THE ROLE OF METABOLISM IN THE TOXICITY OF N-ALKYLFORMAMIDES.

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by

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The industrial solvent N,N-dimethylformamide (DMF) and the investigational anti-tumour agent N-methylformamide (NMF) cause liver damage in rodents and humans. The hepatotoxicity of N-alkylformamides is linked to their metabolism to N-alkylcarbamic acid thioesters. The enzymatic details of this pathway were investigated. Hepatocytes isolated from BALB/c mice which had been pretreated with acetone, an inducer of the cytochrome P-450 isozyme CYP2E1, were incubated with NMF (10mM). NMF caused extensive toxicity (>90%) as determined by lactate dehydrogenase (LDH) release, compared to cells from untreated animals. Incubation of liver cells with NMF for 6 hrs caused 60±17% LDH release whilst in the presence of DMSO (10mM), an alternative substrate for CYP2E1, LDH release was reduced to 20±10%.

The metabolism of NMF to S-(N-methylcarbamoyl)glutathione (SMG) was measured in incubates with liver microsomes from mice, rats or humans. Metabolism of NMF was elevated in microsomes isolated from rats and mice pretreated with acetone, by 339% and 183%, respectively. Pretreatment of animals with 4-methylpyrazole induced the metabolism of NMF to 280% by rat microsomes, but was without effect on NMF metabolism by mouse microsomes. The CYP2E1 inhibitors or alternative substrates diethyl dithiocarbamate (DEDTC), p-nitrophenol (PNP) and dimethyl sulphoxide (DMSO) strongly inhibited the metabolism of NMF in suspensions of rat liver microsomes, at concentrations which did not effect aminopyrine N-demethylation. The rate of metabolism of NMF to SMG in human microsomes correlated ($r>0.8$) with the rate of metabolism of chlorzoxazone, a CYP2E1 probe. A polyclonal antibody against rat CYP2E1 (10mg/nmol P-450) inhibited NMF metabolism in microsomes from rats and humans by 75% and 80%, respectively. The amount of immunoblottable enzyme in human microsomes, determined using an anti-rat CYP2E1 antibody, correlated with the rate of NMF metabolism ($r>0.8$). Purified rat CYP2E1 catalysed the generation of SMG from NMF.

Formation of the DMF metabolite N-hydroxymethyl-N-methylformamide (HMMF) in incubations with rat liver microsomes was elevated by 200% following pretreatment of animals with acetone. Co-incubation with DEDTC (100μM) inhibited HMMF generation from DMF by 88%. Co-incubation of DMF (10mM) with NMF (1mM) inhibited the formation of SMG by 95%. A polyclonal antibody against rat CYP2E1 (10mg/nmol P-450) inhibited generation of HMMF in incubates with rat and human liver microsomes by 68.4% and 67.5%, respectively. Purified rat CYP2E1 catalysed the generation of HMMF from DMF.

Using ionspray tandem mass spectrometry the glutathione conjugate SMG was identified as a biliary metabolite of DMF in rats (0.003% of a dose of 500mg/kg DMF i.p.). Formation of this metabolite was increased five fold after induction of CYP2E1 by acetone, and was inhibited to 20% of control values following pretreatment with disulfiram. Generation of SMG from DMF *in vivo* was shown to exhibit a large kinetic deuterium isotope effect ($K_H/K_D=10.1 \pm 1.3$), which most likely represents the product of 2