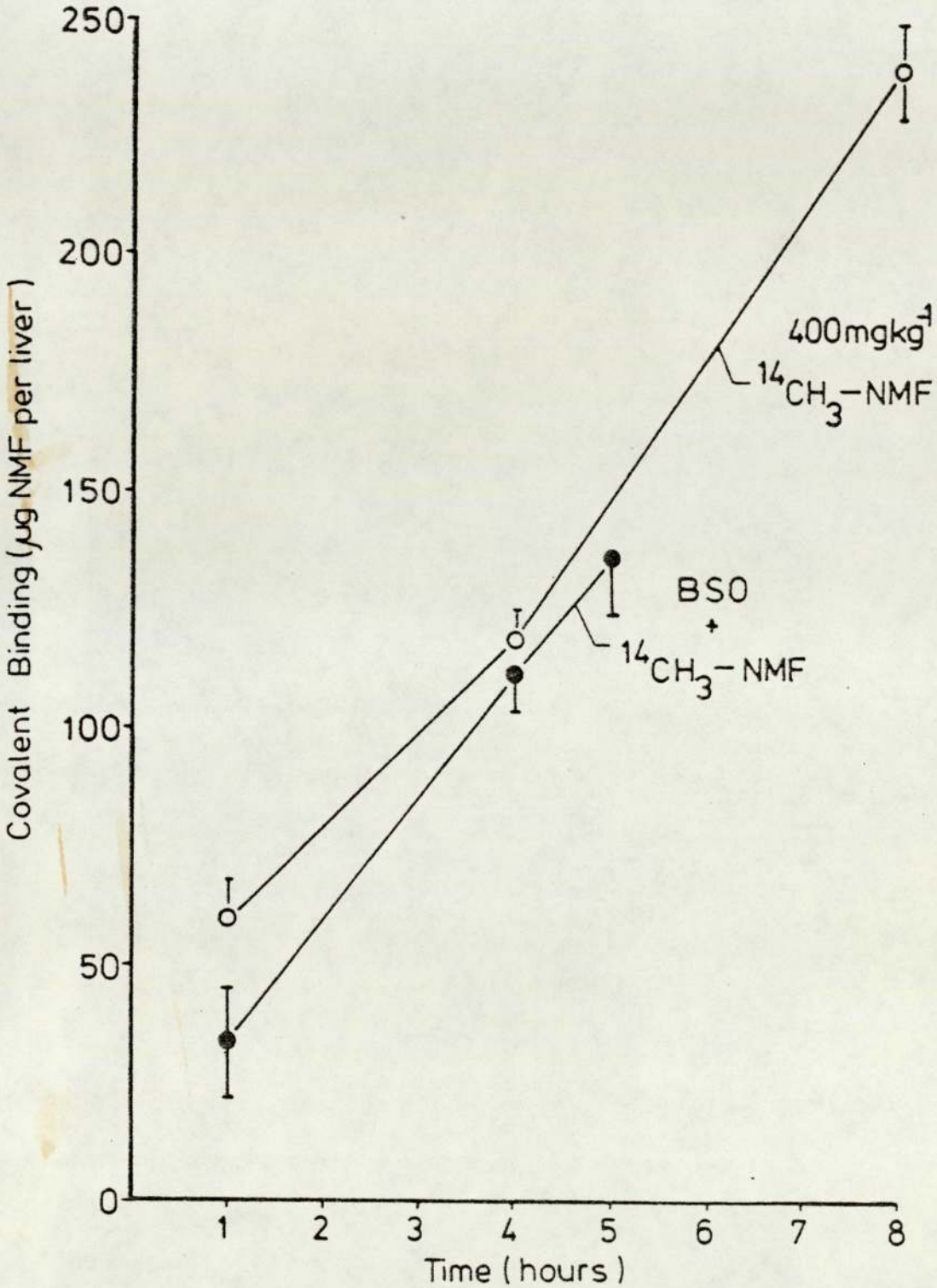


Figure 4.43 Effect of BSO ($1600 mg kg^{-1}$), administered 4h before NMF, upon the binding of $400 mg kg^{-1}$ $^{14}CH_3$-NMF to hepatic macromolecules in Balb/C mice (mean ± s.d; n = 3) (expressed as µg NMF per liver)

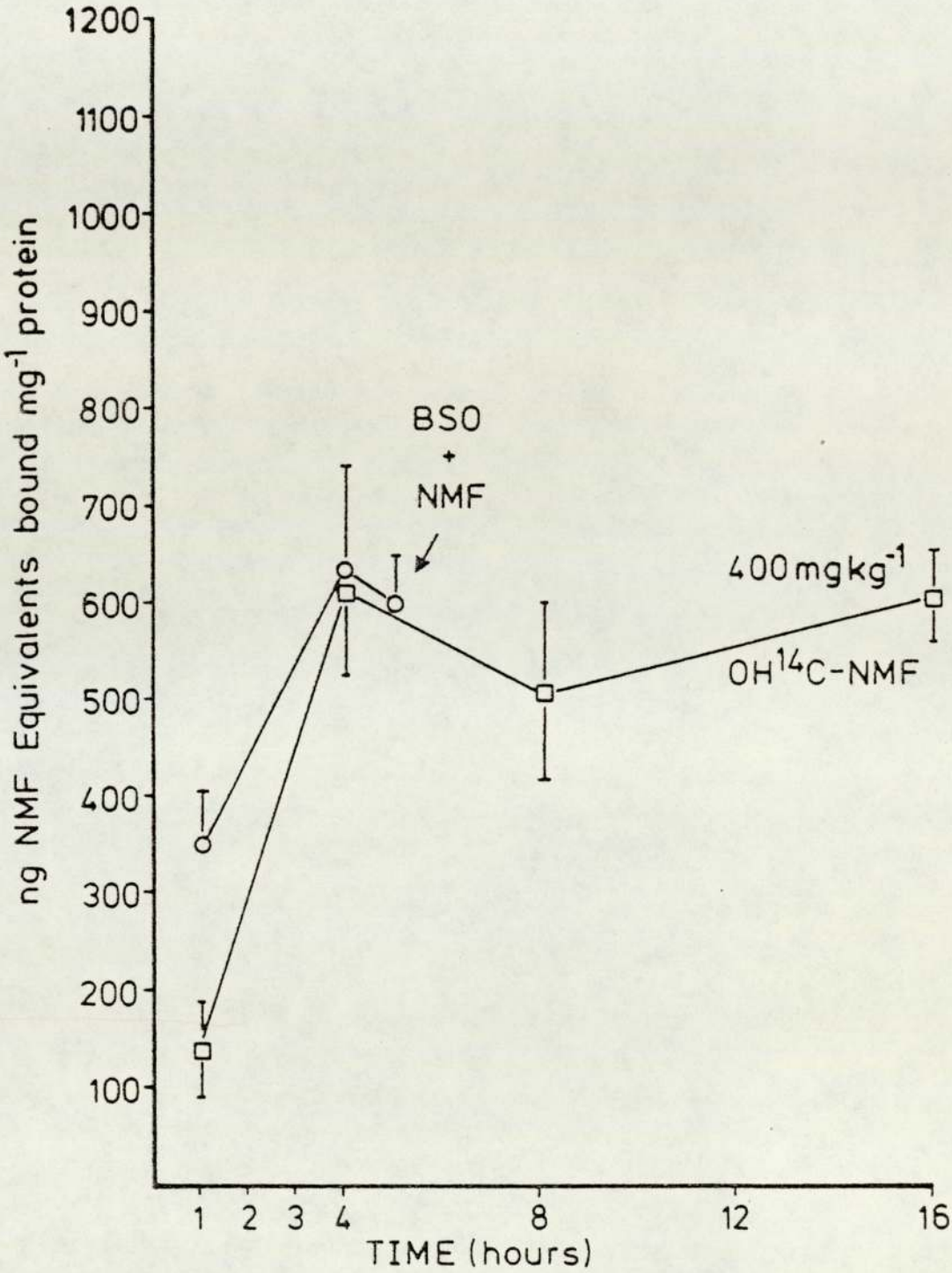


Figure 4.44 Effect of BSO (1600mgkg^{-1}), administered 4h before NMF, upon the binding of 400mgkg^{-1} OH^{14}C-NMF to hepatic macromolecules in Balb/C mice (mean ± s.d; n = 3)

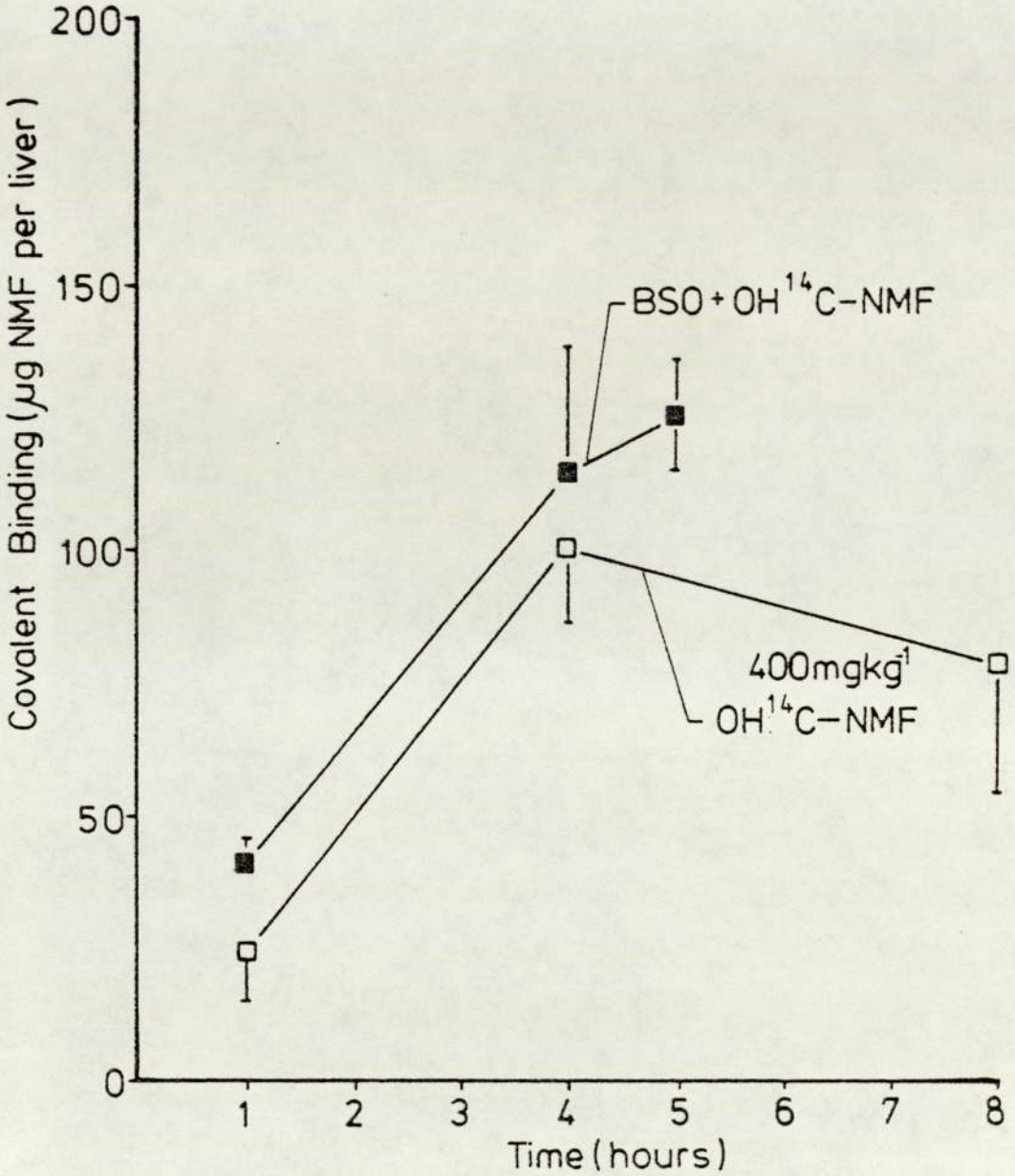


Figure 4.45 Effect of BSO (1600mgkg^{-1}), administered 4h before NMF, upon the binding of 400mgkg^{-1} OH ^{14}C-NMF to hepatic macromolecules in Balb/C mice (mean ± s.d; n = 3) (expressed as µg NMF per liver)

after BSO pretreatment are quantitatively and qualitatively similar to those obtained with NMF alone, but do not exhibit the fall in binding after 4h seen when the results are expressed per milligram of protein.

4.7.3 Discussion

In mice pretreated with SKF525A to inhibit cytochrome P-450 mediated oxidation reactions, the association of $OH^{14}C$-NMF and $^{14}CH_3$-NMF was altered. The reduction in association of $^{14}CH_3$-NMF is in accordance with effects of metabolic inhibitors upon covalent binding of other hepatotoxic drugs (104-106). On the other hand the significant increase in binding of $OH^{14}C$-NMF, caused by SKF525A, 8h after NMF administration is inconsistent with the view that inhibitors of drug metabolism cause a decrease in formation of covalently bound metabolites. SKF525A is known to exhibit biphasic effects upon drug metabolism. In the initial phase cytochrome P-450 is inhibited but after 24 to 48h, or repeated administration of SKF525A, multiple forms of cytochrome P-450 are induced (166, 167). However, it is improbable that the increased association of $OH^{14}C$-NMF, within 8 hours, is due to induction of drug metabolism.

Addition of SKF525A to microsomal incubations, also markedly enhanced covalent binding of $OH^{14}C$-NMF to microsomal protein *in vitro* (Section 4.9). This indicates that SKF525A has a similar effect upon the binding of $OH^{14}C$-NMF both *in vivo* and *in vitro*.

One might speculate that more than one enzyme system competes for NMF as a substrate. By inhibition of one metabolic pathway the biotransformation of NMF by another pathway, probably involving the formyl moiety, may be enhanced. The subsequent increase in association of NMF may represent either covalent binding of a reactive

species, or the metabolic incorporation of radioactivity into hepatic macromolecules.

In an attempt to delineate the relative contribution of incorporation to the irreversible association of NMF with hepatic macromolecules, mice were pretreated with cycloheximide and methotrexate. Cycloheximide inhibited [1 ^{14}C]-Leucine incorporation into hepatic proteins by approximately 50% at 1 and 8h after leucine administration. One hour after NMF administration, the association of OH^{14}C-NMF and $^{14}CH_3$-NMF was reduced to 25% of control values by cycloheximide.

Methotrexate also inhibited $^{14}CH_3$-NMF association with hepatic macromolecules to a greater extent 1h after NMF administration than at 8h. The findings that inhibitors of protein synthesis, and of transport of one carbon units, reduce the association of NMF suggests that the association is in part due to metabolic incorporation of ^{14}C-NMF metabolites into hepatic macromolecules. However, the use of modulators of endogenous metabolism in order to differentiate between covalent binding and incorporation is not unambiguous as these modulators may also alter the disposition of the drug which is being studied. This is particularly true for methotrexate, which is hepatotoxic (168).

In mice pretreated with BSO to deplete GSH to 20% of control values, prior to administration of NMF, the binding of OH^{14}C-NMF or $^{14}CH_3$-NMF was not altered. However, this pretreatment dramatically increased the hepatotoxicity of NMF (Section 4.10). Pretreatment with DEM 0.3mlkg^{-1} had no effect upon OH^{14}C-NMF association with hepatic macromolecules. On the other hand the mice pretreated with DEM (0.7mlkg^{-1}) survived to 16h after NMF administration, and the

association of $^{14}CH_3$-NMF with liver proteins was reduced. Both findings could be due to an inhibitory effect of DEM upon the metabolic activation of NMF, as DEM is known to inhibit the metabolism of certain drugs (86, 169). One may speculate that the binding of NMF metabolites to liver macromolecules reflects the exposure of the liver to reactive species. BSO pretreatment markedly increased the toxicity, but did not alter the binding and presumably the exposure to reactive species. The increase in toxicity caused by BSO may be due to qualitative changes in the nature of binding of the reactive species to hepatic proteins.

In conclusion, the association of ^{14}C-NMF with hepatic macromolecules may be due to both incorporation and covalent binding. The observation that SKF525A increased the binding of $OH^{14}C$-NMF both *in vivo* and *in vitro* (Section 4.9) suggests that SKF525A increases the formation of reactive species. The toxicological implications of this finding will be discussed in Section 4.10.

4.8 Strain differences in the covalent binding of ^{14}C-NMF to hepatic and renal macromolecules

4.8.1 Introduction

The relationship between toxicity, metabolism and covalent binding of an activated species can be studied *in vivo* by modulating the activity of drug metabolising enzymes and by changing intracellular GSH levels (102-105). The link between the covalent binding of a potentially toxic metabolite and the causation of a toxic lesion can also be studied by determining the extent of covalent binding in animal species which display markedly different susceptibilities to the toxin.

The hepatotoxin paracetamol depletes GSH and forms covalent adducts with protein to different degrees in hepatocytes isolated from rats, hamsters, rabbits and dogs. When paracetamol induced hepatotoxicity *in vitro* was compared with the extent of covalent binding in these different hepatocytes a good correlation was established between the two (170). Hepatocytes isolated from the hamster which was the most susceptible of these species, formed more covalently bound adducts of paracetamol, and were depleted of GSH more rapidly than hepatocytes isolated from the other species. Hepatocytes isolated from the relatively resistant rabbit did not form any detectable adducts and retained higher amounts of GSH when incubated with paracetamol.

It has been suggested before that there is a marked strain difference in the susceptibility of mice to the hepatotoxicity of NMF (21). In the BDF_1 mouse which was least susceptible to NMF hepatotoxicity, the extent of GSH depletion is not as great as that

observed in the more susceptible Balb/C mouse (Section 4.4). Therefore it is interesting to establish whether there is also a difference in the extent of covalent binding of NMF in the livers of either resistant or susceptible strains of mice.

Administration of NMF to mice results in a selective lesion of the centrilobular region of the liver (26). The liver and the kidney achieve the highest tissue levels of ^{14}C-NMF (139), however, the kidney displayed no biochemical evidence of damage when NMF ($100mgkg^{-1}$) was administered to Fischer rats daily for ten days (E.S. Harpur, personal communication). It is clearly of interest to compare the extent of covalent binding of NMF in the kidneys with that occurring in the liver, to further establish a role for covalent binding in the hepatotoxicity of NMF.

Therefore the aim of this section is twofold:-

1) To establish the magnitude of covalent binding of NMF in the livers of mice exhibiting different susceptibilities to hepatotoxicity.
2) To measure the covalent binding of NMF in a non-target tissue.

The susceptibility of the different strains to NMF hepatotoxicity will be discussed in Section 4.10.

4.8.2 Results

The binding profile of both OH^{14}C-NMF and $^{14}CH_3$-NMF to renal macromolecules of Balb/C mice was similar to the profile obtained in the liver (Figure 4.46). Binding reached a plateau between 4 and 8 hours after injection of NMF and did not decline significantly within 16 hours after NMF administration. In both liver and kidney $^{14}CH_3$-NMF

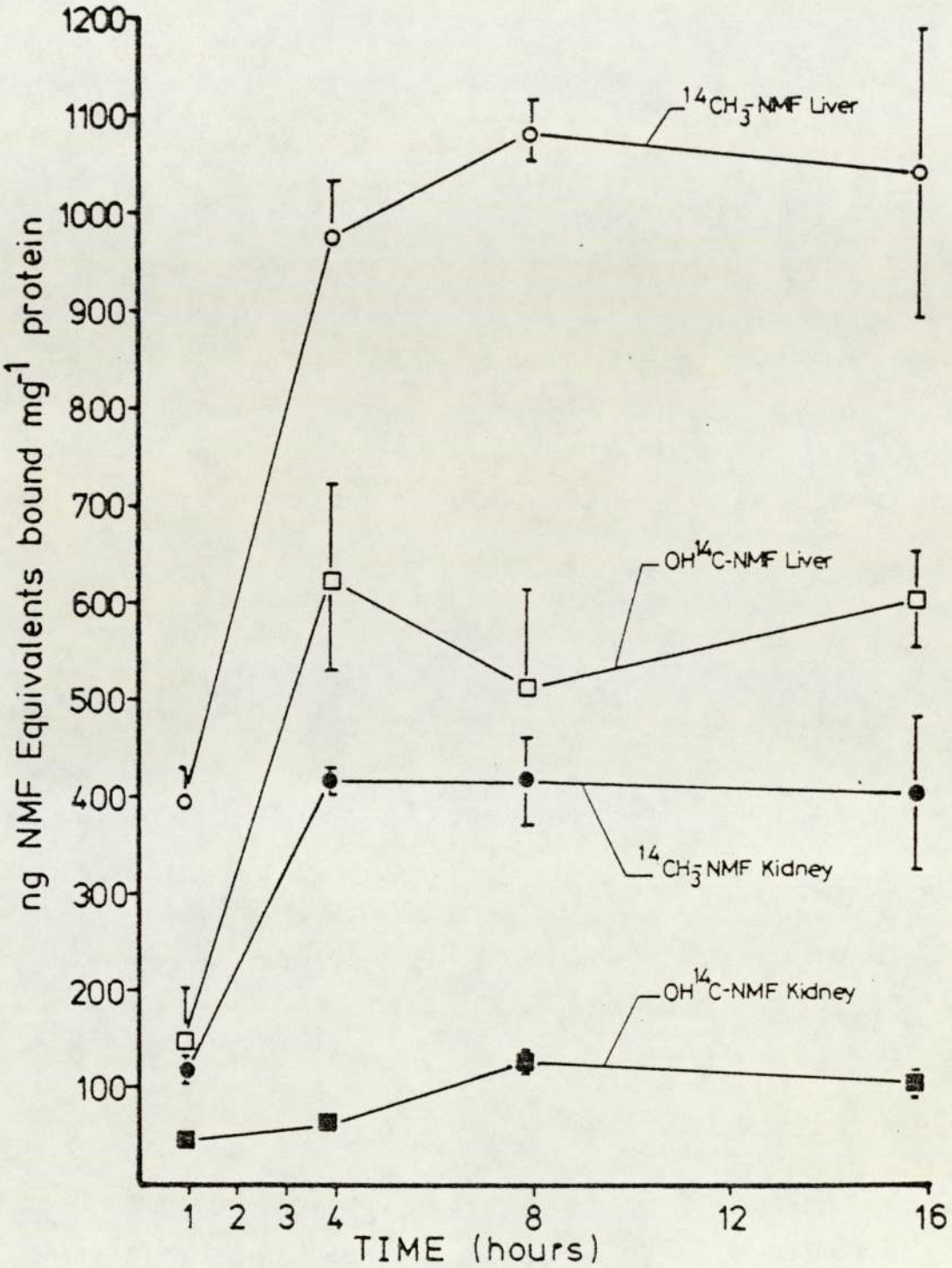


Figure 4.46 Association of $^{14}CH_3$-NMF with the hepatic and renal macromolecules of Balb/C mice after a single i.p. injection of 400mgkg^{-1} ^{14}C-NMF (mean ± s.d; n = 3)

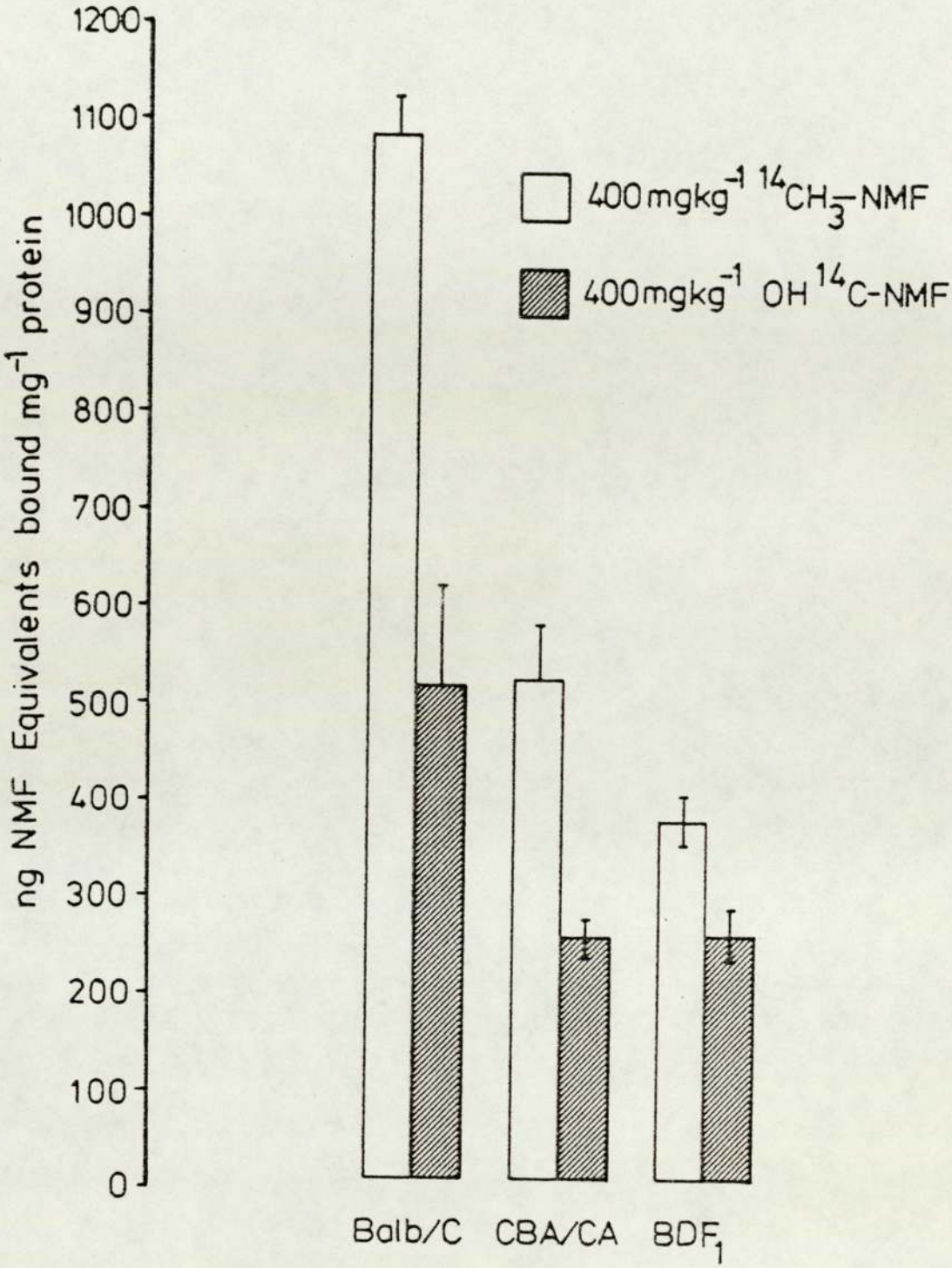


Figure 4.47 Strain difference in association of $OH^{14}C$-NMF and $^{14}CH_3$-NMF with hepatic macromolecules of Balb/C, CBA/CA and BDF_1 mice 8h after a single i.p. injectionof $400mgkg^{-1}$ NMF (mean ± s.d; n = 3)

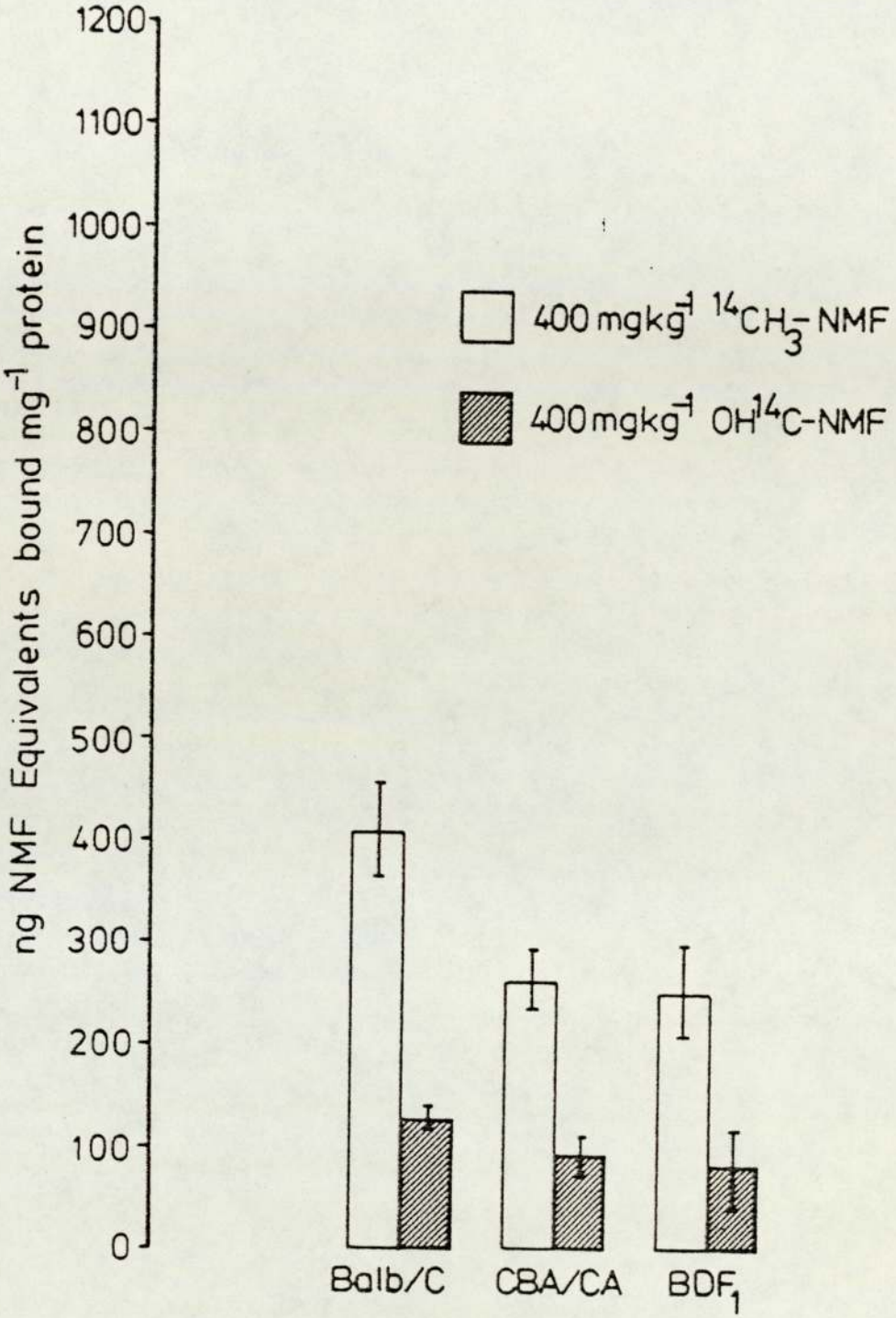


Figure 4.48 Strain differences in associationof $OH^{14}C$-NMF and $^{14}CH_3$-NMF with renal macromolecules of Balb/C, CBA/CA and BDF_1 mice, 8h after a single i.p. dose of $400mgkg^{-1}$ NMF (mean ± s.d; n = 3)

was bound more extensively than $OH^{14}C$-NMF, and the extent of binding of both labels in the kidney was less than half of the levels achieved in the liver.

The strain differences in the association of $^{14}CH_3$-NMF and $OH^{14}C$-NMF with hepatic macromolecules was evaluated in Balb/C, CBA/CA and BDF_1 mice, 8h after a single i.p. dose of 400mgkg^{-1} NMF (Figure 4.47). In all three strains $^{14}CH_3$-NMF was bound more extensively than $OH^{14}C$-NMF. In the Balb/C mouse twice as much $^{14}CH_3$-NMF was bound compared to the CBA/CA mouse ($P<0.001$), which in turn was greater than the binding observed in the BDF_1 mouse ($P<0.05$).

Differences between strains of mouse in the binding of $OH^{14}C$-NMF and $^{14}CH_3$-NMF to renal macromolecules 8h after NMF administration were similar to the differences seen in the binding to hepatic macromolecules (Figure 4.48). As shown previously in the Balb/C mouse (Figure 4.46) the level of binding in the kidneys of the other two strains was also less than half of that in the liver.

4.8.3 Discussion

The results indicate a marked strain difference exists in the association of ^{14}C-NMF with both hepatic and renal macromolecules. This difference in the association of ^{14}C-NMF correlates with the strain difference in susceptibility to NMF hepatotoxicity shown in Section 4.10 and in (21). The Balb/C mouse which is the most susceptible to NMF hepatotoxicity, with a hepatotoxic dose threshold of between 100 and 200mgkg^{-1} NMF (Section 4.10), exhibited the greatest level of covalent binding in the liver. In the case of CBA/CA mouse (hepatotoxic dose threshold between 200 and 400mgkg^{-1}; see Section 4.10) the extent of binding was less than in the Balb/C

mouse, but greater than that in the liver of BDF_1 mice (hepatotoxic dose threshold between 400 and 800mgkg^{-1} NMF (21)). Therefore in different strains of mouse, the susceptibility to NMF induced hepatotoxicity, and the extent of covalent binding of ^{14}C-NMF metabolites appear to be related. In addition hepatic GSH was depleted more extensively in the Balb/C than in other mouse strains (Section 4.4). These findings and the observed strain differences in the metabolism of NMF (Section 4.1), strongly suggest a role for a metabolically generated reactive species, which may deplete hepatic GSH, bind to hepatic macromolecules, and cause toxicity.

In addition to the strain differences in covalent binding there is a marked interorgan difference in binding within strains. The kidney to which NMF does not appear to be toxic exhibits significantly lower levels of binding than the liver. In this context it is interesting to note that 1h after a dose of 400mgkg^{-1} NMF administered to Balb/C mice, renal levels of GSH were depleted by only 30% compared with the more extensive depletion of 59.8% in the liver (4). One may speculate that the extent of binding which may reflect the amount of reactive metabolite present in the kidney is inadequate to evoke a toxic event. Indeed the highest levels of ^{14}C-NMF binding to the kidneys (400ng NMF equivalents mg^{-1} protein) achieved in the Balb/C mouse is of an order of a magnitude similar to that observed in the liver following a non-hepatotoxic dose of 100mgkg^{-1} NMF. To understand the significance of this finding fully it is desirable to characterize the chemical nature of the NMF macromolecule adducts formed, in order to correlate toxicity with covalent modification of a particular target molecule.

In conclusion, the severity of the hepatotoxicity of NMF in different strains of mice is correlated with the extent of binding of ^{14}C-NMF to hepatic macromolecules. NMF binds more extensively to hepatic macromolecules than to the macromolecules of an organ, the kidney, to which NMF is not toxic.

4.9 Covalent binding of NMF to isolated mouse liver microsomes

4.9.1 Introduction

Despite extensive *in vivo* biotransformation of NMF (Section 4.1) it has hitherto proved impossible to demonstrate metabolism *in vitro*, in either suspensions of hepatocytes, or in mixtures containing liver fractions, using gas chromatography as the analytical technique (4, 59). This is contrary to a report in an old paper, which claimed 10% biotransformation of 1.7mM NMF, when incubated with rat liver homogenate for 2 hours (39).

The biochemical events associated with the hepatotoxicity of NMF (Section 4.4) support the view that the hepatotoxicity is mediated via the generation of a reactive species (26). Administration of ^{14}C-NMF to mice resulted in association of radioactivity with hepatic macromolecules, demonstrating that chemically reactive NMF metabolites may have the potential to bind covalently to hepatic tissues.

For a large number of compounds of diverse chemical structure, including paracetamol (108), hexamethylmelamine (14), 1-naphthol (171) and p-chloramphetamine (172) it has been possible to demonstrate covalent binding of a metabolically generated species to microsomal protein *in vitro*. For all of these compounds details of the mechanism of bioactivation *in vitro* have been obtained by the use of inhibitors and inducers of drug metabolising enzymes to modulate the amount of reactive species formed, or by performing incubations in the presence of nucleophiles such as GSH.

Therefore measurement of covalent binding to microsomal protein of a drug such as NMF, which undergoes metabolism *in vitro*

only to a minor extent, or not at all, is a potentially sensitive index of drug metabolism. Consequently measurement of covalently bound radioactivity in incubations of ^{14}C-NMF with liver microsomes was thought to provide definitive evidence for the generation of a reactive species.

In addition, by using this approach it may be possible to establish similarities between the pattern of binding to microsomal protein *in vitro* and the binding of ^{14}C-NMF metabolites to hepatic macromolecules *in vivo*.

Therefore, the aim of this section is twofold:-

1) To evaluate the binding of OH^{14}C-NMF and $^{14}CH_3$-NMF to microsomal protein in *in vitro* experiments.
2) To modify the activity of drug metabolizing enzymes in order to evaluate the biochemical processes which lead to the formation of the species capable of binding to microsomal protein *in vitro* and to relate the findings to the effects of these modifications on the association of ^{14}C-NMF with hepatic macromolecules *in vivo*.

4.9.2 Results

The extent of binding of ^{14}C-NMF to microsomal protein observed after 2h incubation of either OH^{14}C-NMF or $^{14}CH_3$-NMF (7mM) with microsomes isolated from Balb/C mice, is shown in Table 4.5. Incubation of microsomes with $^{14}CH_3$-NMF, in the presence of an NADPH generating system, lead to twice the level of binding obtained with OH^{14}C-NMF. The covalent binding of both OH^{14}C-NMF and $^{14}CH_3$-NMF to microsomal protein exhibited a requirement for NADPH, and was drastically reduced by heat inactivation of the microsomal enzymes.

Table 4.5

Effect of induction and inhibition of cytochrome P-450 on the covalent binding of ^{14}C-NMF metabolites to microsomal protein following 2h incubation with 7mM NMF. (expressed as ng NMF equivalents/mg protein; mean ± s.e. mean)

Experimental conditions	$^{14}CH_3$-NMF	OH^{14}C-NMF
-NADPH	14.5 ± 2.0 (7)	14.4 ± 6.1 (4)
Heat inactivation + NADPH	32.4 ± 5.1 (3)	13.8 ± 2.6 (3)
+ NADPH	$^{+}$123.6 ± 13.2 (9)	$^{+}_{\ddagger}$60.7 ± 9.8 (5)
Phenobarbitone pretreatment (50mgkg^{-1} for 4 days) + NADPH	114.0 ± 4.2 (3)	75.4 ± 10.0 (3)
SKF525A (0.1mM) + NADPH	161.8 ± 22.7 (4)	‡232.0 ± 15.0 (3)

$^{+}$ Significantly different from controls - NADPH, P<0.001

‡ Significantly different, P<0.001

numbers in parentheses indicate the number of replicates of incubations performed in duplicate.

Table 4.6

Effect of GSH (10mM) on the covalent binding of ^{14}C-NMF metabolites to microsomal protein following 2h incubation with 7mM NMF (expressed as ng NMF equivalents/mg protein; mean ± s.e. mean)

Experimental conditions	$^{14}CH_3$-NMF	OH^{14}C-NMF
- NADPH	14.6 ± 2.0 (7)	14.4 ± 6.1 (4)
Heat inactivation + NADPH	32.4 ± 5.1 (3)	13.8 ± 2.6 (3)
+ NADPH	$^{+}_{‡}$123.6 ± 13.2 (9)	$^{+}_{*}$60.7 ± 9.8 (5)
+ NADPH + 10mM glutathione	$_{‡}$10.0 ± 3.5 (3)	$_{*}$13.9 ± 6.2 (3)

$^{+}$ Significantly different from controls, - NADPH, $P<0.001$

‡ Significantly different, $P<0.001$

* Significantly different, $P<0.01$

numbers in parentheses indicate the number of replicates of incubations performed in duplicate.

Table 4.7

In vitro metabolism of aminopyrine by mouse liver microsomes

Incubation conditions	Formaldhyde[a] (nmol/mg protein/min)
Literature value[b]	3.01 ± 0.56 (6)
+NADPH	5.5 ± 0.2 (4)
-NADPH	0.9 ± 0.1 (2)
Heat inactivation + NADPH	1.5 ± 0.007 (2)
Phenobarbitone induced microsomes	7.6 ± 1.3 (4)

a. Measured by the method of Nash (65)

b. Reference 42

Numbers in parentheses indicate the number of replicates of incubations performed in duplicate.

Pretreatment of the mice with phenobarbitone (50mgkg^{-1} for 4 days) prior to isolation of the microsomes did not significantly alter the level of covalent binding of OH^{14}C-NMF or $^{14}CH_3$-NMF.

Incubation of ^{14}C-NMF in the presence of SKF525A (0.1mM) failed to abolish or reduce the NADPH dependent covalent binding of $^{14}CH_3$-NMF to microsomal protein, and increased the covalent binding of OH^{14}C-NMF threefold ($P<0.001$).

Addition of 10mM GSH to the incubation medium abolished the covalent binding observed upon microsomal metabolism of both OH^{14}C-NMF and $^{14}CH_3$-NMF (Table 4.6).

A control experiment ensured that the microsomes used in these assays were metabolically viable. The metabolising capacity of the microsomal preparation was estimated by determining the formation of HCHO as a metabolite of aminopyrine in the presence of an NADPH generating system (Table 4.7). The value obtained for AP demethylation was comparable with literature values (42).

4.9.3 Discussion

The results indicate that ^{14}C-NMF is metabolically activated to a covalently bound species by a pathway that requires NADPH, and is heat labile, indicating the involvement of an enzyme. The extent of binding of $^{14}CH_3$-NMF (2.03 nmol mg^{-1} protein) was comparable with the level of binding reported for metabolites of paracetamol (3.75 nmol mg^{-1} protein) in Balb/C mouse liver microsomes (114).

$^{14}CH_3$-NMF was bound twice as extensively as OH^{14}C-NMF indicating that the molecule is metabolically cleaved. The quantitative difference between the two labels in binding to microsomal protein in vitro is comparable to the quantitative

difference observed in association of $^{14}CH_3$-NMF and $OH^{14}C$-NMF with hepatic macromolecules *in vivo* (Section 4.7).

To evaluate a possible role for cytochrome P-450 in the metabolic activation of NMF, incubations were performed in the presence of an inhibitor of cytochrome P-450, SKF525A (166) or with microsomes which had cytochrome P-450 levels raised by pretreatment of mice with phenobarbitone (136). The phenobarbitone treatment failed to enhance the covalent binding, suggesting a pathway independent of cytochrome P-450.

The presence of SKF525A in the microsomal incubation did not influence the binding of $^{14}CH_3$-NMF but markedly stimulated the binding of $OH^{14}C$-NMF to microsomal protein. This finding is in accordance with the interpretation that activation of NMF is probably independent of cytochrome P-450. It is interesting to note that SKF525A also markedly increased the association of $OH^{14}C$-NMF with hepatic macromolecules *in vivo* (Section 4.7), which is yet another similarity between the *in vivo* association of ^{14}C-NMF and the *in vitro* covalent binding to microsomal protein.

Addition of GSH (10mM) to the incubation media abolished the covalent binding of both labels. This demonstrates that GSH is capable of reacting with metabolite(s) generated from NMF by mouse liver microsomes. This is of particular importance in view of the marked depletion of hepatic GSH caused by NMF (Section 4.4).

The inability of either phenobarbitone to stimulate, or SKF525A to inhibit the microsomal covalent binding of NMF indicates the existence of an activation pathway independent of cytochrome P-450. Even though the available data does not allow inferences as to the enzyme system, or systems which may be responsible for the

formation of a toxic NMF metabolite, a number of speculations are attractive. It is unlikely that cytochrome P-450 is involved in NMF toxification, but one cannot totally exclude a role for this enzyme in the overall metabolism and disposition of NMF. Enzyme systems which may be involved in the metabolic toxification are 1. The Flavin containing monooxygenase (E.C. 1.14.13.8; N,N dimethylaniline N-oxidizing), 2. The Xanthine-Xanthine oxidase system.

The Flavin containing monooxygenase is an attractive candidate for the role in the metabolic activation of NMF as it is not inhibited by SKF525A. The thiono-sulphur containing compound, thioacetamide, is a hepatocarcinogen (173, 174) and causes centrilobular necrosis in the liver (175, 176). The toxicity of thioacetamide is attributed to its sulfine metabolite thioacetamide-S-oxide (177). Thioacetamide-S-oxide is activated to a reactive species that forms an N-ϵ- acetyllysine adduct, by the microsomal flavin containing monoxygenase (178).

The flavin containing monooxygenase first described by Ziegler et al (179, 180) is a NADPH dependent flavoprotein which catalyses the oxidation of nitrogen and sulphur compounds. This system is of particular interest as it is not inhibited by SKF525A, or induced by phenobarbitone (181). One may speculate that the increase in microsomal covalent binding, of $OH^{14}C$-NMF by SKF525A, may represent competition between an enzyme which is sensitive to SKF525A, such as cytochrome P-450, and one or more other enzyme systems which are not sensitive to SKF525A. If any of these systems are responsible for activation, then inhibition of the cytochrome P-450 pathway with SKF525A may increase the covalent binding to microsomes.

Therefore, for the above reasons, the flavin containing monooxygenase may be considered as a candidate for the enzyme which may catalyze the activation of NMF. However, there are a number of reasons to refute the hypothesis that the flavin containing monooxygenase is responsible for NMF activation. Firstly, typical substrates of the FAD monooxygenase are tertiary amines eg. dimethylaniline (179) or substituted thioureas or thioimidazoles (eg. methimazole; N-methyl-2-mercaptoimidazole) (182). An amide such as NMF is more likely to be a substrate for cytochrome P-450 than for the FAD monooxygenase (181).

Secondly, the FAD monooxygenase may be selectively inactivated by incubation of the microsomal preparation for 45 min at 37°C (183, 184). Preincubation at 37°C for 20 min in the absence of NADPH has been reported to lead to a 70% loss of FAD monooxygenase activity. (183).

In a preliminary experiment, preincubation of the microsomal preparation at 37°C for 45 min prior to addition of NADPH and ^{14}C-NMF to the incubation did not alter the extent of covalent binding of either OH^{14}C-NMF or $^{14}CH_3$-NMF to microsomal protein. For these reasons it is unlikely that NMF is bioactivated by the flavin containing monooxygenase.

The second potential candidate enzyme for NMF activation is xanthine oxidase. It has been reported that incubation of the dimethyl analogue of NMF, DMF (5mM) with the hypoxanthine-xanthine oxidase system for sixty minutes leads to the production of 8.5nM HNMF (45), this represented metabolic conversion of 0.17% of the substrate. In the present study 2.03 nmoles of $^{14}CH_3$-NMF metabolites were bound per mg^{-1} protein which represents 0.058% biotransformation of NMF.

It is conceivable that NMF may be biotransformed to a toxic species by the hypoxanthine-xanthine oxidase system or by microsomal cytochrome P-450 reductase stimulated to produce hydroxyl radicals by ferric-EDTA chelates (185). Indeed dimethyl sulphoxide another polar aprotic slvent is demethylated by systems generating hydroxyl radicals (186).

In conclusion NMF is activated by mouse liver microsomes to a covalently bound species by an NADPH requiring enzyme system which appears to be independent of cytochrome P-450. The extent of binding to the microsomal protein is comparable to that seen with paracetamol; and there are certain similarities between the pattern of binding *in vitro* and the association of ^{14}C-NMF with hepatic macromolecules *in vivo*.

4.10 Characterization of the hepatotoxicity of NMF

4.10.1 Introduction

The hepatotoxic potential of NMF has been demonstrated in man and in experimental animals (Section 1.2). In man, reversible transaminites has been shown to be a major toxic effect associated with NMF administration.

In Balb/C mice the hepatotoxicity is characterized by extensive centrilobular necrosis which appeared between 14 and 24h after administration of NMF (187). The activity in plasma of the enzymes sorbitol dehydrogenase (EC 1.1.1.14; SDH), alanine aminotransferase (EC 2.6.1.2; ALT) and aspartate aminotransferase (EC 2.6.1.1; AST) displayed a sharp elevation commencing between 12 and 24h after administration of a hepatotoxic dose of NMF. Elevation of SDH activity in the plasma is indicative of acute liver parenchymal cell damage. In chronic conditions eg. cirrhosis of the liver, SDH levels in the plasma reach an initial peak then fall off (190). In healthy animals SDH is found at highest activity in the liver, with levels three and five times greater than SDH activity in the prostrate and kidney respectively, the other organs in which SDH activity is significant (189). Therefore measurement of SDH in the plasma is used as a relatively specific indicator of hepatocellular damage (144).

AST is present in almost equal concentration in both liver and cardiac muscle, with slightly lower concentrations in skeletal muscle. ALT is present almost exclusively in the liver, with smaller quantities in cardiac and skeletal muscle (190). As a consequence elevation of serum ALT is almost a certain indicator of liver damage, but the serum level of AST will also be raised following traumatic

injury or necrosis, not only in the liver, but also in muscle and heart tissue eg. myocardial infarction.

When liver damage is assessed in terms of raised serum enzyme levels it is desirable to monitor the activity of a panel of enzymes in the plasma rather than a single enzyme for the reasons outlined above. In the case of the hepatotoxicity of NMF the onset of the elevation of SDH, ALT and AST in the plasma of mice correlated well with the onset of histopathological evidence of liver damage (187). There was also a good correlation between histopathological evidence of damage and raised plasma SDH activity in rats administered DMF and NMF (28).

In the present study the nature of the damage has been carefully assessed by histopathological examination of liver sections, and also by measuring the activity of SDH, ALT and AST in the plasma of mice 24h after NMF administration. The use of light microscopy complements the measurement of biochemical parameters as pathological changes may become apparent at lower doses of the hepatotoxin than measurable changes in biochemical indices. In addition identification of the locus of damage within the liver was thought to provide information about the mechanism of hepatotoxicity (188).

For a range of hepatotoxins it is possible to selectively modify the severity of hepatotoxicity in a number of ways. For example the hepatotoxicity of paracetamol may be prevented in experimental animals by pretreatment with N-acetylcysteine. N-acetylcysteine injection is also used clinically to protect against the hepatotoxicity associated with paracetamol overdosage (191). Indeed using this approach it has been possible to alter the NMF induced hepatic GSH depletion with precursors of GSH and with radical

scavenging agents (Section 4.5).

The observation that NMF is metabolically activated to a covalently bound species by mouse liver microsomes is consistent with the view that a reactive species may be responsible for the hepatotoxicity of NMF (see Section 4.9). If this is true then agents which influence the rate of formation of the reactive species should effect the expression of NMF-induced toxicity. Hepatotoxins which are activated by cytochrome P-450 are influenced in the expression of that toxicity by inducers of this enzyme.

The role of the FAD monooxygenase as a potential candidate enzyme responsible for NMF metabolism has been discussed in Section 4.9. The deleterious effect of hepatotoxins that are activated by the FAD monooxygenase (eg. thioacetamide, thiobenzamide) can be reduced by pretreating animals with methimazole, a substrate for the FAD monooxygenase (192, 184).

In view of the above observations, it was considered useful to attempt to modify the severity of NMF induced hepatotoxicity in order to elucidate the mechanism of NMF induced hepatotoxicity. To recapitulate the aims of this section are threefold:-

1) To attempt to modify the toxicity of NMF by pretreating mice with thiol containing compounds, or by depleting GSH, prior to administration of NMF.
2) To attempt to modify the hepatotoxicity of NMF with inducers and inhibitors of drug metabolising enzymes and with radical scavenging agents.
3) To complement studies of strain differences in NMF hepatotoxicity by evaluating its deleterious effects in

CBA/CA mice and comparing them with those seen in Balb/C mice.

4.10.2 Results

To present the results from this study as clearly as possible, the plasma enzyme data are shown and discussed first (see also Appendix II), followed by tabulated histopathological data. The significance of the histopathological finding in relation to the enzyme values will be discussed in 4.10.3.

The threshold dose for NMF induced hepatotoxicity in the Balb/C mouse, as indicated by elevation of the plasma activity of SDH, ALT and AST was between 100 and 200mgkg^{-1} (Figure 4.49). In the CBA/CA mouse the threshold dose for hepatotoxicity was between 200 and 400mgkg^{-1} NMF (Figure 4.50).

Administration of phenobarbitone (50mgkg^{-1}) to Balb/C mice for 4 days prior to administration of a non-hepatotoxic dose of 100mgkg^{-1} NMF did not produce an elevation in plasma enzyme values above control (Figure 4.51). Pretreatment of Balb/C mice with SKF525A did not abolish or reduce the elevation in activity of SDH, ALT or AST in plasma induced by 400mgkg^{-1} NMF (Figure 4.52). Likewise methimazole did not reduce the elevation of SDH, ALT or AST in the plasma of Balb/C mice 24h after a dose of 200mgkg^{-1} NMF (Figure 4.53).

Depletion of hepatic GSH to 18.6% of control values with BSO (see Section 4.3) prior to administration of 100mgkg^{-1} NMF caused a marked elevation in plasma enzyme activity, compared with control, 8h after NMF administration when toxicity was so severe that most mice were morbid and had to be killed (Figure 4.54). Conversley, pretreatment of Balb/C mice with NAC (1200mgkg^{-1} p.o. 20 min before

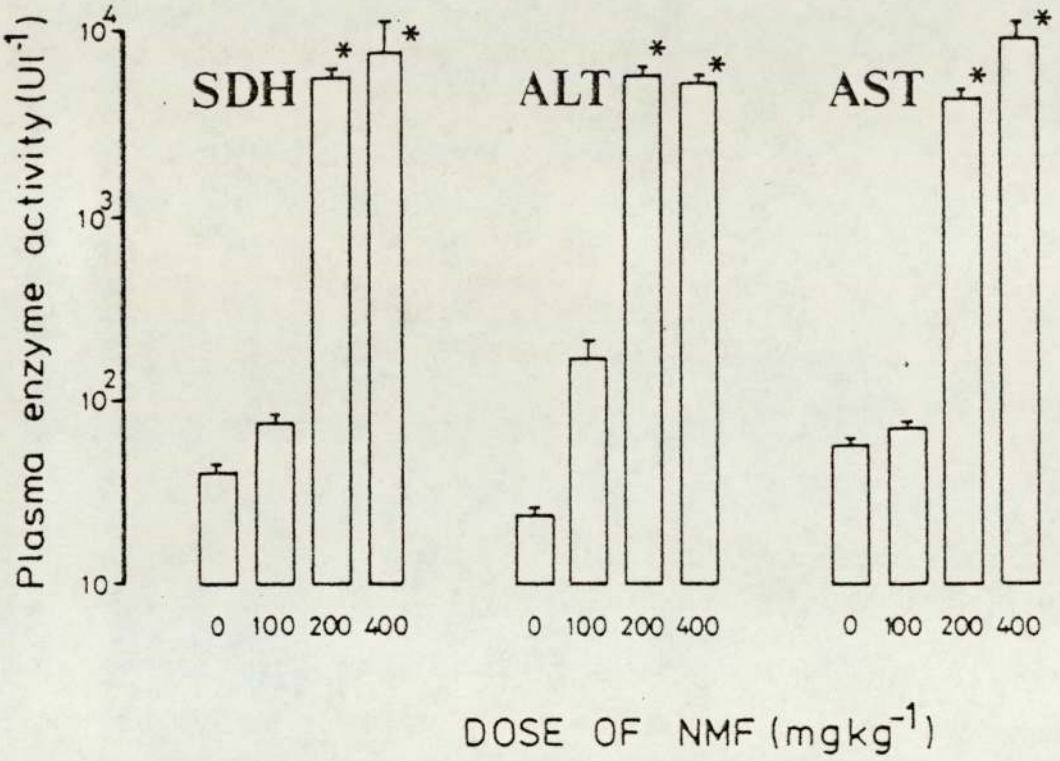


Figure 4.49 Dose related elevation of SDH, ALT and AST in the plasma of Balb/C mice (mean + s.e. mean; $n \geqslant 6$). Significantly different from control (Mann-Whitney U-test) at * $P<0.001$

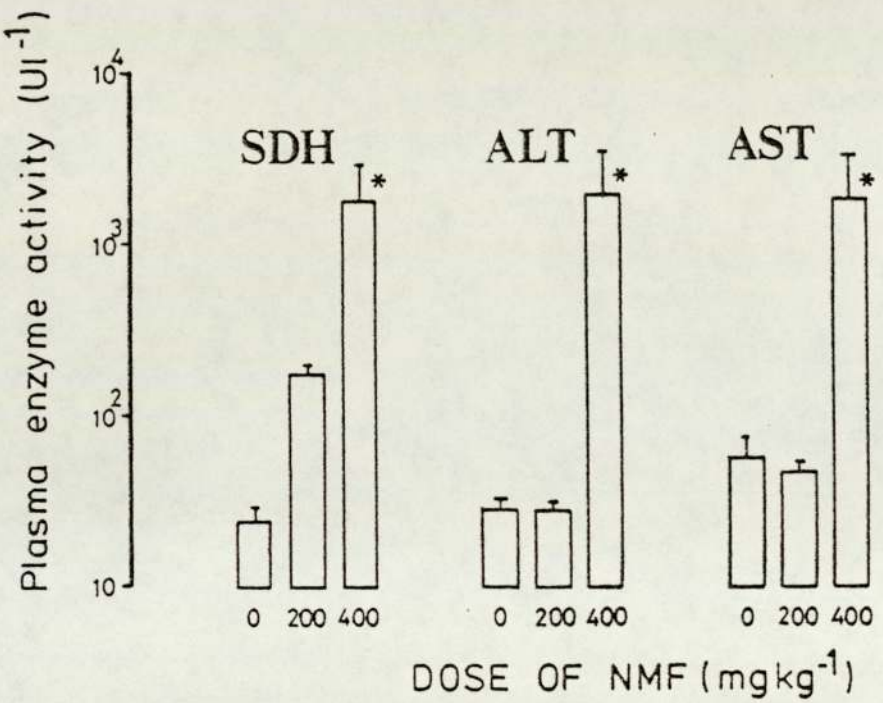


Figure 4.50 Dose related elevation of SDH, ALT and AST in the plasma of CBA/CA mice (mean ± s.e. mean; n=6). Significantly different from control (Mann-Whitney U-test) at * $P<0.001$

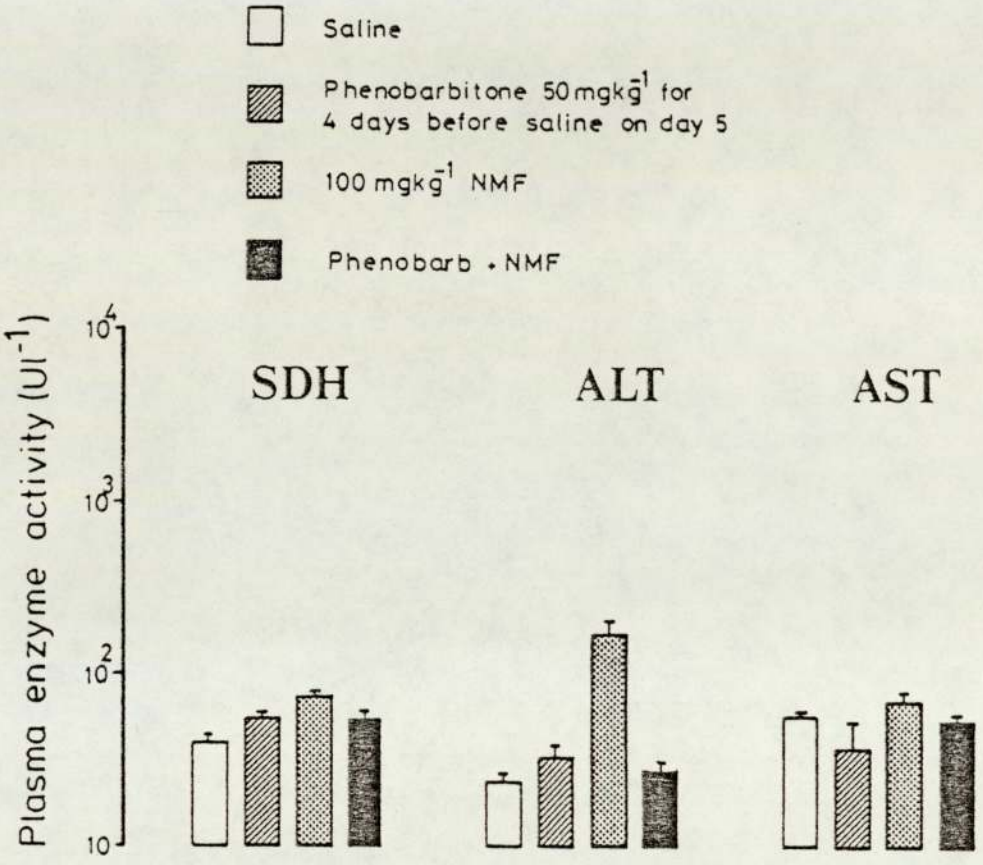


Figure 4.51 Effect of phenobarbitone pretreatment upon the activity of SDH, ALT and AST in the plasma of Balb/C mice 24h after a single i.p. injection of 100mgkg^{-1} NMF (mean ± s.e. mean; n≥6)

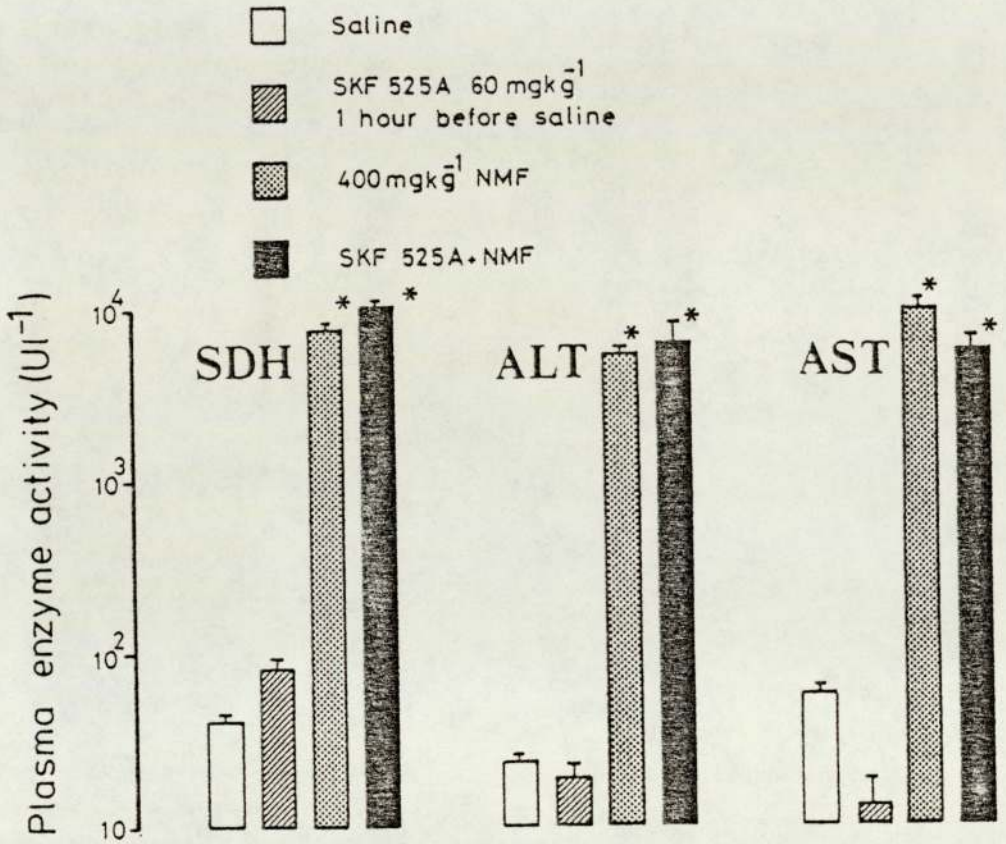


Figure 4.52 Effect of SKF525A pretreatment upon NMF induced elevation of SDH, ALT and AST in the plasma of Balb/C mice 24h[a] after a single i.p. dose of 400mgkg^{-1} NMF (mean ± s.e. mean; n≥6) *significantly different from control (Mann-Whitney U-test) at P<0.001

a, Samples for SKF525A + NMF group, obtained 12h after administration of NMF

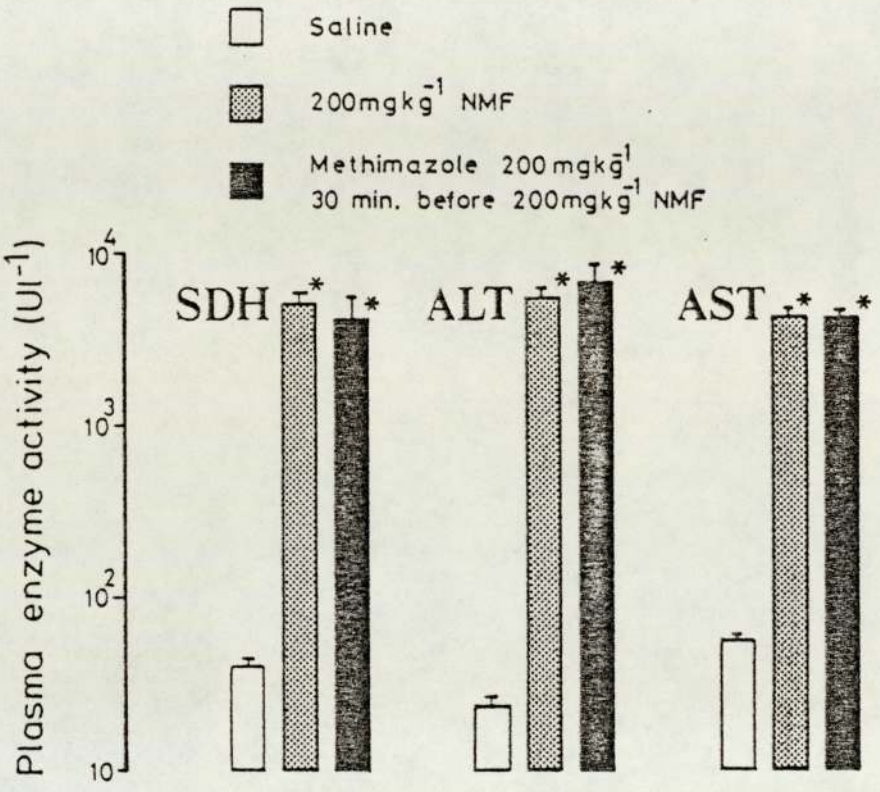


Figure 4.53 Effect of methimazole pretreatment upon NMF induced elevation of SDH, ALT and AST in the plasma of Balb/C mice 24h after a single i.p. dose of 200mgkg^{-1} NMF (mean ± s.e. mean; n≥4) *significantly different from control (Mann-Whitney U-test) at P<0.001

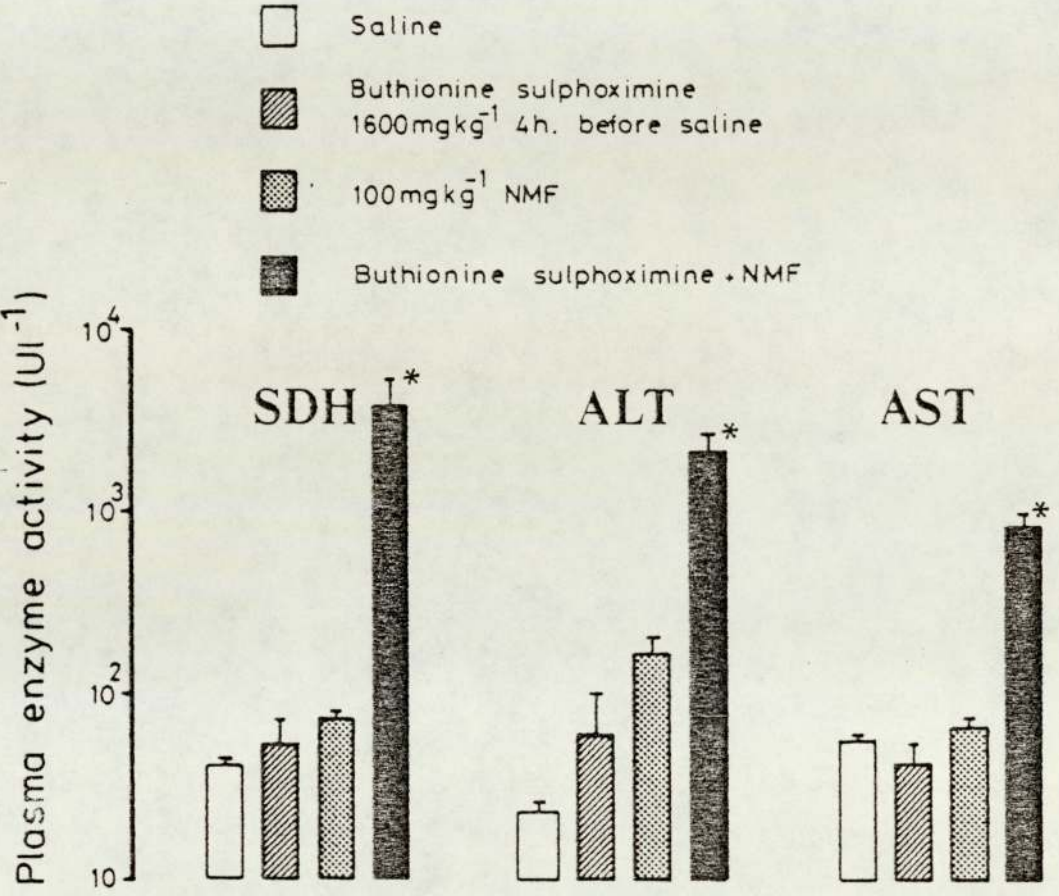


Figure 4.54 Effect of DL-buthionine S,R sulphoximine (BSO) pretreatment upon NMF induced elevation of SDH, ALT and AST in the plasma of Balb/C mice 24h[a] after a single i.p. dose of 100mgkg^{-1} NMF (mean ± s.e. mean; n≥6) *significantly different from control (Mann-Whitney U-test) at P<0.001

a, Sample for BSO and NMF group obtained 8h after NMF administration

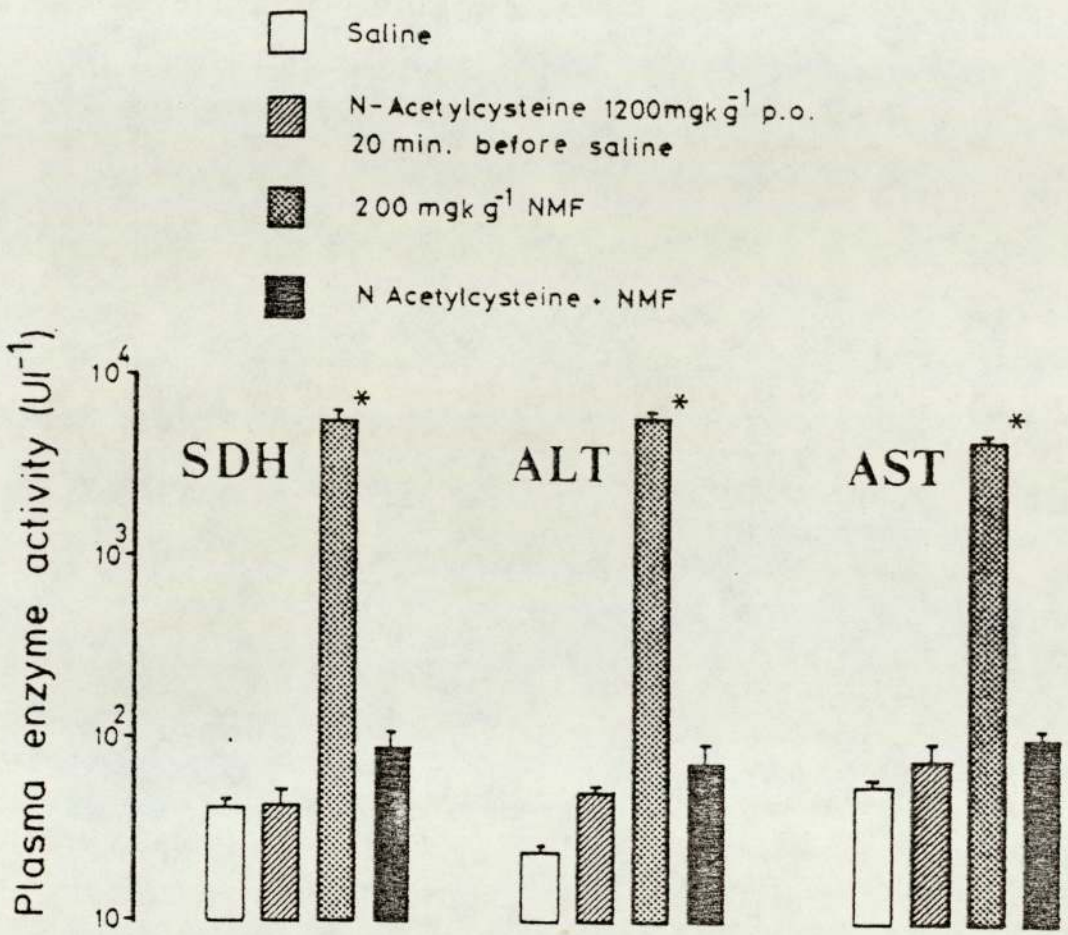


Figure 4.55 Effect of N-acetylcysteine (1200mgkg^{-1}) pretreatment upon NMF induced elevation of SDH, ALT and AST in the plasma of Balb/C mice 24h after a single i.p. dose of 200mgkg^{-1} NMF (mean ± s.e. mean; n≥6) *significantly different from control (Mann-Whitney U-test) at P<0.001

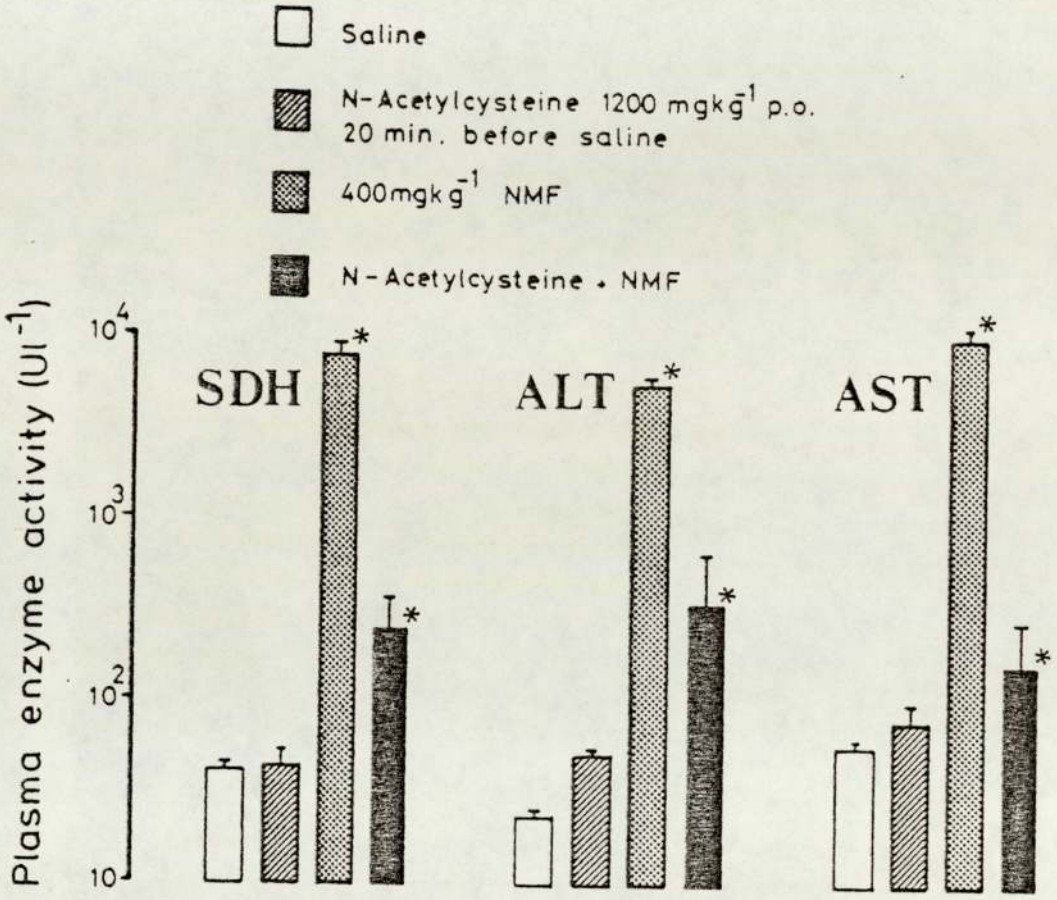


Figure 4.56 Effect of N-acetylcysteine (1200mgkg^{-1}) pretreatment upon NMF induced elevation of SDH, ALT and AST in the plasma of Balb/C mice 24h after a single i.p. dose of 400mgkg^{-1} NMF (mean ± s.e. mean; n = 6) *significantly different (Mann-Whitney U-test) at $P<0.05$

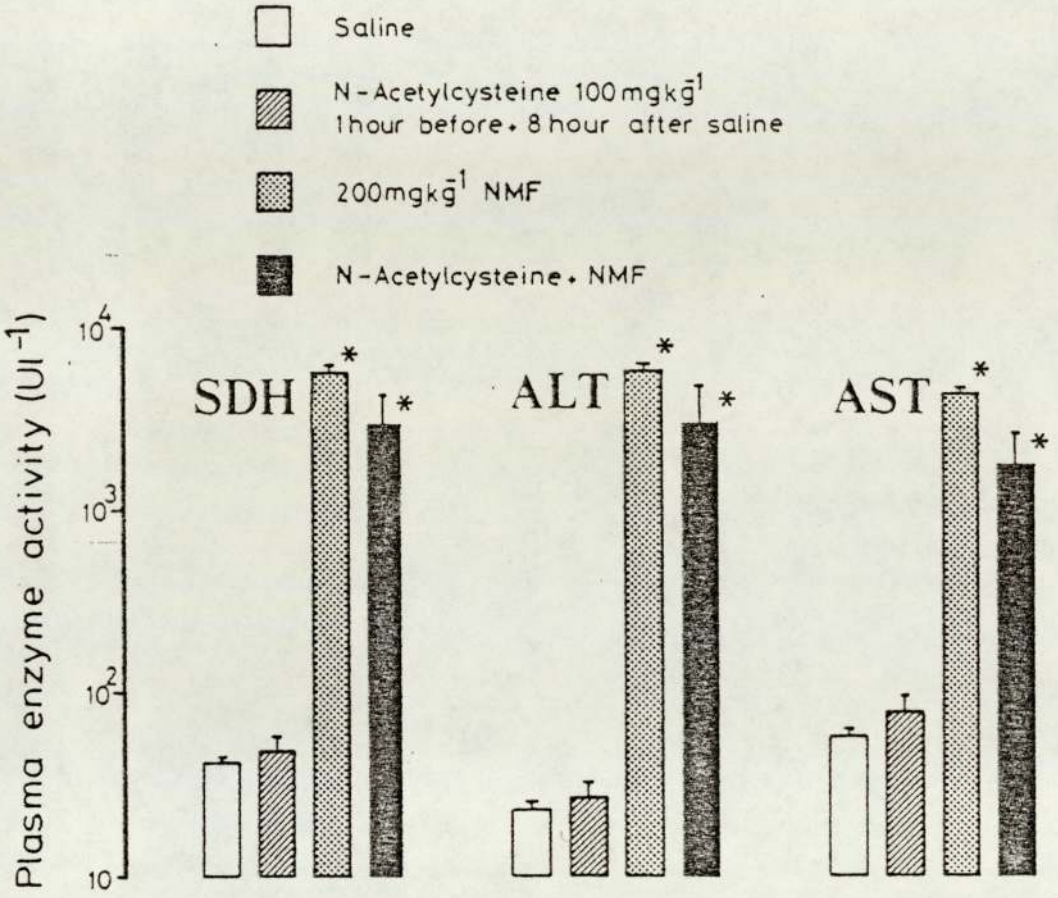


Figure 4.57 Effect of N-acetylcysteine ($100mgkg^{-1}$) pretreatment upon NMF induced elevation of SDH, ALT and AST in the plasma of Balb/C mice 24h after a single i.p. dose of $200mgkg^{-1}$ NMF. (mean ± s.e. mean; n>6) *significantly different from control (Mann-Whitney U-test) at $P<0.001$

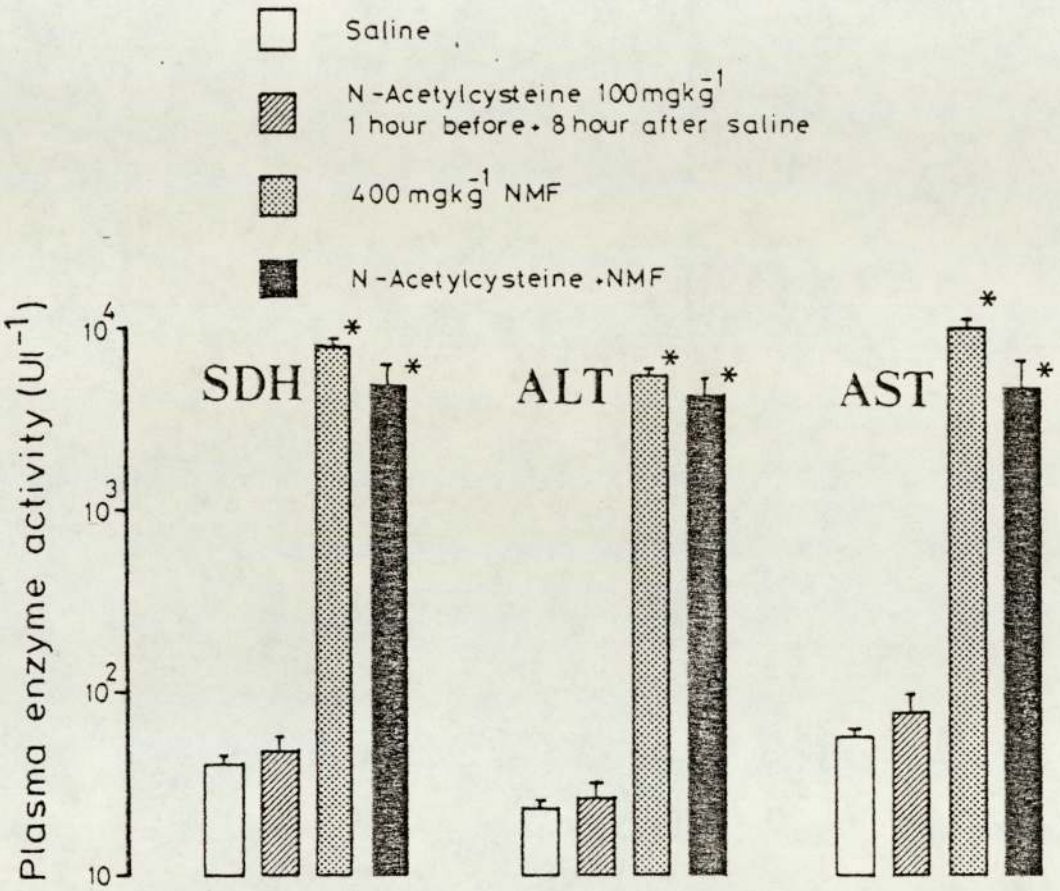


Figure 4.58 Effect of N-acetylcysteine ($100mgkg^{-1}$) pretreatment upon NMF induced elevation of SDH, ALT and AST in the plasma of Balb/C mice 24h after a single i.p. dose of $400mgkg^{-1}$ NMF (mean ± s.e. mean; $n \geq 6$) *significantly different from control (Mann-Whitney U-test) at $P<0.001$

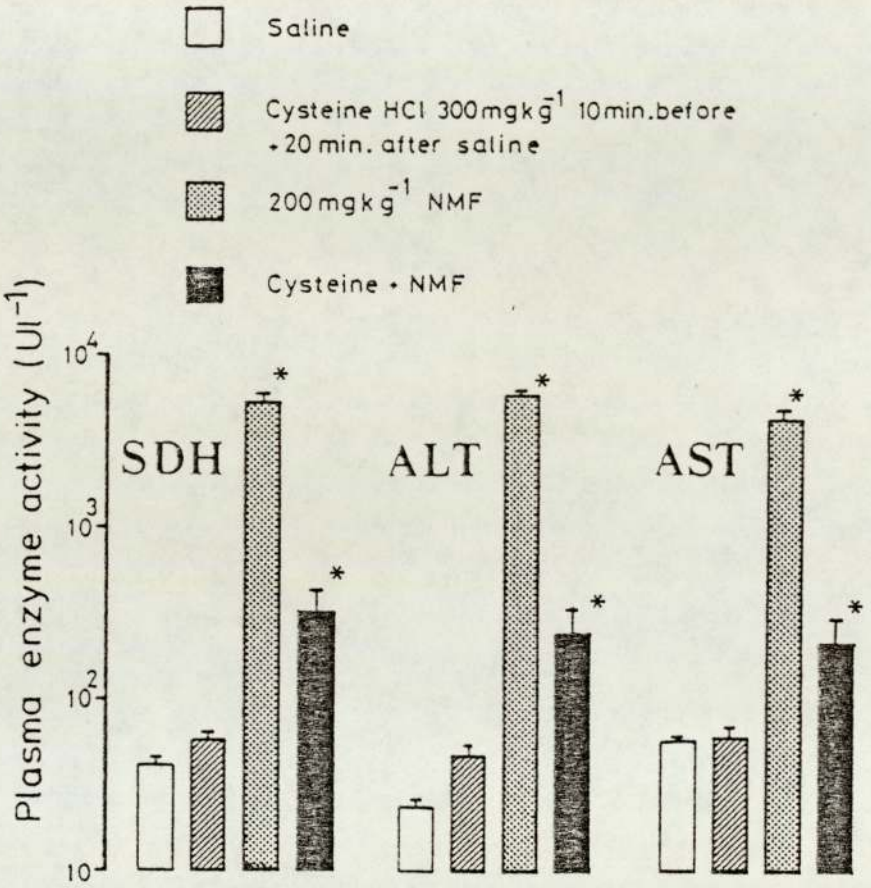


Figure 4.59 Effect of Cysteine pretreatment upon NMF induced elevation of SDH, ALT and AST in the plasma of Balb/C mice 24h after a single i.p. dose of 200mgkg^{-1} NMF (mean ± s.e. mean; n>6) *significantly different (Mann-Whitney U-test) at P<0.05

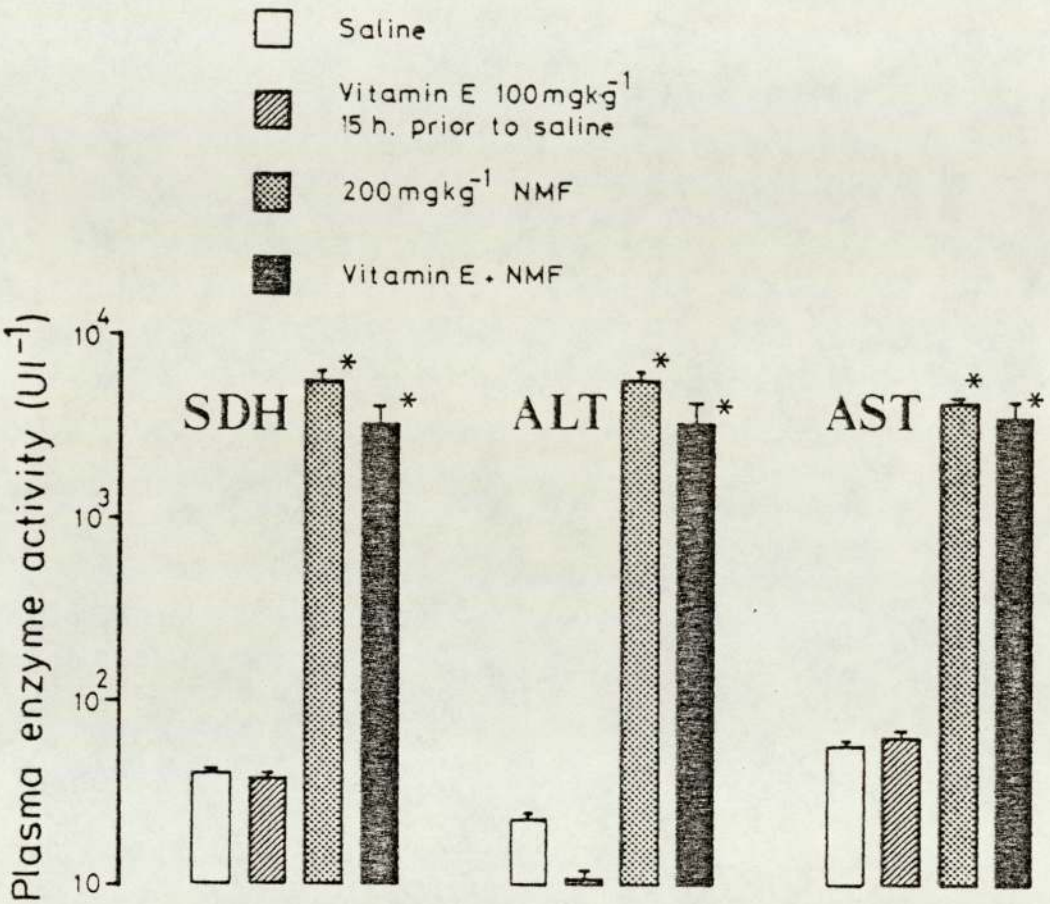


Figure 4.60 Effect of Vitamin E pretreatment upon NMF induced elevation of SDH, ALT and AST in the plasma of Balb/C mice 24h after a single i.p. dose of 200mgkg^{-1} NMF (mean ± s.e. mean; $n \geq 6$) *significantly different from control (Mann-Whitney U-test) at $P<0.001$

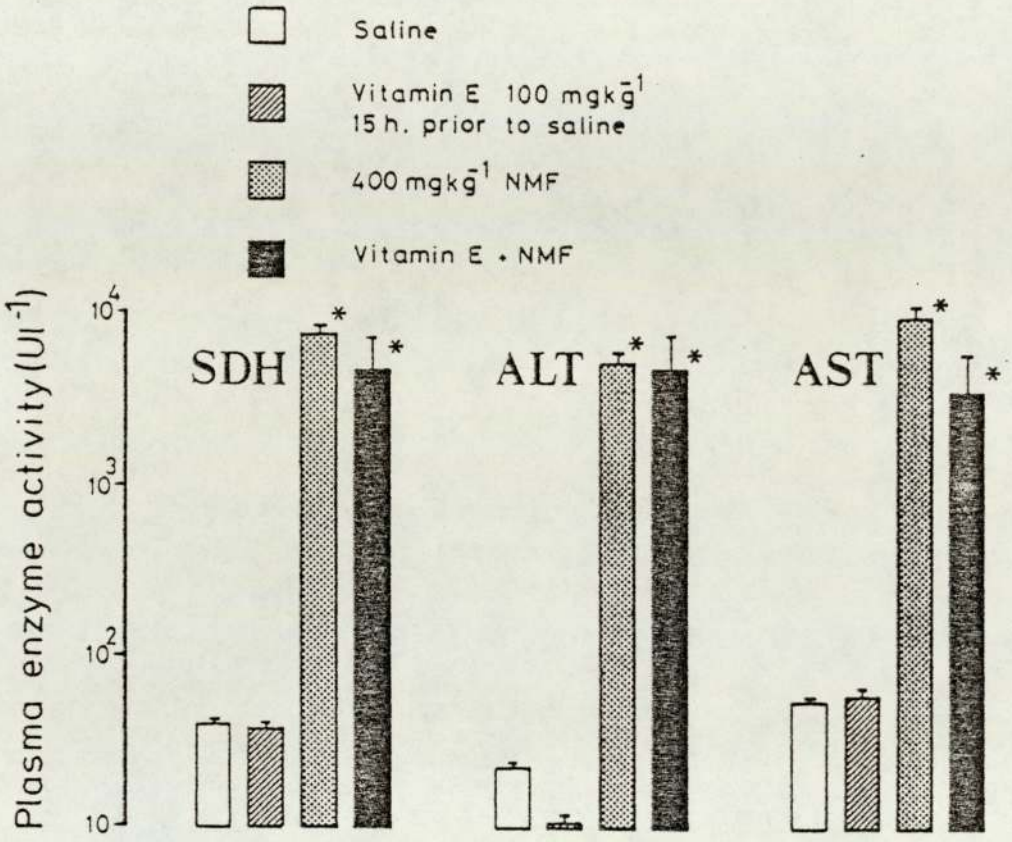


Figure 4.61 Effect of Vitamin E pretreatment upon NMF induced elevation of SDH, ALT and AST in the plasma of Balb/C mice 24h after a single i.p. dose of 400mgkg^{-1} NMF (mean ± s.e. mean; n≥6) *significantly different from control (Mann-Whitney U-test) at P<0.001

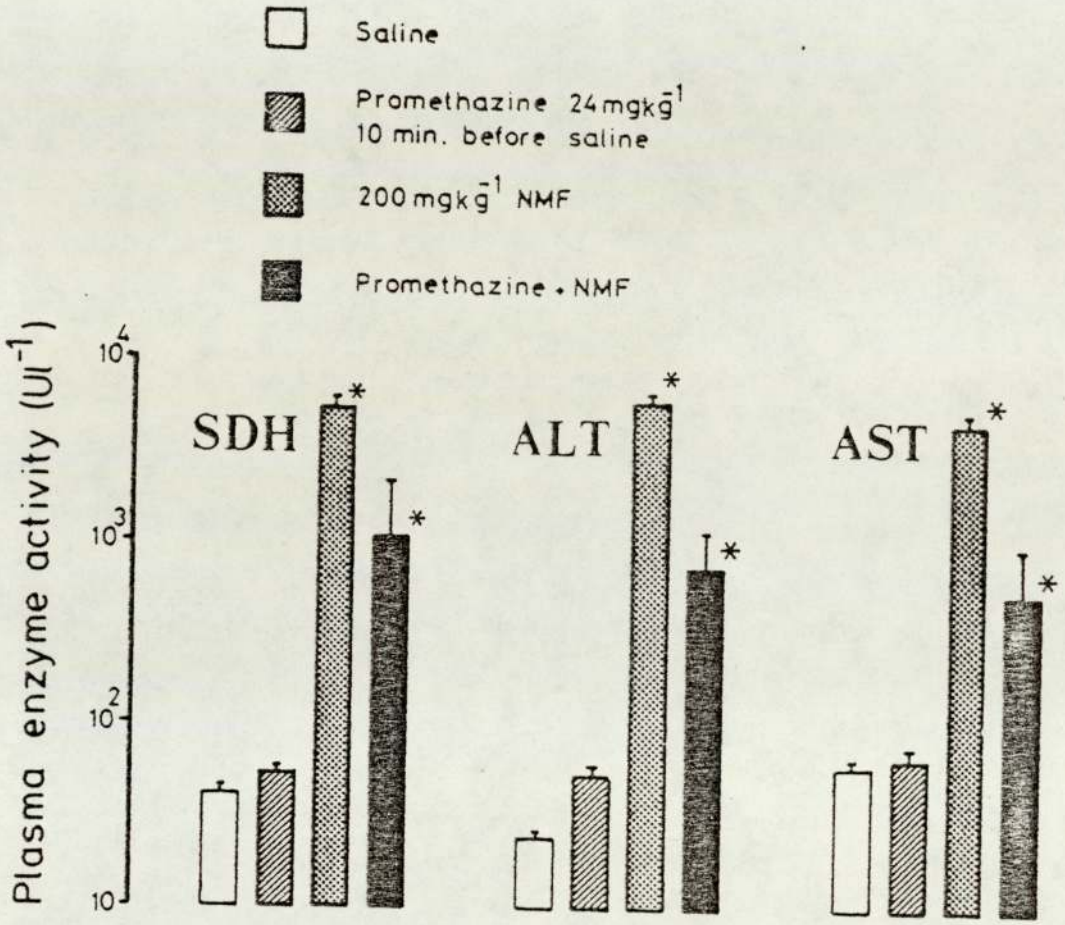


Figure 4.62 Effect of promethazine pretreatment upon NMF induced elevation of SDH, ALT and AST in the plasma of Balb/C mice 24h after a single i.p. dose of 200mgkg^{-1} NMF (mean ± s.e. mean; n≥6) *significantly different (Mann-Whitney U-test) at $P < 0.05$

NMF) completely protected against the hepatotoxicity caused by NMF at 200mgkg^{-1}, and partially against that caused by 400mgkg^{-1} (Mann-Whitney U-test, $P<0.05$) (Figure 4.55 and 4.56). The criteria for selection dosage of the thiol compounds used in this section has previously been described (Section 4.5). NAC at a much lower dose of 100mgkg^{-1} administered 1h before and 8h after NMF failed to protect against the hepatotoxicity of NMF (Figure 4.57 and 4.58). Also cysteine HCl (300mgkg^{-1}) administered to Balb/C mice 10 min before and 20 min after NMF protected against the hepatotoxicity elicited by 200mgkg^{-1} NMF (Mann-Whitney U-test, $P<0.05$) (Figure 4.59).

In addition to the early deaths caused by NMF after pretreatment of mice with BSO, some early deaths were also recorded in other treatment groups. Of the mice administered 200mgkg^{-1} NMF 6 out of a total of 24 mice were killed between 12 and 24h. In the case of 400mgkg^{-1} 1 out of a total of 6 mice was killed between 12 and 24h. When mice pretreated with SKF525A were administered 400mgkg^{-1} NMF, the mice were morbid within 12h of NMF administration and were humanely killed at this time. All mice that were killed within 24h displayed gross liver changes, and had elevated levels of liver enzymes in their plasma.

The radical scavenger Vitamin E (100mgkg^{-1} administered 15h prior to NMF) failed to protect against the hepatotoxicity of either 200 or 400mgkg^{-1} NMF (Figure 4.60 and 4.61). However, promethazine (24mgkg^{-1}) protected partially against the hepatotoxicity of 200mgkg^{-1} NMF (Mann-Whitney U-test, $P<0.05$) (Figure 4.62).

Histopathological examination of liver sections was undertaken by Dr Iona Pratt of University College Dublin, and the findings are presented in Tables 4.8, 4.9a and 4.9b. The division of

the results into each table is based upon the chronological order in which the study was undertaken.

The dominant features of the liver damaged caused by NMF consisted of an extensive haemorrhagic necrosis with inflammatory (neutrophil) response which was predominantly centrilobular in origin and occurred more frequently in subcapsular regions of the liver, indicating that oxygen tension within the liver lobe could be an important factor which contributed to the development of liver damage. This observation was supported by the patchy effect of the necrosis, some lobes being severely affected and others from the same animal showing little change.

The most severely affected animals in the study were those which had received 400mgkg^{-1} NMF and those pretreated with SKF525A prior to administration of 400mgkg^{-1} NMF. The latter group appeared to show marginally more necrosis than the former.

In some animals a variant of the basic necrotic lesion was a severe haemorrhagic lesion without overt cell necrosis, characterized by marked pooling of blood in centrilobular sinusoids, with haemosiderin deposits in sinusoids and hepatocytes. In some animals the whole liver lobule displayed pooling of blood. The haemorrhagic lesion was particularly dominant in animals pretreated with buthionine sulphoximine prior to administration of 100mgkg^{-1} NMF. However in some groups eg. those mice which received 200mgkg^{-1} NMF, some animals showed the haemorrhagic change while others showed necrosis, therefore the two may not be distinctly different in terms of pathnogenesis.

Degeneration of centrilobular hepatocytes was evident in a number of groups (eg. those mice that received 200mgkg^{-1} NMF alone)

without extensive necrosis. The cells were pale, swollen and vacuolated, and in some groups hydropic degeneration (cloudy swelling) was seen.

Eosinophilia and possible hypertrophy of centrilobular hepatocytes was seen in groups which had received 200mgkg^{-1} NMF alone, and also in some groups which displayed enzymatic evidence of protection eg. those pretreated with cysteine or 1200mgkg^{-1} NAC. This change was not seen in control groups and appears to be characteristic of NMF treatment.

Fatty changes in periportal hepatocytes were also evident in animals displaying some necrotic or haemorrhagic changes (see Table 4.9a and 4.9b). In some livers fatty changes could not be distinguished from glycogen accumulation, which can only be elucidated by special stains (PAS for glycogen and Oil Red O for fat on frozen sections).

Accumulation of glycogen particularly in periportal hepatocytes was seen in a number of groups (see Tables 4.8 and 4.9). The marked glycogen accumulation may indicate an inhibitory effect upon oxidative metabolism or glycogenolysis or stimulation of glyconeogenesis. The latter effect could be corticosteroid mediated and stress related. It is interesting to note that periportal glycogen accumulation was apparent after administration of 100mgkg^{-1} NMF, a dose which did not cause a rise in SDH, ALT or AST in the plasma, this manifestation may represent the first pathological change associated with NMF hepatotoxicity. Pretreatment with phenobarbitone prior to administration of 100mgkg^{-1}, prevented the glycogen accumulation (Table 4.8).

HISTOPATHOLOGICAL FINDING / TREATMENT GROUP			Saline	BSO + 100mgkg^{-1} NMF	BSO + Saline	100mgkg^{-1} NMF	400mgkg^{-1} NMF	SKF525A 400mgkg^{-1} NMF	Phenobarbitone + Saline	SKF525A + Saline	Phenobarbitone + 100mgkg^{-1} NMF	Vitamin E + 400mgkg^{-1} NMF	Vitamin E + Saline
NUMBER OF MICE			6	6	6	6	6	6	6	6	6	6	6
LIVER NORMAL			5	0	6	0	0	0	6	5	6	0	6
CENTRILOBULAR NECROSIS	mild	Grade 1+	0	0	0	0	0	1	0	0	0	2	0
		Grade 2+	0	0	0	0	1	1	0	0	0	0	0
	moderate	Grade 3+	0	0	0	0	4	2	0	0	0	1	0
		Grade 4+	0	0	0	0	1	1	0	0	0	1	0
	severe	Grade 5+	0	0	0	0	0	1	0	0	0	0	0
POOLING OF BLOOD IN SINUSOIDS	mild	Grade 1+	0	0	0	0	1	1	0	0	0	1	0
		Grade 2+	0	1	0	0	5	1	0	0	0	0	0
	moderate	Grade 3+	0	5	0	0	0	3	0	0	0	1	0
		Grade 4+	0	0	0	0	0	1	0	0	0	0	0
	severe	Grade 5+	0	0	0	0	0	0	0	0	0	1	0
DEPOSITION OF PIGMENT IN HEPATOCYTES & SINUSOIDS		Grade 1+	0	1	0	0	4	3	0	0	0	0	0
		Grade 2+	0	3	0	0	1	0	0	0	0	0	0
		Grade 3+	0	2	0	0	0	3	0	0	0	2	0
ACCUMULATION OF GLYCOGEN IN PERIPORTAL CELLS		Grade 1+	1	0	0	0	1	1	0	1	0	0	0
		Grade 2+	0	0	0	5	2	4	0	0	0	3	0
		Grade 3+	0	0	0	1	1	1	0	0	0	0	0
VACUOLATION OF PERIPORTAL CELLS		Grade 1+	0	0	0	0	1	1	0	0	0	2	0
		Grade 2+	0	5	0	0	0	0	0	0	0	0	0
		Grade 3+	0	1	0	0	0	0	0	0	0	0	0
POSSIBLE FOCAL CALCIFICATION			0	0	0	0	2	1	0	0	0	0	0

Table 4.8 Histopathological findings in mouse livers from N-methylformamide hepatotoxicity study

HISTOPATHOLOGICAL FINDING / TREATMENT GROUP		Saline	BSO + $100mgkg^{-1}$ NMF	NAC $100mgkg^{-1}$ + $400mgkg^{-1}$ NMF	NAC $100mgkg^{-1}$ + Saline	Vitamine E + $200mgkg^{-1}$ NMF	$200mgkg^{-1}$ NMF	NAC $100mgkg^{-1}$ + $200mgkg^{-1}$ NMF	Cysteine + $200mgkg^{-1}$ NMF	Cysteine + Saline
NUMBER OF MICE		6	3	6	6	19	19	12	9	6
LIVER NORMAL		3	0	0	6	0	0	0	0	0
DIFFUSE GLYCOGEN ACCUMULATION	mild	2	0	0	0	1	0	0	1	2
	moderate	0	0	0	0	0	1	0	0	4
PERIPORTAL GLYCOGEN ACCUMULATION	mild	0	0	0	0	2	0	0	0	0
	moderate	0	0	1	0	0	2 } fat ?	4	1	0
	severe	0	0	4	0	0	2 } fat ?	1	0	0
EOSINOPHILIA AND HYPERTROPHY OF CENTRILOBULAR CELLS	mild	0	1	0	0	0	0	0	1	0
	moderate	0	0	0	0	4	6	2	3	0
DEGENERATIVE CHANGE IN CENTRILOBULAR CELLS WITHOUT APPRECIABLE NECROSIS	mild	0	0	1	0	0	2	1	1	0
	moderate	0	0	0	0	0	0	0	0	0
CENTRILOBULAR NECROSIS WITH INFLAMMATORY CHANGE	mild	1 (focal)	0	0	0	1	1 (focal)	1 (focal)	0	0
	moderate	0	0	4	0	11	5	1	5	0
	severe	0	0	1	0	3	4	3	0	0
MEAN GRADING/ANIMAL FOR CENTRILOBULAR NECROSIS		0	0	2.2	0	1.8	1.6	1.3	1.7	0
BLOOD POOLING AND HAEMORRHAGE IN CENTRILOBULAR SINUSOIDS, WITHOUT APPRECIABLE NECROSIS	mild	0	0	0	0	0	0	0	0	0
	moderate	0	1	0	0	0	0	0	0	0
	severe	0	1	0	0	1	4	2	0	0
MEAN GRADING/ANIMAL FOR CENTRILOBULAR BLOOD POOLING		0	2.3	0	0	0.2	1.0	0.7	0	0
FATTY DEGENERATION OR PERIPORTAL CELLS	mild	0	0	0	0	1	1	0	0	0
	moderate	0	0	0	0	2	4	0	0	0
	severe	0	0	0	0	4	2	3	0	0
VACUOLATION OF PERIPORTAL CELLS (NON-LIPID)	mild	0	0	0	0	0	0	0	0	0
	moderate	0	2	0	0	2	1	2	0	0
GALL BLADDER	normal	1(1)	-	-	-	2 (3)	1 (2)	1 (1)	0 (1)	-
	haemorrhagic and necrotic					1 (3)	1 (2)	0 (1)	1 (1)	-

Table 4.9a Histopathological findings in mouse livers from N-methylformamide hepatotoxicity study

HISTOPATHOLOGICAL FINDING / TREATMENT GROUP		Promethazine + 200mgkg^{-1} NMF	Promethazine + Saline	CBA/CA mice + 400mgkg^{-1} NMF	CBA/CA mice + Saline	Methimazole + 200mgkg^{-1} NMF	CBA/CA mice + 200mgkg^{-1} NMF	NAC 1200mgkg^{-1} + Saline	NAC 1200mgkg^{-1} + 200mgkg^{-1} NMF	NAC 1200mgkg^{-1} + 400 mgkg^{-1} NMF
NUMBER OF MICE		5	6	4	6	4	6	6	10	5
LIVER NORMAL		0	1	1	0	1	0	3	2	0
DIFFUSE GLYCOGEN ACCUMULATION	mild	0	3	0	0	0	5	2	1	0
	moderate	0	1	0	2	0	0	0	0	0
PERIPORTAL GLYCOGEN ACCUMULATION	mild	0	1	0	4	0	1	0	4	1
	moderate	4	0	3	0	0	0	0	0	2
	severe	0	0	0	0	0	0	0	0	0
EOSINOPHILIA AND HYPERTROPHY OF CENTRILOBULAR CELLS	mild	0	0	0	0	0	0	0	0	0
	moderate	0	0	0	0	0	0	0	5	0
DEGENERATIVE CHANGE IN CENTRILOBULAR CELLS WITHOUT APPRECIABLE NECROSIS	mild	0	0	0	0	1	0	0	0	2
	moderate	0	0	0	0	0	0	0	0	1
CENTRILOBULAR NECROSIS WITH INFLAMMATORY CHANGE	mild	2	0	2 (focal)	3 (focal)	0	0	1 (focal)	1 (focal)	0
	moderate	1	0	1	0	1	0	0	0	1
	severe	0	0	0	0	1	0	0	0	0
MEAN GRADING/ANIMAL FOR CENTRILOBULAR NECROSIS		1.0	0	1.0	0.2	1.5	0	0.1	0.1	0.4
BLOOD POOLING AND HAEMORRHAGE IN CENTRILOBULAR SINUSOIDS, WITHOUT APPRECIABLE NECROSIS	mild	0	0	0	0	0	0	0	0	0
	moderate	0	0	0	0	0	0	0	0	0
	severe	0	0	0	0	0	0	0	0	0
MEAN GRADING/ANIMAL FOR CENTRILOBULAR BLOOD POOLING		0	0	0	0	0	0	0	0	0
FATTY DEGENERATION OR PERIPORTAL CELLS	mild	0	0	0	0	0	0	0	0	0
	moderate	0	0	0	0	0	0	0	0	0
	severe	0	0	0	0	0	0	0	0	0
VACUOLATION OF PERIPORTAL CELLS (NON-LIPID)	mild	0	0	0	0	0	0	0	0	0
	moderate	0	0	0	0	0	0	0	0	0
GALL BLADDER	normal	-	-	-	-	-	-	-	-	-
	haemorrhagic and necrotic	-	-	-	-	-	-	-	-	-

Table 4.9b Histopathological findings in mouse livers from N-methylformamide hepatotoxicity study

The gall bladder was only sectioned in a small number of animals (see Table 4.9, total number in parentheses). This organ showed similar changes to liver parenchyma in NMF-treated animals, with mucosal necrosis and marked pooling of blood and submucosal haemorrhage, but again the incidence of damage was patchy. This may indicate that a toxic metabolite of NMF is undergoing bilary excretion.

The marked strain difference in susceptibility to NMF hepatotoxicity shown by differences in elevation of hepatic enzymes was also obvious on histopathological examination, CBA/CA mice showed much less liver damage after 400mgkg^{-1} and 200mgkg^{-1} NMF than did Balb/C mice.

Finally almost complete protection against the histopathological manifestations of NMF hepatotoxicity was achieved by pretreatment with N-acetylcysteine 1200mgkg^{-1} (Table 4.9b).

4.10.3 Discussion

The principle features of the livers damaged by NMF in this study were: 1, necrosis, frequently extensive, of centrilobular hepatocytes; 2, marked pooling of blood in sinusoids, in centrilobular areas of the liver; 3, deposition of pigments in centrilobular hepatocytes and sinusoids; and 4. some vacuolation of periportal hepatocytes and effects upon glycogen accumulation in these cells.

Despite the considerable liver damage seen in this study, there was no evidence of liver regeneration within 24h after NMF administration. In the rat, following carbontetrachloride intoxication, activities of the DNA synthesising enzymes thymidylate synthetase and thymidine kinase increased in the liver (193) between

48 and 72 hours after carbontetrachloride administration. In analogy regeneration in the livers of mice treated with NMF may not be apparent within 24 hours. It has been reported that mice which received NMF daily for 21 days showed an increase in the presence of mitotic cells in the liver indicating regeneration (6).

The most striking finding of the present study was the ability of N-acetylcysteine ($1200mgkg^{-1}$) to protect against the hepatotoxicity of NMF. The need for a large dose of N-acetylcysteine is apparent, as it was not possible to protect with a low dose of $100mgkg^{-1}$. This requirement for high doses of N-acetylcysteine has been established for paracetamol (73). At a dose of $200mgkg^{-1}$ NMF, N-acetylcysteine ($1200mgkg^{-1}$) afforded complete protection against the elevation of SDH, ALT and AST in the plasma. This was supported by the histopathological finding that no necrosis was apparent and the only disturbance seen was a mild periportal glycogen accumulation (Table 4.9b)

Pretreatment with cysteine partially protected against the hepatotoxicity of NMF, however there was some evidence of centrilobular necrosis (Table 4.8). It is worth noting that cysteine and N-acetylcysteine pretreatments that protected against the hepatotoxicity also prevented the NMF induced depletion of hepatic GSH (Section 4.5), which suggests a protective role for GSH, or thiol containing compounds in general, in NMF hepatotoxicity. This role for GSH was further evaluated by depleting hepatic GSH with BSO to 18.6% of control values prior to administration of $100mgkg^{-1}$ NMF. Within 6 to 8 hours of this treatment the mice were so sick that it was necessary to terminate the experiments, and all animals displayed biochemical and histopathological evidence of liver cell dysfunction.

Therefore, by depleting GSH, a non-hepatotoxic dose of NMF became toxic and the pattern of liver damage also changed from one of extensive centrilobular necrosis to an extensive haemorrhagic lesion. The absence of necrosis may be due to the early time of death of the animals at 6 to 8 hours after NMF administration. In previous reports histopathological evidence for NMF induced liver damage was not apparent until 24h after NMF administration (26, 181).

Pretreatment with SKF525A did not ameliorate the severity of hepatic necrosis, on the contrary it exacerbated the toxicity, a finding which supports the contention that NMF is activated by a pathway independent of cytochrome P-450 (see Section 4.9). This is further substantiated by the finding that pretreatment with phenobarbitone did not exacerbate the toxicity, but reduced the periportal glycogen accumulation, which was seen as the first indication of liver dysfunction induced by a dose of $100 mgkg^{-1}$ NMF.

The radical scavengers promethazine and Vitamin E produced contrasting effects. Promethazine protected against NMF induced depletion of GSH (Section 4.5), partially reduced the elevated activities of SDH, ALT and AST in the plasma of mice which had received NMF, and afforded some protection against damage as evidenced by histopathological examination. Vitamin E was unable to protect against either the GSH depletion or the hepatotoxicity. The different effects produced by these two agents may suggest that promethazine affords protection in a way unrelated to its radical scavenging ability. Alternatively the lipophilic properties of vitamin E may dictate an intracellular location, inacessable to the highly water soluble metabolites of NMF.

A previous observation concerning the strain differences in the hepatotoxicity of NMF in mice (21) has been confirmed in this study. The order of suseptibility of the different strains towards NMF-induced hepatotoxicity is Balb/C > CBA/CA >> BDF_1, the difference appears to reflect the ability of certain strains to metabolize NMF (Section 4.1).

Pretreatments which were found to alter the toxicity, also altered the extent of the covalent binding of NMF metabolites to microsomal protein in vitro (Section 4.9). This relationship will be discussed in detail in Section 5.

SECTION 5

GENERAL DISCUSSION

The findings reported in this thesis attempt to elucidate the mechanisms by which N-methylformamide causes centrilobular necrosis in the liver. In Section 4, evidence was provided that NMF is metabolically activated to a reactive species, which appears to be able to deplete hepatic GSH, and which becomes irreversibly associated with hepatic macromolecules. The inter-relationship between hepatic GSH depletion (Section 4.4), covalent binding (Section 4.7), and the hepatotoxicity of NMF (Section 4.10) suggests that the hepatotoxicity of NMF is mediated via generation of a reactive, necrogenic species. In this section the overall conclusions that arise from the results will be discussed together with ideas for further work.

NMF depleted hepatic GSH in a dose related manner (Section 4.4), which was specific to NMF when compared to a range of other non-hepatotoxic formamide derivatives (4). The marked depletion of hepatic GSH, to 20.9% of control values 2 hours after a single i.p dose of 200mgkg^{-1} NMF, suggests that GSH plays a protective role in detoxifying the reactive species responsible for the hepatotoxicity of NMF. In strains of mice less susceptible to NMF induced hepatotoxicity than Balb/C mice, GSH was not depleted to less than 50% of control values. Raised amounts of urinary thioethers were excreted by Balb/C mice administered NMF, this occurred in a dose related manner, which was again specific to NMF when compared with other formamides. Significant amounts of these thioethers were excreted in the urine prior to the onset of toxicity (Section 4.2). A metabolite of NMF of thioether structure has been identified as S-(N-methylcarbamoyl)-N-acetyl-L-cysteine (see Figure 5.1; Kestell, Threadgill, Gescher and Gledhill, manuscript in preparation). This is presumably the mercapturate formed following the reaction of hepatic

GSH with the reactive metabolite of NMF.

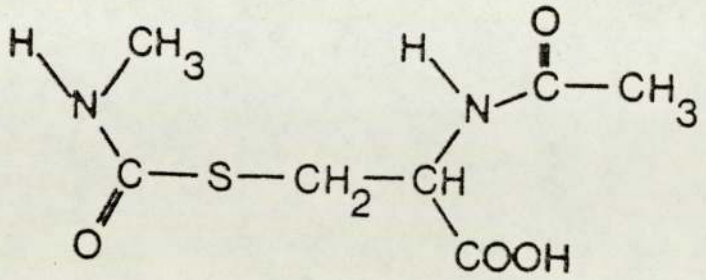


Figure 5.1 Structure of S-(N-methylcarbamoyl)-N-acetyl-L-cysteine

The role of GSH in the hepatotoxicity of NMF was further emphasised by results emanating from experiments in which GSH levels were changed prior to administration of NMF (Section 4.10). Pretreatment of mice with N-acetylcysteine maintained hepatic GSH levels, following NMF administration, at control values, and protected against the hepatotoxicity of NMF. It was also possible to protect partially against the GSH depletion and hepatotoxicity of NMF with cysteine. Conversely pretreatment with BSO to lower hepatic GSH levels prior to NMF administration lowered the hepatotoxic dose threshold of NMF in Balb/C mice to below $100 mgkg^{-1}$.

Mitchell and his co-workers (194) have recently classified compounds that cause tissue injury into several categories according to the chemical nature of their reactive metabolites, and their biological interactions with GSH. In this classification, metabolites of Class B compounds (eg. paracetamol and bromobenzene) are designated as electrophilic species, which are conjugated with GSH *in vivo*. Toxicity caused by these metabolites is potentiated by prior depletion of hepatic glutathione levels. The occurrence of raised thioether levels in the urine of mice treated with NMF, and the potentiation of

NMF hepatotoxicity by BSO, clearly places NMF in Class B together with paracetamol and bromobenzene. The reactive metabolites of compounds in this class also react covalently with proteins, and the toxicity of these compounds may be ameliorated by prior treatment of animals with GSH precursors. The observation that NMF underwent metabolic activation to a covalently bound species by mouse liver microsomes demonstrates the ability of NMF metabolites to react with proteins.

It is clear that NMF is extensively metabolised *in vivo* to methylamine and CO_2 (Section 4.1). However, using chromatographic techniques, it has hitherto proved impossible to demonstrate *in vitro* metabolism of NMF with isolated hepatocytes or liver fractions.

Measurement of the covalent binding of ^{14}C-NMF metabolites to microsomal protein has been shown to be an indicator of NMF metabolism *in vitro* (Section 4.9). The NADPH dependent metabolic activation of NMF to a covalently bound species by mouse liver microsomes, provides definitive evidence that metabolite(s) of NMF bind irreversibly to hepatic proteins. The ability of GSH to protect against the covalent binding of NMF to liver microsomes, and to alleviate NMF hepatotoxicity suggests that GSH protects against NMF induced hepatotoxicity by detoxifying the microsomally generated reactive species.

Further correlations between *in vivo* toxicity and *in vitro* covalent binding were established when the role of metabolic activation in the covalent binding was investigated by pretreating mice with SKF 525A or phenobarbitone. Inhibition of cytochrome P-450, with SKF 525A, failed to alleviate the hepatotoxicity of NMF or abolish the covalent binding of NMF metabolites to microsomal protein.

On the contrary, SKF 525A increased the covalent binding of OH^{14}C-NMF to microsomal protein (Section 4.9) and increased the severity of centrilobular necrosis caused by NMF (Section 4.10). Conversely phenobarbitone prevented the periportal glycogen accumulation, the first pathological change caused by NMF, in the livers of mice which had received 100mgkg^{-1} NMF, but did not change the covalent binding of ^{14}C-NMF to microsomes *in vitro*. These findings, whilst not an exhaustive characterization of the role of cytochrome P-450, are in accordance with the suggestion that cytochrome P-450 is not involved in the activation of NMF to a necrogenic species.

During the search for an NADPH dependent, SKF 525A insensitive enzyme responsible for NMF metabolism, attention was focused upon the flavin containing monooxygenases. But again it was not possible to modify the toxicity of NMF or the *in vitro* covalent binding by inactivation or inhibition of the flavin containing monooxygenase.

Mitchell et al (194) in his classification of reactive species also describes a further group of agents, in Class C, under the heading "organic free radicals" (eg. carbontetrachloride and halothane). Compounds in this category may alkylate proteins and initiate peroxidative damage of lipids. The role of lipid peroxidation in the hepatotoxicity of carbontetrachloride is well established. Previous investigations, carried out at Aston University, into the biochemical mechanisms of NMF induced hepatotoxicity resulted in the observation of raised levels of breakdown products of lipid peroxides in the livers of mice which had received NMF (Section 1.2). However, it was not possible to establish a causal relationship between lipid peroxidation and NMF

hepatotoxicity.

Promethazine has been shown to protect against carbontetrachloride hepatotoxicity (138), and also protected against NMF induced GSH depletion and hepatotoxicity (see Section 4.5 and 4.10). It is surprising that promethazine protected against NMF hepatotoxicity, as the toxicity of class C agents appears not to be enhanced by prior depletion of GSH, or prevented by pretreatment with thiol precursors. It is therefore likely that promethazine may protect against the toxicity by an effect upon NMF metabolism other than that involving its ability to scavenge free radicals.

Administration of ^{14}C-NMF resulted in irreversible association of radioactivity with hepatic macromolecules. In Section 1.5., the role of covalent binding in drug toxicity was discussed and the need for caution in interpretation of the results has been stressed. In the case of NMF this need has to be emphasised as radioactivity derived from ^{14}C-NMF may be incorporated into hepatic proteins, during denovo protein synthesis, as well as becoming covalently bound. However, these are 3 pieces of evidence which suggest that the toxicity of NMF may be mediated by the covalent binding of NMF to hepatic macromolecules <u>in vivo</u>. Firstly, in strains of mice which are less susceptible to NMF hepatotoxicity (Section 4.10) (21) the extent of association of both OH^{14}C-NMF and $^{14}CH_3$-NMF with hepatic macromolecules was lower than that observed in the particularly susceptible Balb/C mouse (Section 4.8). In the less susceptible CBA/CA strain NMF is metabolised less extensively (Section 4.1) and GSH is depleted to a lesser degree. Secondly, in the kidney, apparently not a target organ, the maximum level of binding after a

hepatotoxic dose was below that observed in the liver after an innocuous dose of NMF (Section 4.8). Thirdly, similarities appear to exist between the pattern of binding *in vitro* and the pattern of binding *in vivo*, eg. SKF 525A increased the association of $OH^{14}C$-NMF with hepatic protein both *in vivo* and *in vitro*.

To understand the importance of the covalent binding of NMF metabolites for toxicity, it is necessary to characterize the nature of the covalently bound adduct to unequivocally distinguish between covalent binding and incorporation. For a number of hepatotoxins, adducts formed with liver proteins *in vivo* have been isolated and characterized. Thioacetamide after metabolic activation via thioacetamide-S-oxide reacts with lysine residues in cytosolic proteins to form an N-ϵ-acetyllysine adduct (195). Also, the protein adduct formed between NAPQI, a reactive intermediate of paracetamol, and the cysteine residues of protein has recently been characterised (T.A. Baillie, personal communication). However, despite identification of the adduct, it may still not be possible to draw any conclusions as to how a particular adduct contributes to the hepatotoxicity of a compound. An interesting approach has been used to study the role of DNA methylation in dimethylnitrosamine carcinogenesis. The organ specificity of the carcinogen dimethylnitrosamine changes according to the dose schedule; kidney tumours are induced if DMN is given in a high toxic dose either singly or over a short period, whereas liver is the target organ upon chronic administration of dimethylnitrosamine. By measuring the accumulation of O^6-Methylguanine in target tissues it was possible to assess the relative contribution of this adduct to dimethylnitrosamine hepatocarcinogenesis during chronic dosing (196). In a similar

approach it may be possible to correlate changes in NMF hepatotoxicity with the appearance of specific tissue adducts.

The recent identification of S-(N-methylcarbamoyl) - N-acetylcysteine as a major urinary metabolite of NMF suggested that the reactive species generated from NMF may be a compound such as methylisocyanate (Figure 5.2).

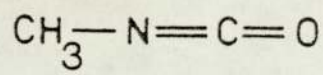


Figure 5.2 Structure of methylisocyanate

In view of this finding, it is possible to speculate upon the likely nature of target proteins to which this reactive species may bind. The reactivity of isocyanates is well established, and isocyanates have been implicated in the cytotoxicity of the antitumour nitrosoureas (197). Inactivation of yeast alcohol dehydrogenase has been reported, following the carbamoylation of sulphur groups on this enzyme by butylisocyanate (198). Also, the enzyme glutathione reductase is inactivated by chloroethylisocyanate derived from bis (2-chroroethyl) nitrosourea (199). If methylisocyanate is the reactive species generated from NMF, the ability of isocyanates to react with thiol groups may account for the marked depletion of hepatic GSH observed after NMF administration (Section 4.4). However, definitive proof of the nature of the reactive species will be difficult to

obtain.

To summarize, the work described in this thesis provides evidence for the contention that NMF is metabolised to a reactive species which binds to microsomal protein. The ability of GSH to protect against the toxicity *in vivo* and covalent binding *in vitro* implicate GSH in a protective role, and suggest that NMF may be grouped together with compound such as paracetamol and bromobenzene in class B of Mitchell's classification (194) of compounds that cause tissue damage. To unequivocally establish a role for covalent binding *in vivo* in the hepatotoxicity of NMF it might be helpful to identify the nature of the adduct formed *in vivo* and the function of possible target proteins.

APPENDIX I

Tabulated results from

mass balance study

MASS BALANCE STUDY
CUMULATIVE EXCRETION OF 14C-NMF (as a percentage of the dose administered)
Excretion of 100 ag/kg OH14C-NMF by Balb/C mice

Animal Dose of NMF Dose of 14C-NMF		A 115 ag/kg 11.67 µCi	B 75 ag/kg 7.61 µCi	C 110 ag/kg 11.93 µCi	Mean +/- s.e.mean 100.00 +/- 15.45 10.40 +/- 1.72
Urine	Time 12 h	20.10	22.02	20.41	20.84 +/- 0.78
	24 h	23.04	27.18	22.64	24.28 +/- 1.78
	36 h	24.51	28.83	23.34	25.50 +/- 0.90
	48 h	25.39	29.25	24.09	26.24 +/- 1.89
	60 h	25.79	29.46	24.47	26.57 +/- 1.83
	72 h	26.01	29.61	24.48	26.83 +/- 1.75
Breath	Time 12 h	63.72	45.36	53.40	54.19 +/- 6.50
	24 h	67.00	47.03	55.59	56.54 +/- 7.07
	36 h	68.25	47.82	57.62	57.89 +/- 7.22
	48 h	69.01	48.61	58.12	58.58 +/- 7.24
	60 h	69.33	49.13	58.60	59.02 +/- 7.17
	72 h	69.53	49.36	59.75	59.49 +/- 7.15
Faeces	Time 12 h	1.68	0.01	0.32	0.67 +/- 0.62
	24 h	1.72	2.70	0.96	1.80 +/- 0.62
	36 h	2.35	2.79	1.21	2.14 +/- 0.57
	48 h	2.91	3.45	1.34	2.56 +/- 0.77
	60 h	3.07	3.86	1.36	2.76 +/- 0.91
	72 h	3.12	4.13	1.41	2.88 +/- 0.99
Cage	Time 72 h	1.06	1.05	0.44	0.85 +/- 0.25
Carcass	Time 72 h	1.93	2.16	2.30	2.12 +/- 0.13
Total balance at 72 hours		101.64	86.31	88.56	92.17 +/- 5.87

Cage = Residual radioactivity recovered from metabolism cage at 72h
carcass = Residual radioactivity in carcass at 72h

MASS BALANCE STUDY
CUMULATIVE EXCRETION OF 14C-NMF (as a percentage of the dose administered)
Excretion of 100 mg/kg 14CH3-NMF by Balb/C mice

Animal		F	G	I	Mean +/- s.e.mean
Dose of NMF		114 mg/kg	107 mg/kg	103 mg/kg	108.00 +/- 5.56
Dose of 14C-NMF		10.31 µCi	9.64 µCi	10.58 µCi	10.17 +/- 0.34
Urine	Time 12 h	47.83	42.73	47.45	46.03 +/- 2.01
	24 h	53.06	51.78	56.21	53.68 +/- 1.61
	36 h	54.96	53.76	58.36	55.69 +/- 1.69
	48 h	56.53	55.74	59.06	57.11 +/- 1.23
	60 h	56.97	56.94	59.52	57.94 +/- 1.21
	72 h	57.04	ND	59.84	58.44 +/- 1.63
Breath	Time 12 h	22.36	20.49	17.74	20.19 +/- 1.64
	24 h	24.53	24.59	22.02	23.72 +/- 1.04
	36 h	25.82	26.29	24.75	25.62 +/- 0.59
	48 h	26.75	27.77	26.60	27.04 +/- 0.45
	60 h	27.71	28.10	27.19	27.71 +/- 0.19
	72 h	28.44	ND	27.56	28.00 +/- 0.44
Faeces	Time 12 h	1.64	2.42	5.28	3.11 +/- 1.35
	24 h	2.43	5.68	5.59	4.06 +/- 1.62
	36 h	2.92	6.41	5.91	5.08 +/- 1.37
	48 h	3.02	7.12	6.16	5.43 +/- 1.52
	60 h	3.22	7.39	6.38	5.66 +/- 1.54
	72 h	3.43	ND	6.69	5.06 +/- 1.64
Cage	Time 72 h	0.91	0.83	0.06	0.60 +/- 0.33
Carcass	Time 72 h	7.16	14.28	5.32	8.92 +/- 3.35
Total balance at 72 hours		96.98	107.54	99.42	101.31 +/- 3.91

Cage = Residual radioactivity recovered from metabolism cage at 72h
carcass = Residual radioactivity in carcass at 72h
ND = Not determined

MASS BALANCE STUDY
CUMULATIVE EXCRETION OF 14C-NMF (as a percentage of the dose administered)
Excretion of 400 mg/kg OH14C-NMF by Balb/C mice

Animal		K	M	N	Mean +/- s.e.mean
Dose of NMF		420 mg/kg	420 mg/kg	420 mg/kg	420.00 +/- 0.00
Dose of 14C-NMF		10.76 µCi	11.71 µCi	11.71 µCi	11.39 +/- 0.39
Urine	Time 12 h	18.73	19.31	17.56	18.60 +/- 0.69
	24 h	28.37	35.73	26.42	30.18 +/- 3.47
	36 h	34.21	38.27	30.49	34.32 +/- 2.75
	48 h	36.33	38.73	31.49	35.52 +/- 2.61
	60 h	37.37	39.01	31.92	36.10 +/- 2.62
	72 h	37.80	39.17	32.15	36.37 +/- 2.62
Breath	Time 12 h	37.48	32.25	35.74	35.15 +/- 1.88
	24 h	54.57	46.82	52.57	50.65 +/- 2.37
	36 h	56.74	47.60	54.78	53.04 +/- 3.40
	48 h	57.58	48.14	55.60	53.78 +/- 3.50
	60 h	56.12	48.44	55.98	53.51 +/- 3.10
	72 h	59.65	48.58	56.22	54.82 +/- 4.00
Faeces	Time 12 h	1.19	2.43	2.93	2.18 +/- 0.63
	24 h	2.69	2.97	3.25	2.97 +/- 0.19
	36 h	3.45	3.23	3.82	3.50 +/- 0.29
	48 h	4.89	3.38	4.07	4.15 +/- 0.75
	60 h	5.88	3.83	4.22	4.65 +/- 1.08
	72 h	6.89	4.15	4.37	5.14 +/- 1.52
Cage	Time 72 h	0.18	0.93	0.02	0.37 +/- 0.34
Carcass	Time 72 h	1.18	1.20	0.89	1.09 +/- 0.12
Total balance at 72 hours		105.69	94.00	93.61	97.77 +/- 4.86

Cage = Residual radioactivity recovered from metabolisa cage at 72h
carcass = Residual radioactivity in carcass at 72h

MASS BALANCE STUDY
CUMULATIVE EXCRETION OF 14C-NMF (as a percentage of the dose administered)
Excretion of 400 ag/kg 14CH3-NMF by Balb/C mice

Animal Dose of NMF Dose of 14C-NMF		H 440 ag/kg 16.44 µCi	D 400 ag/kg 14.96 µCi	O 408 ag/kg 10.20 µCi	Mean +/- s.e.mean 416.03 +/- 14.99 13.86 +/- 2.04
Urine	Time 12 h	40.34	22.07	33.54	31.98 +/- 6.52
	24 h	56.52	53.16	51.25	53.64 +/- 1.88
	36 h	63.25	62.11	55.36	60.24 +/- 3.01
	48 h	65.42	63.58	58.06	62.33 +/- 2.73
	60 h	66.16	64.69	59.03	63.29 +/- 2.65
	72 h	66.75	65.29	59.54	63.89 +/- 2.69
Breath	Time 12 h	9.66	10.03	7.47	9.05 +/- 0.98
	24 h	16.24	18.10	13.82	16.06 +/- 1.52
	36 h	18.40	20.36	16.87	18.54 +/- 1.24
	48 h	19.71	21.06	18.38	19.72 +/- 0.95
	60 h	20.65	21.74	19.75	20.71 +/- 0.70
	72 h	21.23	21.76	20.40	21.13 +/- 0.45
Faeces	Time 12 h	4.03	0.11	4.93	3.02 +/- 1.82
	24 h	7.34	5.16	6.29	6.26 +/- 0.77
	36 h	8.09	6.15	9.16	7.80 +/- 1.08
	48 h	8.72	7.79	10.21	8.91 +/- 0.86
	60 h	12.25	8.30	10.51	10.53 +/- 1.21
	72 h	12.61	9.00	11.50	11.03 +/- 1.31
Cage	Time 72 h	0.63	0.78	0.12	0.51 +/- 0.25
Carcass	Time 72 h	4.16	3.97	8.03	5.38 +/- 1.62
Total balance at 72 hours		105.38	100.81	99.60	101.93 +/- 2.16

Cage = Residual radioactivity recovered from metabolism cage at 72h
carcass = Residual radioactivity in carcass at 72h

MASS BALANCE STUDY
CUMULATIVE EXCRETION OF 14C-NMF (as a percentage of the dose administered)
Excretion of 400 ag/kg OH14C-NMF by CBA/CA mice

Dose of NMF (Nominal) Dose of 14C-NMF (Nominal)		400 ag/kg 10 μCi	400 ag/kg 10 μCi	400 ag/kg 10 μCi	Mean +/- s.e.mean ND ND
Urine	Time 12 h	39.8	39.5	36.2	38.5 +/- 1.4
	24 h	47.2	44.3	46.4	45.9 +/- 1.1
	48 h	48.8	46.3	50.1	48.4 +/- 1.4
	72 h	49.4	46.9	50.7	49.0 +/- 1.4
Breath	Time 12 h	26.7	23.8	28.4	26.3 +/- 1.6
	24 h	38.9	37.1	44.7	40.2 +/- 2.8
	48 h	39.9	37.5	47.0	41.5 +/- 3.5
	72 h	40.0	37.6	47.7	42.7 +/- 3.7
Faeces	Time 12 h	2.4	2.9	2.0	2.4 +/- 0.3
	24 h	10.4	11.6	3.2	8.4 +/- 3.2
	48 h	11.3	14.5	3.7	9.8 +/- 3.9
	72 h	11.4	17.4	4.8	11.2 +/- 4.5
Cage	Time 72 h	1.3	0.6	0.9	0.9 +/- 0.3
Carcass	Time 72 h	ND	ND	1.0	ND
Total balance at 72 hours		102.1	102.5	105.1	103.2 +/- 1.1

Cage = Residual radioactivity recovered from metabolism cage at 72h
carcass = Residual radioactivity in carcass at 72h
ND = Not determined

MASS BALANCE STUDY
CUMULATIVE EXCRETION OF 14C-NMF (as a percentage of the dose administered)
Excretion of 400 mg/kg 14CH3-NMF by CBA/CA mice

Dose of NMF (Nominal) Dose of 14C-NMF (Nominal)		400 mg/kg 10 µCi	400 mg/kg 10 µCi	400 mg/kg 10 µCi	Mean +/- s.e.mean ND ND
Urine	Time 12 h	42.6	40.1	52.0	44.9 +/- 4.4
	24 h	69.5	58.4	73.0	66.9 +/- 5.4
	48 h	77.4	62.0	76.5	71.9 +/- 6.1
	72 h	79.3	63.2	77.3	73.2 +/- 6.2
Breath	Time 12 h	5.9	8.4	8.5	7.6 +/- 1.0
	24 h	11.2	13.3	12.6	12.4 +/- 0.7
	48 h	14.5	14.1	13.0	13.8 +/- 0.7
	72 h	14.8	14.6	13.1	14.2 +/- 0.6
Faeces	Time 12 h	0.2	4.9	1.4	2.2 +/- 1.7
	24 h	0.5	12.2	1.6	4.7 +/- 4.6
	48 h	2.3	12.7	4.8	6.6 +/- 3.9
	72 h	2.3	13.0	4.8	6.7 +/- 4.0
Cage	Time 72 h	1.8	1.2	1.1	1.4 +/- 0.3
Carcass	Time 72 h	3.6	2.7	3.7	3.3 +/- 0.5
Total balance at 72 hours		101.8	94.7	100.0	98.2 +/- 2.2

Cage = Residual radioactivity recovered from metabolism cage at 72h
carcass = Residual radioactivity in carcass at 72h
ND = Not determined

APPENDIX II

Tabulated plasma enzyme values

from hepatotoxicity study

N-METHYLFORMAMIDE HEPATOTOXICITY STUDY

Dose related elevation of sorbitol dehydrogenase, alanine aminotransferase and aspartate aminotransferase in the plasma of male Balb/C mice 24 hours after a single i.p. dose of NMF

	Sorbitol dehydrogenase				Alanine aminotransferase				Aspartate aminotransferase			
GROUP	A	L	T	M	A	L	T	M	A	L	T	M
TREATMENT	Saline	100mg/kg	200mg/kg	400mg/kg	Saline	100mg/kg	200mg/kg	400mg/kg	Saline	100mg/kg	200mg/kg	400mg/kg
ANIMAL 1	53.06	70.75	ND	12982.00	ND	24.10	1133.00	8242.00	ND	74.71	506.10	11423.40
2	53.06	57.80	2730.00	9101.00	21.69	38.50	3085.00	7181.00	ND	57.84	2434.00	8868.00
3	51.50	73.90	3863.00	3087.00	21.69	448.10	1012.00	1682.00	ND	60.25	7000.00	12050.00
4	54.60	107.70	199.00	12092.00	31.30	296.40	115.00	10459.00	ND	103.60	602.50	13640.00
5	57.80	62.70	1741.00	7686.00	ND	274.70	1591.00	4145.00	ND	48.20	915.80	4974.00
6	57.80	136.70	8923.00	2894.00	24.10	60.20	11810.00	1009.00	ND	ND	8795.00	7856.00
7	25.72	57.80	6485.00		33.70	19.20	9158.00		69.89	ND	5543.00	
8	32.16	62.00	8191.00		21.70	43.40	11583.00		53.02	ND	8049.00	
9	28.94	136.70	ND		26.50	361.50	ND		38.59	ND	ND	
10	28.90	59.50	5055.00		14.46	ND	723.00		40.97	ND	795.30	
11	30.50	57.80	ND		28.92	ND	ND		84.35	ND	ND	
12	28.90	136.70	4993.00		28.92	ND	5880.00		62.66	ND	3229.00	
13		62.70	686.00			ND	84.06			ND	72.90	
14		64.30	5975.00			ND	3731.00			ND	3070.00	
15		57.80	8040.00			ND	13560.00			ND	7944.00	
16			12490.00				12370.00				6834.00	
17			12000.00				12430.00				6828.00	
Mean	41.90	75.30	5812.00	7974.00	23.30	174.00	5884.00	5453.00	58.20	68.90	4174.00	9801.90
SD	4.07	8.70	3848.70	4314.63	1.89	59.9	5297.00	3781.00	7.87	10.80	3215.00	3174.30
S.E.M.	1.23	2.24	436.90	593.39	0.63	28.3	471.94	475.27	3.52	5.40	350.92	1419.59
SE % of mean	2.94	2.97	7.52	7.44	2.70	16.28	8.02	8.72	6.05	7.84	8.41	14.48
Number	12.00	15.00	14.00	6.00	10.00	9.00	15.00	6.00	6.00	5.00	15.00	6.00

KEY:
ND=Not determined, insufficient plasma sample obtained

N-METHYLFORMAMIDE HEPATOTOXICITY STUDY

Dose related elevation of sorbitol dehydrogenase, alanine aminotransferase and aspartate aminotransferase in the plasma of male CBA/CA mice 24 hours after a single i.p. dose of NMF

	Sorbitol dehydrogenase			Alanine aminotransferase			Aspartate aminotransferase		
GROUP TREATMENT	DD Saline	EE 200mg/kg	CC 400mg/kg	DD Saline	EE 200mg/kg	CC 400mg/kg	DD Saline	EE 200mg/kg	CC 400mg/kg
ANIMAL 1	15.10	24.10	1616.00	26.92	25.30	1225.00	92.90	53.10	1204.00
2	31.50	205.00	1264.00	10.23	29.80	1189.00	12.30	44.40	1096.00
3	29.30	120.00	306.50	37.30	28.70	133.50	66.06	58.60	135.90
4	20.40	171.00	93.81	32.70	26.40	53.00	80.08	48.70	185.10
5	28.40	149.00	5574.00	36.20	27.60	7242.00	58.80	43.20	6433.00
6	30.70	187.00	ND	27.30	27.60	ND	52.17	42.10	ND
Mean	24.94	178.80	1770.80	28.40	27.56	1968.50	59.50	48.40	1810.80
SD	6.91	42.50	2219.30	9.90	1.59	3000.00	27.90	6.42	2631.10
S.E.M.	3.09	19.00	1109.60	4.43	0.71	1500.00	12.47	2.87	1315.50
SE % of mean	12.39	10.63	62.66	15.60	2.58	76.20	20.96	5.93	72.65
n	6.00	6.00	5.00	6.00	6.00	5.00	6.00	6.00	5.00

KEY:
ND=Not determined, insufficient plasma sample obtained

N-METHYLFORMAMIDE HEPATOTOXICITY STUDY

Effect of phenobarbitone pretreatment upon the elevation of sorbitol dehydrogenase, alanine aminotransferase, and aspartate aminotransferase in the plasma of male Balb/C mice after a single i.p. dose of 100mg/kg NMF

	Sorbitol dehydrogenase				Alanine aminotransferase				Aspartate aminotransferase			
GROUP TREATMENT	A Saline	O PB	L 100mg/kg	I 100mg/kg +PB	A Saline	O PB	L 100mg/kg	I 100mg/kg +PB	A Saline	O PB	L 100mg/kg	I 100mg/kg +PB
ANIMAL 1	53.06	53.06	70.75	27.30	ND	36.15	24.10	16.87	ND	53.02	74.71	53.02
2	53.06	57.88	57.80	41.81	21.69	ND	38.50	21.69	ND	ND	57.84	60.25
3	51.50	70.75	73.90	57.80	21.69	48.20	448.10	38.56	ND	139.70	60.25	48.20
4	54.60	49.84	107.70	69.10	31.30	19.28	296.40	28.92	ND	53.02	103.60	55.40
5	57.80	53.06	62.70	53.00	ND	31.33	274.70	33.74	ND	81.94	48.20	43.40
6	57.80	56.28	136.70	64.32	24.10	28.92	60.20	31.83	ND	96.40	ND	57.80
7	25.72		57.80		33.70		19.20		69.89		ND	
8	32.16		62.00		21.70		43.40		53.02		ND	
9	28.94		136.70		26.50		361.50		38.59		ND	
10	28.90		59.50		14.46		ND		40.97		ND	
11	30.50		57.80		28.92		ND		84.35		ND	
12	28.90		136.70		28.92		ND		62.66		ND	
13			62.70				ND				ND	
14			64.30				ND				ND	
15			57.80				ND				ND	
16												
17												
Mean	41.90	56.81	75.30	52.22	23.30	32.77	174.00	28.52	58.20	34.81	68.90	53.02
SD	4.07	7.38	8.70	15.44	1.89	10.60	59.90	7.98	7.87	35.90	10.80	6.27
S.E.M.	1.23	3.30	2.24	6.90	0.63	5.30	28.30	3.56	3.52	17.95	5.40	2.80
SE % of mean	2.94	5.80	2.97	13.22	2.70	16.17	8.02	12.51	6.05	51.50	7.84	5.28
Number	12.00	6.00	15.00	6.00	10.00	5.00	9.00	6.00	6.00	5.00	5.00	6.00

KEY:
ND=Not determined, insufficient plasma sample obtained
Group O= Phenobarbitone 50mg/kg for 4 days prior to saline
Group L= 100mg/kg saline
Group I= Phenobarbitone 50mg/kg for 4 days prior to 100mg/kg NMF

N-METHYLFORMAMIDE HEPATOTOXICITY STUDY

Effect of SKF525A pretreatment upon the elevation of sorbitol dehydrogenase, alanine aminotransferase, and aspartate aminotransferase in the plasma of male Balb/C mice after a single i.p. dose of 400mg/kg NMF

	Sorbitol dehydrogenase				Alanine aminotransferase				Aspartate aminotransferase			
GROUP	A	G	M	N	A	G	M	N	A	G	M	N
TREATMENT	Saline	SKF525A	400mg/kg	400mg/kg +SKF525A	Saline	SKF525A	400mg/kg	400mg/kg +SKF525A	Saline	SKF525A	400mg/kg	400mg/kg +SKF525A
ANIMAL 1	53.06	ND	12982.00	3216.00	ND	ND	8242.00	11206.00	ND	2.41	11423.40	6965.00
2	53.06	56.28	787.90	11899.00	21.69	4.82	7181.00	1662.00	ND	2.41	8868.00	1542.00
3	51.50	62.71	3087.00	12703.00	21.69	14.46	1682.00	5639.00	ND	7.23	12050.00	6840.00
4	54.60	127.00	12092.00	15919.00	31.30	21.69	10459.00	9567.00	ND	31.30	13640.00	8025.00
5	57.80	77.18	7686.00	12140.00	ND	31.30	4145.00	8266.00	ND	14.50	4974.00	7230.00
6	57.80	83.62	2894.00	6464.00	24.10	24.10	1009.00	508.50	ND	21.70	7856.00	3494.00
7	25.72				33.70				69.89			
8	32.16				21.70				53.02			
9	28.94				26.50				38.59			
10	28.90				14.46				40.97			
11	30.50				28.92				84.35			
12	28.90				28.92				62.66			
Mean	41.90	81.35	7974.00	10390.50	23.30	19.27	5453.00	6141.40	58.20	13.25	9801.90	5682.00
SD	4.07	27.70	4314.63	4650.00	1.89	10.00	3781.00	4650.00	7.87	11.50	3174.30	2561.20
S.E.M.	1.23	12.38	593.39	1898.30	0.63	4.47	475.27	1898.30	3.52	4.69	1419.00	1045.60
SE % of mean	2.94	15.22	7.44	18.27	2.70	23.2	8.72	30.91	6.05	35.40	14.48	18.40
Number	12.00	5.00	6.00	6.00	10.00	5.00	6.00	6.00	6.00	6.00	6.00	6.00

KEY:
ND=Not determined, insufficient plasma sample obtained
Group G= SKF525A 60mg/kg 1 h prior to saline
Group M= 400mg/kg NMF
Group N= SKF525A 60mg/kg 1 h prior to 400mg/kg NMF

N-METHYLFORMAMIDE HEPATOTOXICITY STUDY

Effect of methimazole pretreatment upon the elevation of sorbitol dehydrogenase, alanine aminotransferase, and aspartate aminotransferase in the plasma of male Balb/C mice after a single i.p. dose of 200mg/kg NMF

	Sorbitol dehydrogenase			Alanine aminotransferase			Aspartate aminotransferase		
GROUP	A	T	BB	A	T	BB	A	T	BB
TREATMENT	Saline	200mg/kg	200mg/kg +Meth	Saline	200mg/kg	200mg/kg +Meth	Saline	200mg/kg	200mg/kg +Meth
ANIMAL 1	53.06	ND	6907.00	ND	1133.00	8780.00	ND	506.10	7130.00
2	53.06	2730.00	584.70	21.69	3085.00	7867.00	ND	2434.00	442.00
3	51.50	3863.00	8065.00	21.69	1012.00	10480.00	ND	7000.00	8497.00
4	54.60	199.00	589.90	31.30	115.00	728.20	ND	602.50	496.70
5	57.80	1741.00		ND	1591.00		ND	915.80	
6	57.80	8923.00		24.10	11810.00		ND	8795.00	
7	25.72	6485.00		33.70	9158.00		68.89	5543.00	
8	32.16	8191.00		21.70	11583.00		53.02	8049.00	
9	28.94	ND		26.50	ND		38.59	ND	
10	28.90	5055.00		14.46	723.00		40.97	795.30	
11	30.50	ND		28.92	ND		84.35	ND	
12	28.90	4993.00		28.92	5880.00		62.66	3229.00	
13		686.00			84.06			72.90	
14		5975.00			3731.00			3070.00	
15		8040.00			13560.00			7944.00	
16		12490.00			12370.00			6834.00	
17		12000.00			12430.00			6828.00	
Mean	41.90	5812.00	4036.50	23.30	5884.00	6951.30	58.20	4174.00	4141.40
SD	4.07	3848.70	4010.00	1.89	5297.00	4288.70	7.87	3215.00	4276.00
S.E.M	1.23	436.90	1793.00	0.63	471.94	1918.00	3.52	350.92	1912.60
SE % of mean	2.94	7.52	27.59	2.70	8.02	27.59	6.05	8.41	46.18
n	12.00	15.00	4.00	10.00	15.00	4.00	6.00	15.00	4.00

KEY:
ND=Not determined, insufficient plasma sample obtained
Group BB= Methimazole 200mg/kg 30 min prior to 200mg/kg NMF
Group T= 200mg/kg NMF

N-METHYLFORMAMIDE HEPATOTOXICITY STUDY

Effect of buthionine sulfoximine pretreatment upon the elevation of sorbitol dehydrogenase, alanine aminotransferase, and aspartate aminotransferase in the plasma of male Balb/C mice after a single i.p. dose of 100mg/kg NMF

	Sorbitol dehydrogenase				Alanine aminotransferase				Aspartate aminotransferase			
GROUP TREATMENT	A Saline	E BSO	L 100mg/kg	D 100mg/kg +BSO	A Saline	E BSO	L 100mg/kg	D 100mg/kg +BSO	A Saline	E BSO	L 100mg/kg	D 100mg/kg +BSO
ANIMAL 1	53.06	127.00	70.75	ND	ND	274.70	24.10	ND	ND	97.36	74.71	ND
2	53.06	53.06	57.80	2830.00	21.69	24.10	38.50	1021.80	ND	36.87	57.84	992.90
3	51.50	33.70	73.90	12397.00	21.69	21.69	448.10	4506.70	ND	34.52	60.25	698.90
4	54.60	35.40	107.70	5354.00	31.30	14.46	296.40	2361.80	ND	24.15	103.60	366.30
5	57.80	38.50	62.70	ND	ND	19.04	274.70	ND	ND	32.63	48.20	ND
6	57.80	51.50	136.70	1511.00	24.10	18.32	60.20	313.30	ND	42.54	ND	332.60
7	25.72		57.80	2638.60	33.70		19.20	ND	69.89		ND	1010.10
8	32.16		62.00	3800.40	21.70		43.40	2250.00	53.02		ND	1503.40
9	28.94		136.70	1205.30	26.50		361.50	3215.20	38.59		ND	354.30
10	28.90		59.50	2610.30	14.46		ND	798.80	40.97		ND	1225.40
11	30.50		57.80	2879.40	28.92		ND	1750.90	84.35		ND	987.60
12	28.90		136.70	ND	28.92		ND	2710.70	62.66		ND	ND
13			62.70				ND				ND	
14			64.30				ND				ND	
15			57.80				ND				ND	
16												
17												
Mean	41.90	56.52	75.30	3913.60	23.30	62.05	174.00	2103.20	58.20	44.67	68.90	830.16
SD	4.07	35.40	8.70	3404.70	1.89	104.20	59.90	1307.17	7.87	26.49	10.80	418.25
S.E.M.	1.23	15.83	2.24	1203.70	0.63	45.59	28.30	462.34	3.52	11.85	5.40	147.87
SE % of mean	2.94	28.01	2.97	30.75	2.70	73.47	8.02	21.98	6.05	26.52	7.84	17.81
Number	12.00	6.00	15.00	9.00	10.00	6.00	9.00	9.00	6.00	6.00	5.00	9.00

KEY:
ND=Not determined, insufficient plasma sample obtained
Group E= Buthionine sulfoximine 1600mg/kg 4 h prior to saline
Group L= 100mg/kg NMF
Group D= Buthionine sulfoximine 1600mg/kg 4 h prior to 100mg/kg NMF

N-METHYLFORMAMIDE HEPATOTOXICITY STUDY

Effect of N-acetylcysteine pretreatment upon the elevation of sorbitol dehydrogenase, alanine aminotransferase, and aspartate aminotransferase in the plasma of male Balb/C mice after a single i.p. dose of 200mg/kg NMF

	Sorbitol dehydrogenase				Alanine aminotransferase				Aspartate aminotransferase			
GROUP TREATMENT	A Saline	FF NAC	T 200mg/kg	GG 200mg/kg +NAC	A Saline	FF NAC	T 200mg/kg	GG 200mg/kg +NAC	A Saline	FF NAC	T 200mg/kg	GG 200mg/kg +NAC
ANIMAL 1	53.06	36.70	ND	37.06	ND	43.25	1133.00	39.62	ND	132.40	506.10	63.00
2	53.06	54.68	2730.00	159.20	21.69	60.10	3085.00	208.80	ND	93.44	2434.00	70.26
3	51.50	55.86	3863.00	50.75	21.69	57.93	1012.00	40.12	ND	187.08	7000.00	70.26
4	54.60	40.42	199.00	70.84	31.30	54.27	115.00	59.36	ND	75.93	602.50	198.30
5	57.80	42.65	1741.00	46.72	ND	48.36	1591.00	49.03	ND	119.80	915.80	119.50
6	57.80	24.55	8923.00	190.20	24.10	48.41	11810.00	166.70	ND	122.00	8795.00	159.60
7	25.72		6485.00	93.69	33.70		9158.00	49.14	69.89		5543.00	75.80
8	32.16		8191.00	65.18	21.70		11583.00	56.90	53.02		8049.00	128.3
9	28.94		ND	154.66	26.50		ND	44.30	38.59		ND	63.60
10	28.90		5055.00	51.08	14.46		723.00	38.57	40.97		795.30	76.90
11	30.50		ND		28.92		ND		84.35		ND	
12	28.90		4993.00		28.92		5880.00		62.66		3229.00	
13			686.00				84.06				72.90	
14			5975.00				3731.00				3070.00	
15			8040.00				13560.00				7944.00	
16			12490.00				12370.00				6834.00	
17			12000.00				12430.00				6828.00	
Mean	41.90	42.47	5812.00	91.94	23.30	52.05	5884.00	75.25	58.20	121.75	4174.00	102.55
SD	4.07	11.72	3848.70	55.47	1.89	6.46	5297.00	60.50	7.87	38.20	3215.00	47.00
S.E.M.	1.23	5.24	436.90	18.49	0.63	2.88	471.94	20.15	3.52	17.08	350.92	15.66
SE % of mean	2.94	12.30	7.52	20.11	2.70	5.53	8.02	28.68	6.05	14.02	8.41	15.27
Number	12.00	6.00	14.00	10.00	10.00	6.00	14.00	10.00	6.00	6.00	15.00	10.00

KEY:
ND=Not determined, insufficient plasma sample obtained
Group FF= N-Acetylcysteine 1200mg/kg p.o. 20 min prior to saline
Group T= 200mg/kg NMF
Group GG= N-acetylcysteine 1200mg/kg p.o. 20 min prior to 200mg/kg NMF

N-METHYLFORMAMIDE HEPATOTOXICITY STUDY

Effect of N-acetylcysteine pretreatment upon the elevation of sorbitol dehydrogenase, alanine aminotransferase, and aspartate aminotransferase in the plasma of male Balb/C mice after a single i.p. dose of 400mg/kg NMF

	Sorbitol dehydrogenase				Alanine aminotransferase				Aspartate aminotransferase			
GROUP TREATMENT	A Saline	FF NAC	M 400mg/kg	HH 400mg/kg + NAC	A Saline	FF NAC	M 400mg/kg	HH 400mg/kg +NAC	A Saline	R NAC	M 400mg/kg	HH 400mg/kg +NAC
ANIMAL 1	53.06	36.70	12982.00	8246.00	ND	43.25	8242.00	15180.00	ND	142.20	11423.40	6560.00
2	53.06	54.68	787.90	1770.80	21.69	60.10	7181.00	1161.80	ND	86.76	8868.00	1260.00
3	51.50	55.86	3087.00	210.00	21.69	57.93	1682.00	115.20	ND	53.02	12050.00	71.45
4	54.60	40.42	12092.00	4318.00	31.30	54.27	10459.00	3613.00	ND	69.89	13640.00	1270.00
5	57.80	42.65	7686.00	198.00	ND	48.36	4145.00	96.70	ND	ND	4974.00	294.20
6	57.80	24.55	2894.00	160.40	24.10	48.41	1009.00	198.28	ND	45.79	7856.00	93.90
7	25.72				33.70				69.89			
8	32.16				21.70				53.02			
9	28.94				26.50				38.59			
10	28.90				14.46				40.97			
11	30.50				28.92				84.35			
12	28.90				28.92				62.66			
Mean	41.90	42.47	7974.00	2483.80	23.30	52.05	5453.00	3394.16	58.20	79.50	9801.90	1591.60
SD	4.07	11.72	4314.63	3251.70	1.89	6.46	3781.00	5929.20	7.87	38.40	3174.30	2495.40
S.E.M.	1.23	5.24	593.39	1458.16	0.63	2.88	475.27	2651.60	3.52	19.20	1419.00	1119.00
SE % of mean	2.94	12.30	7.44	58.70	2.70	5.53	8.72	78.12	6.05	24.15	14.48	70.30
Number	12.00	6.00	6.00	6.00	10.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00

KEY:
ND=Not determined, insufficient plasma sample obtained
Group FF= N-Acetylcysteine 1200mg/kg p.o. 20 min prior to saline
Group M= 400mg/kg NMF
Group HH= N-Acetylcysteine 1200mg/kg p.o. prior to 400mg/kg NMF

N-METHYLFORMAMIDE HEPATOTOXICITY STUDY

Effect of N-acetylcysteine pretreatment upon the elevation of sorbitol dehydrogenase, alanine aminotransferase, and aspartate aminotransferase in the plasma of male Balb/C mice after a single i.p. dose of 200mg/kg NMF

	Sorbitol dehydrogenase				Alanine aminotransferase				Aspartate aminotransferase			
GROUP TREATMENT	A Saline	R NAC	T 200mg/kg	U 200mg/kg +NAC	A Saline	R NAC	T 200mg/kg	U 200mg/kg +NAC	A Saline	R NAC	T 200mg/kg	U 200mg/kg +NAC
ANIMAL 1	53.06	46.60	ND	54.67	ND	36.15	1133.00	24.10	ND	142.20	506.10	48.20
2	53.06	43.40	2730.00	131.85	21.69	28.92	3085.00	60.25	ND	86.76	2434.00	43.30
3	51.50	43.40	3863.00	638.40	21.69	21.69	1012.00	441.03	ND	53.02	7000.00	200.30
4	54.60	30.50	199.00	59.49	31.30	36.15	115.00	33.74	ND	69.89	602.50	53.02
5	57.80	ND	1741.00	857.06	ND	ND	1591.00	855.50	ND	ND	915.80	657.90
6	57.80	80.40	8923.00	693.01	24.10	16.87	11810.00	532.60	ND	45.79	8795.00	419.30
7	25.72		6485.00	5023.00	33.70		9158.00	16364.00	69.89		5543.00	5977.00
8	32.16		8191.00	9468.00	21.70		11583.00	12900.00	53.02		8049.00	9237.00
9	28.94		ND	194.00	26.50		ND	149.40	38.59		ND	506.10
10	28.90		5055.00	37.20	14.46		723.00	14.46	40.97		795.30	79.53
11	30.50		ND	1380.00	28.92		ND	1084.50	84.35		ND	7300.00
12	28.90		4993.00	4244.00	28.92		5880.00	ND	62.66		3229.00	ND
13			686.00				84.06				72.90	
14			5975.00				3731.00				3070.00	
15			8040.00				13560.00				7944.00	
16			12490.00				12370.00				6834.00	
17			12000.00				12430.00				6828.00	
Mean	41.90	48.70	5812.00	2986.10	23.30	27.95	5884.00	2949.00	58.20	79.40	4174.00	1722.20
SD	4.07	18.70	3848.70	4299.00	1.89	8.62	5297.00	5833.00	7.87	38.40	3215.00	3202.50
S.E.M.	1.23	9.35	436.90	1296.20	0.63	4.31	471.94	1758.70	3.52	19.20	350.92	965.60
SE % of mean	2.94	19.15	7.52	43.40	2.70	15.42	8.02	59.63	6.05	24.15	8.41	56.00
Number	12.00	5.00	14.00	12.00	10.00	5.00	14.00	11.00	6.00	5.00	15.00	11.00

KEY:
ND=Not determined, insufficient plasma sample obtained
Group R and U= N-Acetylcysteine 100mg/kg i.p. 1 h before and 8 h after saline (R) and 200mg/kg NMF (U), respectively
Group T= 200mg/kg NMF

N-METHYLFORMAMIDE HEPATOTOXICITY STUDY

Effect of N-acetylcysteine pretreatment upon the elevation of sorbitol dehydrogenase, alanine aminotransferase, and aspartate aminotransferase in the plasma of male Balb/C mice after a single i.p. dose of 400mg/kg NMF

	Sorbitol dehydrogenase				Alanine aminotransferase				Aspartate aminotransferase			
GROUP TREATMENT	A Saline	R NAC	M 400mg/kg	P 400mg/kg + NAC	A Saline	R NAC	M 400mg/kg	P 400mg/kg +NAC	A Saline	R NAC	M 400mg/kg	P 400mg/kg +NAC
ANIMAL 1	53.06	46.60	12982.00	5370.00	ND	36.15	8242.00	6748.00	ND	142.20	11423.40	7037.00
2	53.06	43.40	787.90	916.50	21.69	28.92	7181.00	120.50	ND	86.76	8868.00	168.70
3	51.50	43.40	3087.00	2412.00	21.69	21.69	1682.00	1687.00	ND	53.02	12050.00	795.30
4	54.60	30.50	12092.00	2251.00	31.30	36.15	10459.00	1879.00	ND	69.89	13640.00	898.90
5	57.80	ND	7686.00	5418.00	ND	ND	4145.00	5952.00	ND	ND	4974.00	5784.00
6	57.80	80.40	2894.00	9519.00	24.10	16.87	1009.00	9567.00	ND	45.79	7856.00	11977.00
7	25.72				33.70				69.89			
8	32.16				21.70				53.02			
9	28.94				26.50				38.59			
10	28.90				14.46				40.97			
11	30.50				28.92				84.35			
12	28.90				28.92				62.66			
Mean	41.90	48.80	7974.00	4727.10	23.30	27.95	5453.00	4321.10	58.20	79.50	9801.90	4443.00
SD	4.07	18.70	4314.63	3306.60	1.89	8.62	3781.00	3647.70	7.87	38.40	3174.30	4678.10
S.E.M.	1.23	9.35	593.39	1478.70	0.63	4.31	475.27	729.54	3.52	19.20	1419.00	2092.10
SE % of mean	2.94	19.15	7.44	31.28	2.70	15.42	8.72	16.88	6.05	24.15	14.48	47.08
Number	12.00	5.00	6.00	6.00	10.00	5.00	6.00	6.00	6.00	6.00	6.00	6.00

KEY:
ND=Not determined, insufficient plasma sample obtained
Group R and P= N-Acetylcysteine 100mg/kg i.p. 1 h before and 8 h after saline (R) and 400mg/kg NMF (P), respectively
Group M= 400mg/kg NMF

N-METHYLFORMAMIDE HEPATOTOXICITY STUDY

Effect of cysteine pretreatment upon the elevation of sorbitol dehydrogenase, alanine aminotransferase, and aspartate aminotransferase in the plasma of male Balb/C mice after a single i.p. dose of 200mg/kg NMF

	Sorbitol dehydrogenase				Alanine aminotransferase				Aspartate aminotransferase			
GROUP TREATMENT	A Saline	Y Cysteine	T 200mg/kg	X 200mg/kg +Cysteine	A Saline	Y Cysteine	T 200mg/kg	X 200mg/kg +Cysteine	A Saline	Y Cysteine	T 200mg/kg	X 200mg/kg +Cysteine
ANIMAL 1	53.06	65.30	ND	7382.00	ND	40.42	1133.00	6868.00	ND	96.40	506.10	6308.00
2	53.06	56.00	2730.00	7163.00	21.69	55.95	3085.00	6748.00	ND	57.80	2434.00	6434.00
3	51.50	59.70	3863.00	ND	21.69	49.07	1012.00	ND	ND	57.80	7000.00	ND
4	54.60	57.80	199.00	592.60	31.30	71.66	115.00	150.10	ND	65.07	602.50	118.20
5	57.80	56.20	1741.00	6611.00	ND	46.45	1591.00	4522.00	ND	45.79	915.80	4098.00
6	57.80	59.70	8923.00	ND	24.10	28.98	11810.00	ND	ND	43.38	8795.00	ND
7	25.72		6485.00	4056.00	33.70		9158.00	3023.00	69.89		5543.00	1575.00
8	32.16		8191.00	338.00	21.70		11583.00	182.50	53.02		8049.00	81.66
9	28.94		ND	79.20	26.50		ND	50.19	38.59		ND	78.40
10	28.90		5055.00	126.80	14.46		723.00	61.70	40.97		795.30	73.40
11	30.50		ND	74.60	28.92		ND	46.20	84.35		ND	49.30
12	28.90		4993.00	4544.00	28.92		5880.00	2869.00	62.66		3229.00	1793.00
13			686.00				84.06				72.90	
14			5975.00				3731.00				3070.00	
15			8040.00				13560.00				7944.00	
16			12490.00				12370.00				6834.00	
17			12000.00				12430.00				6828.00	
Mean	41.90	59.11	5812.00	3096.70	23.30	48.75	5884.00	2452.10	58.20	61.04	4174.00	2060.00
SD	4.07	3.43	3848.70	3183.00	1.89	14.4	5297.00	2798.10	7.87	19.14	3215.00	2608.00
S.E.M.	1.23	2.53	436.90	1061.10	0.63	6.45	471.94	932.60	3.52	8.56	350.92	869.00
SE % of mean	2.94	2.56	7.52	34.26	2.70	13.23	8.02	38.03	6.05	14.02	8.41	42.18
Number	12.00	6.00	14.00	10.00	10.00	6.00	15.00	10.00	6.00	6.00	15.00	10.00

KEY:
ND=Not determined, insufficient plasma sample obtained
Group Y and X= Cysteine HCl 300mg/kg 10 min before and 20 min after saline (Y) and 200mg/kg NMF (X), respectively
Group T= 200mg/kg NMF

N-METHYLFORMAMIDE HEPATOTOXICITY STUDY

Effect of vitamin E pretreatment upon the elevation of sorbitol dehydrogenase, alanine aminotransferase, and aspartate aminotransferase in the plasma of male Balb/C mice 24 h after a single i.p. dose of 200mg/kg NMF

	Sorbitol dehydrogenase				Alanine aminotransferase				Aspartate aminotransferase			
GROUP	A	J	T	S	A	J	T	S	A	J	T	S
TREATMENT	Saline	Vitamin E	200mg/kg	200mg/kg + Vit E	Saline	Vitamin E	200mg/kg	200mg/kg + Vit E	Saline	Vitamin E	200mg/kg	200mg/kg + Vit E
ANIMAL 1	53.06	45.02	ND	3003.00	ND	9.64	1133.00	4495.00	ND	96.40	506.10	6250.00
2	53.06	36.98	2730.00	3842.00	21.69	9.64	3085.00	3972.00	ND	57.80	2434.00	4940.00
3	51.50	43.42	3863.00	3954.00	21.69	9.64	1012.00	4314.00	ND	57.80	7000.00	5663.00
4	54.60	38.59	199.00	1876.00	31.30	14.46	115.00	139.80	ND	65.07	602.50	915.80
5	57.80	30.55	1741.00	304.70	ND	14.46	1591.00	192.80	ND	45.79	915.80	118.10
6	57.80	41.80	8923.00	7734.00	24.10	7.23	11810.00	10242.00	ND	43.38	8795.00	7953.00
7	25.72		6485.00	8064.00	33.70		9158.00	11013.00	69.89		5543.00	9808.00
8	32.16		8191.00	ND	21.70		11583.00	ND	53.02		8049.00	ND
9	28.94		ND	1774.00	26.50		ND	1662.90	38.59		ND	1638.00
10	28.90		5055.00	3595.00	14.46		723.00	2988.40	40.97		795.30	4531.00
11	30.50		ND	2809.00	28.92		ND	2892.00	84.35		ND	2389.00
12	28.90		4993.00	212.20	28.92		5880.00	90.92	62.66		3229.00	64.60
13			686.00	7001.00			84.06	5132.00			72.90	7380.00
14			5975.00	834.20			3731.00	721.00			3070.00	339.00
15			8040.00	1238.00			13560.00	1058.00			7944.00	283.00
16			12490.00	ND			12370.00	ND			6834.00	ND
17			12000.00	4647.00			12430.00	1075.00			6828.00	205.00
Mean	41.90	39.93	5812.00	3392.50	23.30	10.84	5884.00	3332.50	58.20	61.04	4174.00	3474.50
SD	4.07	5.26	3848.70	2564.00	1.89	2.95	5297.00	3416.70	7.87	19.14	3215.00	3303.40
S.E.M.	1.23	0.55	436.90	685.20	0.63	1.31	471.94	913.15	3.52	8.56	350.92	882.80
SE % of mean	2.94	1.37	7.52	20.20	2.70	12.17	8.02	27.40	6.05	14.02	8.41	25.40
Number	12.00	6.00	14.00	15.00	10.00	6.00	15.00	15.00	6.00	6.00	15.00	15.00

KEY:
ND=Not determined, insufficient plasma sample obtained
Group J=Vitamin E 100mg/kg 15 h prior to saline
Group T= 200mg/kg NMF
Group S= Vitamin E 100mg/kg 15 h prior to 200mg/kg NMF

N-METHYLFORMAMIDE HEPATOTOXICITY STUDY

Effect of vitamin E pretreatment upon the elevation of sorbitol dehydrogenase, alanine aminotransferase,and aspartate aminotransferase in the plasma of male Balb/C mice 24 h after a single i.p. dose of 400mg/kg NMF

	Sorbitol dehydrogenase				Alanine aminotransferase				Aspartate aminotransferase			
GROUP	A	J	M	K	A	J	M	K	A	J	M	K
TREATMENT	Saline	Vitamin E	400mg/kg	400mg/kg + Vit E	Saline	Vitamin E	400mg/kg	400mg/kg + Vit E	Saline	Vitamin E	400mg/kg	400mg/kg + Vit E
ANIMAL 1	53.06	45.02	12982.00	12092.00	ND	9.64	8242.00	11543.00	ND	96.40	11423.40	11688.00
2	53.06	36.98	787.90	787.90	21.69	9.64	7181.00	144.60	ND	57.80	8868.00	151.80
3	51.50	43.42	3087.00	10066.00	21.69	9.64	1682.00	14146.00	ND	57.80	12050.00	7760.00
4	54.60	38.59	12092.00	1398.00	31.30	14.46	10459.00	819.40	ND	65.07	13640.00	532.60
5	57.80	30.55	7686.00	3987.00	ND	14.46	4145.00	2313.60	ND	45.79	4974.00	1301.00
6	57.80	41.80	2894.00	241.00	24.10	7.23	1009.00	168.70	ND	43.38	7856.00	74.71
7	25.72				33.70				69.89			
8	32.16				21.70				53.02			
9	28.94				26.50				38.59			
10	28.90				14.46				40.97			
11	30.50				28.92				84.35			
12	28.90				28.92				62.66			
Mean	41.90	39.93	7974.00	4762.00	23.30	10.84	5453.00	4855.80	58.20	61.04	9801.90	3584.60
SD	4.07	5.26	4314.63	5099.00	1.89	2.95	3781.00	6291.90	7.87	19.14	3174.30	4934.20
S.E.M.	1.23	0.55	593.39	2280.30	0.63	1.31	475.27	2813.80	3.52	8.56	1419.00	2206.60
SE % of mean	2.94	1.37	7.44	47.80	2.70	12.17	8.72	57.94	6.05	14.02	14.48	61.50
Number	12.00	6.00	6.00	6.00	10.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00

KEY:
ND=Not determined, insufficient plasma sample obtained
Group J= Vitamin E 100mg/kg 15 h prior to saline
Group M= 400mg/kg NMF
Group K= Vitamin E 100mg/kg 15 h prior to 400mg/kg NMF

N-METHYLFORMAMIDE HEPATOTOXICITY STUDY

Effect of promethazine pretreatment upon the elevation of sorbitol dehydrogenase, alanine aminotransferase, and aspartate aminotransferase in the plasma of male Balb/c mice after a single i.p. dose of 200mg/kg NMF

	Sorbitol dehydrogenase				Alanine aminotransferase				Aspartate aminotransferase			
GROUP TREATMENT	A Saline	AA PROM	T 200mg/kg	Z 200mg/kg +PROM	A Saline	AA PROM	T 200mg/kg	Z 200mg/kg +PROM	A Saline	AA PROM	T 200mg/kg	Z 200mg/kg +PROM
ANIMAL 1	53.06	49.80	ND	773.20	ND	52.30	1133.00	548.80	ND	44.30	506.10	359.40
2	53.06	45.00	2730.00	357.00	21.69	36.70	3085.00	141.50	ND	54.80	2434.00	132.80
3	51.50	57.80	3863.00	102.90	21.69	60.30	1012.00	65.10	ND	81.17	7000.00	77.20
4	54.60	41.80	199.00	94.60	31.30	31.50	115.00	80.30	ND	61.40	602.50	68.80
5	57.80	56.30	1741.00	5186.00	ND	50.70	1591.00	3281.00	ND	67.30	915.80	2086.00
6	57.80	78.40	8923.00	195.60	24.10	81.80	11810.00	133.50	ND	92.70	8795.00	340.60
7	25.72		6485.00		33.70		9158.00		69.89		5543.00	
8	32.16		8191.00		21.70		11583.00		53.02		8049.00	
9	28.94		ND		26.50		ND		38.59		ND	
10	28.90		5055.00		14.46		723.00		40.97		795.30	
11	30.50		ND		28.92		ND		84.35		ND	
12	28.90		4993.00		28.92		5880.00		62.66		3229.00	
13			686.00				84.06				72.90	
14			5975.00				3731.00				3070.00	
15			8040.00				13560.00				7944.00	
16			12490.00				12370.00				6834.00	
17			12000.00				12430.00				6828.00	
Mean	41.90	54.85	5812.00	1118.20	23.30	52.22	5884.00	708.30	58.20	66.94	4174.00	510.80
SD	4.07	13.10	3848.70	2008.70	1.89	17.90	5297.00	1273.00	7.87	17.64	3215.00	782.20
S.E.M.	1.23	5.86	436.90	898.30	0.63	8.03	471.94	569.30	3.52	7.89	350.92	349.80
SE % of mean	2.94	10.68	7.52	80.30	2.70	15.37	8.02	80.37	6.05	11.78	8.41	68.40
Number	12.00	6.00	14.00	6.00	10.00	6.00	14.00	6.00	6.00	6.00	15.00	6.00

KEY:
ND=Not determined, insufficient plasma sample obtained
Group AA= Promethazine 24mg/kg 10 min prior to saline
Group T= 200mg/kg NMF
Group Z= Promethazine 24mg/kg 10 min prior to 200mg/kg NMF

REFERENCES

REFERENCES

1. Clarke, D.A., Philips, F.S., Sternberg, S.E., Barclay, R.K. and Stock, C.C. (1953)
Effects of N-methylformamide and related compounds in sarcoma 180.
Proc.Soc.Exp.Biol.Med., 84, 203-207.

2. Furst, A., Cutting., W.C. and Cross, H. (1955)
Retardation of growth of Ehrlich ascites tumour by formamides and related compounds.
Cancer Res., 15, 294-299.

3. Hickman, J.A. (1978)
Investigation of the mechanism of action of antitumour dimethyltriazines. Biochimie., 60, 997-1002.

4. Gescher, A., Gibson, N.W., Hickman, J.A., Langdon, S.P., Ross, D. and Atassi, G. (1982)
N-Methylformamide:Antitumour activity and metabolism in mice.
Br. J. Cancer., 45, 843-850.

5. Gate, E.N., Threadgill, M.D., Stevens, M.F.G., Chubb, D., Vickers, L.M., Langdon, S.P., Hickman, J.A. and Gescher, A. (1985)
Structural studies on bioactive compounds Part 5 : A structure antitumour-activity study on analogues of NMF. (submitted for publication).

6. Dexter, D.L., Spremulli, E.N., Matook, G.M., Diamond, I. and Calabresi, P. (1982)
Inhibition of the growth of human colon cancer xenografts by polar solvents.
Cancer Res., 42, 5028-5022.

7. Lomax, N.R., and Narayan, V.L., in Chemical Structures of Interest to the Division of Cancer Treatment. Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, National Cancer Institute (1981).

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

9. Overton, M., Hickman, J.A., Threadgill, M.D., Vaughan, K. and Gescher, A. (1985)
The generation of potentially toxic, reactive iminium ions from the oxidative metabolism of xenobiotic N-alkyl compounds.
Biochem. Pharmac. 34, 2055-2061

10. Nguyen, T.L., Grueneke, L.D. and Castagnoli, N. (1979)
Metabolic dealkylation of nicotine. Trapping of a reactive iminium species with cyanide ion.
J. Med. Chem., 19, 1168-1169

11. Nguyen, T.L., Grueneke, L.D. and Castagnoli, N. (1979)
Metabolic oxidation of nicotine to chemically reactive intermediates.
J. Med, Chem., 22, 259-263.

12. Zeigler, R., Ho, B., and Castagnoli, N. (1981)
Trapping of metabolically generated electrophilic species with cyanide ion: Metabolism of methapyrilene.
J. Med. Chem., 24, 113-1138.

13. Ketterer, B., Srai, S.K.S., Waynforth, B., Tullis, D.L., Evans, F.E. and Kadlubar, F.F. (1982)
Formation of N-(glutathion-S-methylene)-4-aminoazobeneze following metabolic oxidationof the N-methyl group of the carcinogen N-methyl-4-aminoazobenzene.
Chem. Biol. Interact., 38, 287-302.

14. Ames, M.M., Sanders, M.E. and Teide, W.S. (1983)
Role of N-Methyololpentamethylmelamine in the Metabolic Activation of Hexamethylmelamine.
Cancer Res., 43, 500-504.

15. Cooksey, P., Gate, E.N., Gescher, A., Hickman, J.A., Langdon, S.P. and Stevens, M.F.G. (1983)
Cytotoxicity and antitumour activity of N-methylformamide, a putative metabolite of N-methylformamide.
Biochem. Pharmacol., 32, 3037-3043.

16. Collins, S.J., Ruscetti, F.W., Gallagher, R. and Gallo, R. (1978) Terminal differentiation of human promyelocytic leukaemia cells induced by dimethylsulfoxide and other polar compounds.
Proc. Natl. Acad. Sci. U.S.A., 75, 2458-2462.

17. Spemulli, E.N. and Dexter, D. (1984)
Polar Solvents: A Novel Class of antineoplastic agent.
Journal of Clinical Oncology, 2, 227-241.

18. Brindley, C., Gescher, A., Harpur, E.S., Ross, D., Slack, J.A., Threadgill, M.D. and Whitby, H. (1982)
Studies of the pharmacology of N-methylformamide in mice.
Cancer Treat. Rep., 66, 1597-1965.

19. Brindley, C., Kestell, P., Gescher, A. and Slack, J.A. (1983)
Plasma levels of N-methylformamide following i.v. and oral administration in man.
Br. J. Cancer, 48, 115.

20. Newman, K.N., Meeks, R.G. and Frick, S. (1981)
Phase 1 report of Task 1 preclinical Intravenous Toxicity Study of N-methylformamide (NSC-3051) in CDF_1 mice and beagle dogs.
Southern Research Institute, Birmingham, Alabama.

21. Langdon, S.P., Chubb, D., Gescher, A., Hickman, J.A. and Stevens, M.F.G. (1985)
Studies on the toxicity of the antitumour agent N-methylformamide in mice.
Toxicology, 34, 173-183.

22. McVie, J.G., ten Bokkel Huinink, W.W., Simonetti, G. and Dubbelman, R. (1984)
Phase 1 trial of N-methylformamide.
Cancer Treat. Rep., 68, 607-610.

23. Wiemann, M.C., Cummings, F.J., Posner, M.R., Weens, J.H., Crabtree, G.W., Birmingham, B.K., Moore, A. and Calabresi, P. (1985)
Phase 1 clinical and pharmacological trial of oral N-methylformamide (NMF).
Proc. Am. Soc. Clin. Oncol., 4, 38.

24. Tauer, K., Kemeny, P. and Hollander, P. (1985)
Phase II trial of N-methylformamide (NMF) in patients with advanced colorectal carcinoma.
Proc. Am. Assoc. Cancer. Res., 26, 169.

25. Laird Myers., W.P., Karnofsky., D.A. and Curchenal, J.H. (1956)
The hepatotoxicity of N-methylformamide in man.
Cancer, 9, 945-954.

26. Whitby, H., Gescher, A. and Levy, L. (1984)
An investigation of the mechanism of the hepatotoxicity of the antitumour agent N-methylformamide in mice.
Biochem. Pharmacol., 33, 295-302.

27. Scailteur, V., Buchet, J.P. and Lauwerys, R. (1981)
Relationship between dimethylformamide metabolism and toxicity.
In "Organ-directed toxicity, chemical indices and mechanisms (eds. S.S. Brown and D.S. Davies) p.163-174, Pergamon, Oxford.

28. Scailteur, V., and Lauwerys, R. (1984)
In vivo metabolism of dimethylformamide and relationship to toxicity in the male rat.
Arch. Toxicol., 56, 87-91.

29. Lundberg, I., Lundberg, A. and Kronevi, I. (1981)
Some observations on Dimethylformamide Hepatotoxicity.
Toxicology, 22, 1-7.

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

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

32. Eberling, C.L. (1980)
Formic acid and derivatives (DMF). in Encyclopedia of chemical technology, 3rd Edition, Volume 11, 263-268. eds. Marks, A.F., Othemar, D.F., Overberger, C.G. and Seaborg, G.T. John wiley & Sons, New York. 1980.

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

34. Chivers, C.P. (1978)
Disulfiram effect from inhalationof Dimethylformamide.
The Lancet (Letter). Feb. 11. p331.

35. Lyle, W.H., Spence., T.W.M., McKinneley, W.M. and Duckers, K. (1979)
Dimethylformamide and alcohol intolerance.
Brit. J. Industr. Med., 30, 63-66.

36. Hanasono, G.K., Fuller, R.W., Broddle, W.D. and Gibson, W.R. (1977)
Studies on the Effects of N,N-Dimethylformamide on Ethanol Disposition and on Monoamine Oxidase Activity in Rats.
Toxicol. Appl. Pharmacol., 39, 461-472.

37. Elovara, E., Marselos, M., and Vainio, H. (1983)
N,N-Dimethylformamide-induced effects on hepatic and renal xenobiotic enzymes with emphasis on aldehyde metabolism in the rat.
Acta. Pharmacol. et toxicol., 53, 159-165.

38. Sharkawi, M. (1979)
Inhibition of alcohol dehydrogenase by dimethylformamide and dimethylsulfoxide.
Toxicol. Lett., 4, 493-497.

39. Barnes, J.R. and Ranta, K.E. (1972)
The metabolism of dimethylformamide and dimethylacetamide.
Toxicol. Appl. Pharmacol., 23, 271-276.

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

41. Kestell, P., Gescher, A. and Slack, J.A. (1985)
The fate of N-methylformamide in mice: Routes of elimination and characterisation of metabolites.
Drug. Metab. Dispos., in press.

42. Scailteur, V., de Hoffman. E., Buchet, J.P., and Lauwerys, R. (1984)
Study on in vivo and in vitro metabolism of dimethylformamide in male and female rats.
Toxicology, 29, 221-234.

43. Barnes, J.R. and Henry, N.W. (1974)
The detemination of N-methylformamide and N-methylacetamide in urine.
Am. Ind. Hyg. Assoc. J., 35, 84-87.

44. Brindley, C., Gescher, A. and Ross, D. (1983).
Studies of the metabolism of dimethylformamide in mice.
Chem. Biol. Interactions, 45, 387-392.

45. Scailteur, V. and Lauwerys, R. (1984).
In vivo and in vitro oxidative biotransformation of dimethylformamide in rat.
Chem. Biol. Interactions, 50, 327-337.

46. Brodie, B.B., Reid, W.D., Cho, A.K., Sipes, G., Krishna, G. and Gillette, J.R. (1971).
Possible mechanism of liver necrosis caused by aromatic organic compounds.
Proc. Natl. Acd. Sci. U.S.A., 68, 160-164.

47. Gooding, P.E., Chayen, J., Sawyer, B.C., and Slater, T.F. (1978)
Cytochrome P-450 Distribution in Rat liver and the effects of sodium phenobarbitone administration.
Chem. Biol. Interact., 20, 299-310.

48. Smith, M.T., Loveridge, N., Wills, E.D. and Chayen, J. (1979).
The distribution of glutathione in the rat liver lobule.
Biochem. J., 182, 103-108.

49. Whitby, H., Chahwala, S.B. and Gescher, A. (1984)
Investigation of the mechanism of hepatotoxicity of N-methylformamide in mice: Effects on calcium sequestration in hepatic microsomes and mitochondria and on hepatic plasma membrane potential.
Biochem. Biophys. Res. Commun., 125, 712-718.

50. Matook, G.M. Spremulli, E.N., Birmingham, B.K., Calabresi, P. and Griffiths, W.C. (1984)
Residue analysis of N-methylformamide: Organ distribution.
J. Analyt. Toxicol., 8, 146-148.

51. Kimmerle, G. and Eben, A. (1975).
Metabolism studies of N,N-dimethylformamide II studies in persons.
Int. Arch. Arbeeitsmed., 34, 127-136.

52. Eben, A. and Kimmerle, G. (1976)
Metabolism studies of N,N-dimethylformamide III studies about the influence of ethanol in persons and laboratory animals.
Int. Arch. Occup. Environ. Hlth., 36, 243-265.

53. Lauwerys, R.R., Rivits, A., Lhoir, M., Rigolet, P., Houbeau, D., Buchet, J.P. and Roels, H.A. (1980).
Biological surveillance of workers exposed to dimethylformamide and the influence of skin protection on its percutaneous absorption.
Int. Arch. Occup. Environ. Health., 45, 189-203.

54. Yonemoto, J. and Suzuki, S. (1980)
Relationship of exposure to dimethylformamide vapour and the metabolite, methylformamide, in urine of workers.
Int. Arch. Occup. Environ. Hlth., 46, 159-165.

55. Krivanek, N.D., McLaughlin, M. and Fayerweather, W.E. (1978)
Monomethylformamide levels in human urine after repetitive exposure to dimethylformamide vapour.
J. Occup. Med., 20, 179-182.

56. Maxfield, M.E., Barnes, J.R., Azar, A. and Trochimowicz, H.T. (1975).
Urinary excretion of metabolite following experimental human exposures to DMF and DMAC.
J. Occup. Med., 17, 506-511.

57. Gescher, A. and Raymont, C. (1981)
Studies on the metabolism of N-methyl containing antitumour agents. $^{14}CO_2$ breath analysis after administration of ^{14}C labelled N-methyl drugs, formaldehyde and formate in mice.
Biochem. Pharmacol., 30, 1245-1251.

58. Gross, N.J., Getz, G.S. and Rabinowitz, M. (1969)
Apparent turnover of mitochondrial phospholipids in the tissues of rat.
J. Biol. Chem., 244, 1552-1556.

59. Brindley, C.J. (1983)
Ph.D. Thesis, Aston University, Birmingham.

60. Mackenzie, C.G. and Frisell, W.R. (1958)
The metabolism of dimethylglcine by liver mitochrondria.
J. Biol. Chem., 323, 417-427.

61. Osborn, M.J., Talbert, P.T. and Huennekens, F.M. (1960)
The structure of active formaldehyde (N^5,N^{10}-methylene-tetrahydrofolic acid)
J. Am. Chem. Soc., 82, 4921-4927.

62. Harper's Review of Biochemistry, 18th Edition (1981). Ed Martin, D.W., Mayes, P.A. and Rodwell, V.W. Lange, Los Altos, California.

63. Waydhas, C., Weigl, K. and Sies, H. (1978)
The disposition of formaldehyde and formate arising from drug N-demethylation dependent on cyt.-P-450 in hepatocytes and in perfused rat liver.
Eur. J. Biochem., 89, 143-150.

64. Krebs, H.A., Hems, R. and Tyler, B. (1976)
The regulation of folate and methionine metabolism.
Biochem, J., 158, 341-353.

65. Nash, T. (1953)
The colorimetric estimation of formaldehyde by means of the Hantzsch reaction.
Biochem. J., 55, 416-421.

66. Larson, A., Orrenius, S., Holmgren, A., Mannervik, B., eds. (1983)
Functions of Glutathione, Biochemical, Physiological, Toxicological and Clinical Aspects.
Raven Press, New York.

67. Meister, A. and Anderson, M.E. (1983)
Glutathione.
Ann. Rev. Biochem., 52, 711-760.

68. Meister, A. (1984)
New aspects of glutathione biochemistry and transport: selective alteration of glutathione metabolism.
Fed. Proc., 53, 3031-3042.

69. Orrenius, S. and Jones, D.P. (1978)
Function of Glutathione in Drug Metabolism.
in Functions of Glutathione in liver and kidney. (Proceedings in Life Sciences) eds. Sies, H. and Wendel, A.
Springer. New York. 164-175.

70. Wills, E.D. (1981)
The role of glutathione in drug metabolism and the protection of the liver against toxic metabolites.
in Testing for Toxicity. eds. Gorrod, J.W., Taylor and Francis.
London. 95-109.

71. Jollow, D.J., Mitchell, J.R., Zampaglione, N. and Gillette, J.R. (1974)
Bromobenzene induced liver necrosis. Protective role of glutathione and evidence for 3,4-bromobenzene epoxide as the hepatotoxic metabolite.
Pharmacology, 11, 151-169.

72. Mitchell, J.R., Jollow, D.J., Potter, W.Z., Gillette, J.R. and Brodie, B.B. (1973)
Acetaminophen induced hepatic necrosis. IV Protective role of glutathione.
J. Pharmacol. Exp. Therap., 187, 211-217.

73. Corcoran, G.B., Racz, W.J., Smith, C.V. and Mitchell, J.R. (1985)
Effects of N-Acetylcysteine on Acetaminophen Covalent Binding and Hepatic Necrosis in Mice.
J. Pharmacol. Exp. Therap., 232, 864-872.

74. Dawson, J.R., Norbeck, K., Anundi, I. and Moldeus, P. (1984)
The effectiveness of N-acetylcysteine in isolated hepatocytes, against the toxicity of paracetamol, acrolein, and paraquat.
Arch. Toxicol., 55, 11-15.

75. Habig, W.H., Pabst, M.J. and Jakoby, W.B. (1974)
Glutathione S-Transferases: The first enzymatic step in mercapturic acid formation.
J. Biol. Chem., 249, 7130-7139.

76. Chasseaud, L.F. (1979)
The role of glutathione and glutathione-S-tranferases in the metabolism of chemical carcinogens and other electrophilic agents.
Adv. Can. Res., 29, 175-274.

77. Jakoby, W.B., Ketterer, B. and Mannervik, B. (1984)
Glutathione transferases: Nomenclature.
Biochem, Pharmacol., 33, 2539-2540.

78. Wendel, A. and Feuerstein, S. (1981)
Drug-induced lipid peroxidation in mice-I: Modulation by monooxygenase activity, glutathione and selenium status.
Biochem. Pharmacol., 30, 2513-2520.

79. Younes, M. and Siegers, C.P. (1984)
Interrelation between lipid peroxidation and other hepatotoxic events.
Biochem. Pharmacol., 33, 2001-2003.

80. Hill, K.E. and Burk, R.F. (1984)
Influence of vitamin E and selenium on glutathione-dependent protection against microsomal lipid peroxidation.
Biochem. Pharmacol., 33, 1065-1068.

81. Beall, J.R. and Ulsamer, A.G. (1984)
Formaldehyde and hepatotoxicity: A review.
J. Toxicol. Environ. Health., 14, 1-21.

82. Ulsamer, A.G., Beall, J.R., Kang, H.K. and Frazier, J.A. (1984)
Overview of health effects of formaldehyde, in Harard Assessment of Chemicals: current developments, vol. 3.
Academic Press, New York (1984) 337-400.

83. d´ a.Heck, H. and Casanova-Schmitz, M. (1984)
Biochemical toxicology of formaldehyde.
Rev. Biochem. Toxicol, 6, 155-189. Ed Hodgson, E., Bend, J.R., Philpot, R.M. Elsevier New York.

84. Krieter, P.A., Ziegler, D.M., Hill, K.E. and Burk, R.F. (1985)
Studies on the biliary efflux of GSH from rat liver due to the metabolism of aminopyrine.
Biochem. Pharmacol., 34, 955-960.

85. Plummer, J.L., Smith, B.R., Sies, H. and Bend, J.R. (1981)
Chemical depletion of glutathione in vivo, in Methods in Enzymology vol 77. Detoxification and drug metabolism: Conjugation and related systems. Ed. W.B. Jakoby. Academic Press. London and New York. 1981. 50-59.

86. Anders, M.W. (1978)
Inhibition and enhancement of microsomal drug metabolism by diethylmaleate.
Biochem. Pharmacol., 27, 1098-1101.

87. Griffith, O.W. (1981)
Depletion of glutathione by inhibition of biosynthesis, in Methods in Enzymology vol 77. Detoxification and drug metabolism: Conjugation and related systems. Ed. W.B. Jakoby. Academic Press. London and New York. 1981. 59-64.

88. Drew, R. and Miners, J.O. (1984)
The effects of buthionine sulphoximine (BSO) on glutathione depletion and xenobiotic biotransformation.
Biochem. Pharmacol., 33, 2989-2994.

89. Lauterburg, B.H., Adams, J.D. and Mitchell, J.R. (1984)
Hepatic glutathione homeostasis in the rat: efflux accounts for glutathione turnover.
Hepatology, 4, 586-590.

90. Meredith, M.J. and Reed, D.J. (1982)
Status of the mitochondrial pool of glutathione in the isolated hepatocyte.
J. Biol. Chem., 257, 3747-3753.

91. Reed, D.J., Brodie, A.E. and Meredith, M.J. (1983)
Cellular heterogeneity in the status and functions of cysteine and glutathione.
in Functions of Glutathione: Physiological, Toxicological, and Clinical Aspects. ed. Larsson, A., Orrenius, S., Holmgren, A. and Mannervik, B. Raven Press, New York. (1983) 39-49.

92. White, R.D., Norton, R. and Bus, J.S. (1984)
The effect of buthione sulfoximine, an inhibitor of glutathione synthesis, on hepatic drug metabolism in the male mouse.
Toxicol. Lett., 23, 25-32.

93. Williamson, J.M. and Meister, A. (1982)
New substrates of 5-oxo-L-prolinase.
J. Biol. Chem., 257, 12039-12042.

94. Nagasawa, H.T., Goon, D.J.W., Muldoon, W.P. and Zera, R.T. (1984)
2-Substituted thiazolidine-4(R)-carboxylic acids as prodrugs of L-cysteine. Protection of mice against acetaminophen hepatotoxicity.
J. Med. Chem., 27, 591-596.

95. Williamson, J.M., Boettcher, B. and Meister, A. (1982)
An intracellular cysteine delivery system that protects against toxicity by promoting glutathione synthesis.
Proc. Natl. Acad. Sci. USA, 79, 6246-6249.

96. Williamson, J.M. and Meister, A. (1981)
Stimulation of hepatic glutathione formation by administration of L-2-oxo-thiazolidine-4-carboxylate, a 5-oxo-L-prolinase substrate.
Proc. Natl. Acad. Sci. USA, 78, 936-939.

97. Puri, R.N. and Meister, A. (1983)
Transport of glutathione, as γ-glutamylcysteinylglycyl ester, into liver and kidney.
Proc. Natl. Acad. Sci. USA, 80, 5258-5260.

98. Inskeep, P.B. and Guengerich, F.P. (1984)
Glutathione-mediated binding of dibromoalkanes to DNA: specificity of rat glutathione-S-transferases and dibromoalkane structure.
Carcinogenesis 5, 805-808.

99. Wolf, C.R., Berry, P.N., Nash, J.A., Green, T. and Lock, E.A. (1984)
Role of microsomal and cytosolic glutathione S-transferases in the conjugation of hexachloro-1:3-butadiene and its possible relevance to toxicity.
J. Pharmacol. Exp. Therap., 228, 202-228.

100. Miller, E.C. and Miller, J.A. (1966)
Mechanism of chemical carcinogenesis: Nature of proximate carcinogens and interactions with macromolecules.
Pharmacol. Rev. 18, 805.

101. Miller, J.A. (1970)
Carcinogenesis by chemicals: An overview - G.H.A. Clowes Memorial Lecture.
Cancer. Res., 30, 559-576.

102. Gorrod, J.W. (1981)
Covalent binding as an indicator of drug toxicity.
In Testing for Toxicity. Ed. J.W. Gorrod. Taylor and Francis Ltd. London pp 77-93.

103. Pohl, L.R. and Branchflower, R.V. (1981)
Covalent binding of electrophilic metabolites to macromolecules.
In Methods in Enzymology, 77, Academic Press 1981 pp43-50.

104. Gillette, J.R. (1974)
A perspective on the role of chemically reactive metabolites of foreign compounds in toxicity I. Correlation of changes in covalent binding of reactivity metabolites with changes in the incidence and severity of toxicity.
Biochem. Pharmacol., 23, 2785-2794.

105. Gillette, J.R. (1974)
A perspective on the role of chemically reactive metabolites of foreign compounds in toxicity II. Alterations in the kinetics of covalent binding.
Biochem. Pharmacol., 23, 2927-2938.

106. Mitchell, J.R., Jollow, D.J., Potter, W.Z., Davis, D.C., Gillette, J.R. and Brodie, B.B. (1973)
Acetaminophen-induced hepatic necrosis.
I., Role of drug metabolism.
J. Pharmacol. Exp. Ther., 187, 185-194.

107. Jollow, D.J., Mitchell, J.R., Potter, W.Z., Davis, D.C., Gillette, J.R. and Brodie, B.B. (1973)
Acetaminophen-induced hepatic necrosis.
II. Role of covalent binding in vivo.
J. Pharmacol. Exp. Ther., 187, 195-202.

108. Potter, W.Z., Davis, D.C., Mitchell, J.R., Jollow, D.J., Gillette, J.R. and Brodie, B.B. (1973)
Acetaminophen-induced hepatic necrosis.
III. Cytochrome P-450-mediated covalent binding in vivo.
J. Pharmacol. Exp. Ther., 187, 203-210.

109. Mitchell, J.R., Jollow, D.J., Potter, W.Z., Gillette, J.R. and Brodie, B.B. (1973)
Acetaminophen-induced hepatic necrosis.
IV. Protective role of glutathione.
J. Pharmacol. Exp. Ther., 187, 211-217.

110. Ioannides, C., Hall, D.E., Mulder, D.E., Steele, C.M., Spickett, J., Delaforge, M. and Parke, D.V. (1983)
A comparison of the protective effects of N-acetylcysteine and S-carboxymethylcysteine against paracetamol-induced hepatotoxicity.
Toxicology, 28, 313-321

111. Tredger, J.M., Smith, H.M., Davis, M. and Williams, R. (1981)
In vitro interactions of sulfur-containing compounds with the hepatic mixed-function oxidase system in mice: Effects on paracetamol activation and covalent binding.
Toxicol. Appl. Pharmacol., 59, 111-121.

112. Massey, T.E. and Racz, W.J. (1981)
Effects of N-acetylcysteine on metabolism, covalent binding, and toxicity of acetaminophen in isolated mouse hepatocytes.
Toxicol. Appl. Pharmacol., 60, 220-228.

113. Hinson, J.A., Monks, T.J., Hong, M., Highet, R.J. and Pohl, L.R. (1982)
3-(Glutathion-S-yl)-acetaminophen: a biliary metabolite of acetaminophen.
Drug Metab. Dispos., 10, 47.

114. Streeter, A.J., Dahlin, D.C., Nelson, S.D. and Baillie, T.A. (1984)
The covalent binding of acetaminophen to protein. Evidence for cysteine residues as major sites of arylation in vitro.
Chem. Biol. Interactions, 48, 349-366.

115. Hoffman, K.J., Streeter, A.J., Axworthy, D.B. and Baillie, T.A. (1985)
Structural characterization of the major covalent adduct formed in vitro between acetaminophen and bovine serum albumin.
Chem. Biol. Interactions, in press.

116. Devalia, J.L., Ogilvie, R.C. and McLean, A.E.M. (1982)
Dissociation of cell death from covalent binding of paracetamol by flavones in a hepayocyte system.
Biochem. Pharmacol., 31, 3745-2749.

117. Devalia, J.L. and McLean, A.E.M. (1983)
Covalent binding and the mechanism of paracetamol toxicity.
Biochem. Pharmacol., 32, 2602-2603.

118. Streeter, A.J., Bjorge, S.M., Axworthy, D.B., Nelson, S.D. and Baillie, T.A. (1984)
The microsomal metabolism and site of covalent binding to protein of 3´-hydroxyacetanilie, a nonhepatotoxic positional isomer of acetaminophen.
Drug Metab. Dispos., 12, 565-576.

119. Dahlin, D.C., Miwa, G.T., Lu, A.Y.H. and Nelson, S.D. (1984)
N-acetyl-p-benzoquinone imine: a cytochrome P-450 mediated oxidation production of acetaminophen.
Proc. Natl. Acad. Sci., 81, 1327-1331.

120. Woodward, K.N. and Timbrell, J.A. (1984)
Acetylhydrazine hepatotoxicity: The role of covalent binding.
Toxicology, 30, 65-74.

121. Schnell, R.G., Bozigian, H.P., Davies, M.H., Merrick, B.A. and Johnson, K.L. (1983)
Circadian Rhythm in Acetaminophen Toxicity. Role of Non protein Sulfhydryls.
Toxicol. Appl. Pharmacol., 71, 353-361.

122. Grady, D and Stott (1965)
U.K. Patent, 1.092. 632.

123. Peng, C.T. (1977)
Sample preparation in liquid scintillation counting, Review 17, The Radiochemical Centre Amersham, England.

124. Soluene-350, Technical publication.
Packard Instrument Company, Inc. 1974. Berkshire, England.

125. Threadgill, M.D. and Gate, E.N. (1983)
Labelled compounds of interest as antitumour agents I: N-methylformamide and N,N-dimethylformamide.
Journal of Labelled Compounds and Radiopharmaceuticals XX, 447-451.

126. Bircher, J. and Preisig, R., (1981)
Exhalation of Isotopic CO_2 in Methods in Enzymology. Volume 77, Detoxification and Drug Metabolism: Conjugation and Related Systems. Ed. W.B. Jokoby. Academic Press. New York 1981. 3-9.

127. Lauterburg, B.H. and Bircher, J. (1976) Expiratory measurement of maximal aminopyrine demethylation in vivo.
J. Pharmacol. Exp. Ther., 197, 501-509.

128. Seuter-Berlage, F., van Dorp, H.L., Kosse, H.G.J. and Henderson, P.H. (1977)
Urinary mercapturic acid excretion as a biological parameter of exposure to alkylating agents.
Int. Arch. Occup. Environ. Health, 39, 45-51.

129. van Doorn, R., Bos, R.P., Leijdekkers, Ch-M., Wagenaar-Zegers, M.A.P., Theuws, J.L.G. and Henderson, P.Th. (1979)
Thioether concentration and mutagenicity of urine from cigarette smokers.
Int. Arch. Occup. Environ. Health, 43, 156-166.

130. Baba, S., Watanabe, Y., Gejyo, F. and Arakawa, M. (1984)
High-performance liquid chromatographic determination of serum aliphatic amines in chronic renal failure.
Clinica Chimica Acta. 136, p.49-56.

131. Taussky, H. and Kurzmann, G. (1954)
A microcolorimetric determination of creatinine in urine by the jaffe reaction.
J. Biol. Chem., 208, 853-861.

132. Ellman, G.L. (1959)
Tissue sulfhydryl groups.
Arch. Biochem. Biophys., 82, 70-77.

133 Tietze, Frank (1969)
Enzymic method for quantitative determination of nanogram amounts of total and oxidised glutathione: Applications to blood and other mammalian tissue.
Analytical Biochemistry, 27, 502-522.

134. Akerboom, T.P.M. and Sies, H. (1981)
Assay of glutathione, glutathione disulfide and glutathione mixed disulfides in biological samples.
Methods in enzymology, 77, 373-382.

135. Oshino, N. and Chance, B. (1976)
Properties of glutathione release observed during reduction of organic hydroperoxides, demethylation of aminopyrine and oxidation of some substances in perfused rat liver and their implications for the physiological function of catalase.
Biochem, J., 162, 509-525.

136. Conney, A.H., Davison, C., Gastell, R. and Burns, J.J. (1960)
Adaptive increases in drug metabolising enzymes induced by phenobarbital and other drugs.
J. Pharmac. Exp. Ther., 130, 1-8.

137. Cadenas, E., Muller, A., Bridelius, R., Esterbauer, H. and Sies, H. (1983)
Effects of 4-hydroxynonenal on isolated hepatocytes.
Biochem, J., 214, 479-487.

138. Reddrop, C.J., Cheesman, K.H. and Slater, T.F. (1983)
Correlations between common tests for assessment of liver damage: indices of the hepatoprotective activity of promethazine in carbon tetrachloride hepatotoxicity.
Cell Biochemistry and Function, 1, 55-63.

139. Barlow, T.J. (1982)
MSc Thesis, Aston University, Birmingham.

140. Mukundrai, M. (1982)
MSc Thesis, Aston University, Birmingham.

141. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951)
Protein measurement with the Folin-Phenol reagent.
J. Biol. Chem., 193, 265-275.

142. Kornbrust, D.J., Bus, J.S., Doerjer, G. and Swenberg, J.A. (1982)
Association of inhaled [^{14}C]methyl chloride with macromolecules from various rat tissues.
Toxicol. Appl. Pharmacol., 65, 122-134.

143. Nebert, D.W. (1978)
Genetic differences in microsomal electron transport: The Ah locus.
in Methods in Enzymology Vol 52 (c). Eds. Sidney Fleisher and Lester Packer. Academic Press. New York. 1978 p226-240.

144. Rose, C.I. and Henderson, A.R. (1975)
Reaction-rate assay of serum sorbitol dehydrogenase activity at 37^{o}C.
Clin. Chem., 21, 1619-1626.

145. Kachmar, J.F. and Moss, D.W. (1976)
Enzymes - The Transminases
In Fundamentals of Clin. Chem. (Ed. Tyetz, N.W.)
W.B.Saunders Co. Philadelphia; pp672-681.

146. Conover, W.J. (1980) Practical Non-Parametric statistics 2nd Edition, John Wiley. New York, pp216-223.

147. Hantzsch, A.R. (1882)
Ueber die synthese pyridinartiger verbindungen aus acetessigather und aldehydammoniak.
Justus Leibig's Ann. Chem., 215, 1-82.

148. Werringloer, J. (1978)
Assay of Formaldehyde generated during microsomal oxidation reactions.
In: Methods in Enzymology Vol 52 (c). Eds. Sidney Fleisher and Lester Packer. Academic Press, New York, 1978 pp297-302.

149. Carelton, H.M. (1980)
Carelton's Histological Technique 5th Edition.
Oxford University Press, Oxford 1980, pp137-142.

150. Levine, W.G. and Finklestein, T.T. (1979)
A role for liver glutathione in the hepatobiliary fate of N,N-dimethyl-4-aminoazobenzene.
J. Pharmacol. Exp. Ther., 208, 399-405.

151. Lauterburg, B.H., Vaishnav, Y., Stillwell, W.G. and Mitchell, J.K., (1980)
The effect of age and glutathione depletion on hepatic glutathione turnover in vivo determined by Acetaminophen probe analysis.
J. Pharmacol. Exp. Ther., 213, 54-58.

152. Barhart, J. L. and Coombes, B. (1978)
Choleresis associated with metabolism and biliary excretion of diethylmaleate in the rat and dog.
J. Pharmacol. Exp. Ther., 206, 614-623.

153. Green, M.D. and Fischr, L.J. (1984)
Hepatotoxicity of acetaminophen in neonatal and young rats.
Toxicol. Appl. Pharmacol., 74, 125-133.

154. Freeman, R.W., MacDonald, J.S., Olson, R.D., Boerth, R.C., Oates, J.A. and Harbison, R.D. (1980)
Effect of sulfhydryl-containing compounds on the antitumour effects of adriamycin.
Toxicol. Appl. Pharmacol., 54, 168-175.

155. Olson, R.D., MacDonald, J.S., vanBoxtel, C.J., Boerth, R.C., Harbison, R.D., Slonim, A.E., Freeman, R.W. and Oates, J.A. (1980)
Regulatory role of glutathione and soluble sulfhydryl groups in the toxicity of adriamycin.
J. Pharmacol. Exp. Ther., 215, 450-454.

156. Kato, R., Chiesara, E. and Vassanelli, P. (1964)
Further studies on the inhibition and stimulation of microsomal drug-metabolishing enzymes of rat liver by various compounds.
Biochem. Pharmacol., 13, 69-83.

157. Anders, M.W. and Mannering, G.J. (1966)
Kinetics of the inhibition of the N-demethylation of ethylmorphine by SKF525A and related compounds.
Molecular Pharmacology, 2, 319-327.

158. Videla, L.A. (1984)
Chemically induced antioxidant-sensitive respiration.
FEBS, 178, 119-122.

159. Burk, R.F., Patel, K. and Lane, J.M. (1983)
Reduced glutathione protection against rat liver microsomal injury by carbon tetrachloride dependence on O_2.
Biochem.J., 215, 441-445.

160. Pohl, L.R., Schulick, R.D., Highet, R.J., and George, J.W. (1983)
Reductive-oxygenation mechanism of metabolism of carbon tetrachloride to phosgene by cytochrome P-450.
Molecular Pharmacology, 25, 318-321.

161. Corongui, F.P., Vargliolo, S., Milia, A., Cheesman, R.H. and Slater, T.F. (1985)
Antioxidants and lipid peroxidation.
Biochem. Pharmacol., 34, 397-398.

162. Burke, M.D. (1981)
Cytochrome P-450: A pharmacological necessity or a biological curiosity?
Biochem. Pharmacol., 30, 181-187.

163. Albano, E., Poli, G., Chiarpotto, E., D´Anelli, N. Dianzani, M.U. (1981)
Promethazine protection against paracetamol induced glutathione depletion in isolated hepatocytes.
IRCS Medical Science, 9, 961-962.

164. Lauterburg, B.H., Corcoran, G.B. and Mitchell, J.R. (1983)
Mechanism of action of N-acetylcysteine in the protection against the hepatotoxicity of acetaminophen in rats in vivo.
J. Clin. Invest., 71, 980-991.

165. Jollow, D.J., Potter, W.Z., Hashimoto, M., Davis, D.C. and Mitchell, J.R. (1972)
Acetaminophen-induced hepatic necrosis IV. Role of glutathione.
Fed. Proc. Fed. Am. Soc. Exp. Biol., 31, 539.

166. Fernandez, G., Villarreul, M.C. and Castro, J.A. (1978)
Mechanism of the drug-metabolizing enzymes´ induction by 2-diethylaminoethyl -2-2-diphenylvalerate-HCl (SKF525A).
Toxicol. Appl. Pharmacol., 46, 315-321.

167. Bornheim. L.M., Peters, P.G. and Franklin, M.R. (1983)
The induction of multiple forms of cytochrome P-450 by SKF525A.
Chem. Biol. Interactions, 47, 45-55.

168. Dahl, M.G.C., Gregory, M.M. and Scheuer, P.J. (1971)
Liver damage due to methotrexate in patients with psoriasis.
Br. Med. J., 1, 625.

169. Suarez, K.A., Griffin, K., Kopplin, R.P. and Bhonsle, P. (1981)
Protective effect of diethylmaleate pretreatment on carbon tetrachloride hepatotoxicity.
Toxicol. Appl. Pharmacol., 57, 318-324.

170. Green, C.E., Dabbs, J.E. and Tyson, C.A. (1984)
Metabolism and cytotoxicity of acetaminophen in hepatocytes isolated from resistant and susceptible species.
Toxicol. Appl. Pharmacol., 76, 139-149.

171. D´Arcy Doherty, M. and Cohen, G.M. (1984)
Metabolic activation of 1-Naphthol by rat liver microsomes to 1,4-naphthoquinone and covalent binding species.
Biochem. Pharmacol, 33, 3209-3212.

172. Ames, M.M., Nelson, S.D., Lovenberg, W. and Sasame, H.A. (1977)
Metabolic activation of para-chloroamphetamine to a chemically reactive metabolite.
Communications in Psychopharmacology, 1, 455-460.

173. Fitzhugh, D.G. and Nelson, A.A. (1948)
Liver tumours in rats fed thiourea or thioacetamide.
Science, 108, 626-628.

174. Ambrose, A.M., DeEds, F. and Ratter, L.F. (1949)
Toxicity of thioacetamide in rats.
J. Ind. Hyg. Toxicol., 31, 158-161.

175. Gupta, D.N. (1956)
Nodular cirrhosis and metastasising tumour produced in the livers of rats by prolonged feeding with thioacetamide.
J. Path. Bact., 72, 415-425.

176. Trennery, P.N. and Waring, R.H. (1983)
Early changes in thioacetamide-induced liver damage.
Toxicol. Lett., 19, 299-307.

177. Hunter, A.L., Holscher, M.A. and Neal, R.A. (1977)
Thioacetamide induced hepatic necrosis I: Involvement of the mixed function oxidase system.
Pharmacol. Exp. Ther., 200, 439-448.

178. Dyroff, M.C. and Neal, R.A. (1982)
Studies of the mechanism of metabolism of thioacetamide S-oxide by rat liver microsomes.
Molecular Pharmacology, 23, 219-227.

179. Bold, M.S. and Ziegler, D.M. (1973)
Dimethylaniline N-oxidase and aminopyrine N-demethylase activities of human liver tissue.
Xenobiotica, 3, 179-189.

180. Ziegler, D.M. (1980)
Microsomal flavin-containing monooxygenase:Oxygenation of nucleophilic nitrogen and sulfur compounds
In: Enzymatic Basis of Detoxification Volume 1.
Ed: W.B. Jakoby. Academic Press, London (1980) p.201-227.

181. Gorrod, J.W. (1978)
The current status of the pKa concept in the differentiation of enzymic N-oxidation.
In: Enzymatic Oxidation of Nitrogen.
Ed: J.W. Gorrod, Elsevier, L (1978) p.201-210.

182. Poulsen, L.L., Hyslop, R.M. and Ziegler, D.M. (1979)
S-Oxygenation of N-substituted thioureas catalyzed by the pig liver microsomal FAD-containing monooxygenase.
Arch. Biochem. Biophys., 198, 78-88.

183. Gudzinowicz, M.J. and Neal, R.A. (1981)
Differentiation of rat liver microsomal amine oxidase and cytochrome P-450 catalysed metabolism of thioacetamide (TAA) and thioacetamide S-oxide (TASO).
Toxicologist, 1, 67.

184. Kedderis, G.L. and Rickert, D.E. (1984)
Loss of rat liver microsomal cytochrome P-450 during methimazole metabolism.
Drug Metab. Dispos., 13, 58-61.

185. Winston, G.W., Feierman, D.E. and Cederbaum, A.I. (1984)
The role of iron chelates in hydroxyl radical production by rat liver microsomes, NADPH-cytochrome P-450 reductase and xanthine oxidase.
Arch. Biochem. Biophys., 232, 378-390.

186. Klein, S.M., Cohen, G. and Cederbaum, A.I. (1981)
Production of formaldehyde during metabolism of dimethyl sulfoxide by hydroxyl radical generating systems.
Biochemistry, 20, 6006-6012.

187. Whitby, H.M. PhD thesis (1982)
Aston University, Birmingham.

188. Zimmerman, H.J. (1978)
Vulnerability of the liver to toxic injury.
In: Hepatotoxicity - the adverse effects of drugs and other chemicals on the liver.
Appleton-Century-Crofts, New York; pp.32-46.

189. Gerlach, U. and Hiby, W. (1974)
Sorbitol dehydrogenase
In: Methods of Enzymatic Analysis 2, Ed. H.U. Bergmayer, Academic Press, New York, N.Y., p.569-573.

190. Woodman, D.D. (1981)
Plasma enzymes in drug toxicity
In: Testing for Toxicity. Ed. J.W. Gorrod. Taylor and Francis, London, p.145-156.

191. Prescott, L.F., (1979)
The nephrotoxicity and hepatotoxicity of antipyretic analgesics.
Br. J. Clin. Pharmac., 1, 453-462.

192. Chieli, E. and Malvaldi, G. (1985)
Role of the P-450 dependent and FAD-containing monooxygenases in the bioactivation of thioacetamide, thiobenzamide and their sulphoxides.
Biochem. Pharmacol., 34, 395-396.

193. Nakata, R., Tsukamoto, I., Miyoshi, M. and Kojo, S. (1985)
Liver regeneration after carbon tetrachloride intoxication in the rat.
Biochem. Pharmacol., 34, 586-588.

194. Smith, C.V., Hughes, H, Lauterburg, B.H. and Mitchell, J.R. (1983)
Chemical Nature of Reactive Metabolites determines. Their biological Interactions with Glutathione in Functions of Glutathione. Biochemical, Physiological, Toxicological, and clinical aspects. Eds. A. Larsson, S. Orrenius, A. Holmgren and B. Mannervik.
Raven Press, New York, 1983. pp. 125-137.

195. Dyroff, M.C., and Neal, R.A. (1981)
Identification of the Major Protein Adduct Formed in Rat Liver after Thioacetamide Administration.
Cancer. Res., 41, 3430-3435.

196. Marginson, G.P., Marginson, J.M. and Montesano, R. (1977)
Accumulation of O^6-Methylguanine in Non-Target-Tissue Deoxyribonucleic Acid during Chronic Administration of Dimethylnitrosamine.
Biochem. J., 165, 463-468.

197. Gibson, N.W. and Hickman, J.A. (1982)
The role of isocyanates in the toxicity of antitumour haloalkynitrosoureas.
Biochem. Pharmacol., 31, 2795-2800.

198. Twu, J. and Wold, F. (1973)
Butylisocyanate - an active site specific reagent for yeast alcohol dehydrogenase.
Biochem., 12, 381-386.

199. Babson, J.R. and Reed, D.J. (1978)
Inactivation of glutathione reductase by 2-chloroethyl nitrosourea-derived isocyanate.
Biochem. Biophys. Res. Commun., 83, 754-762.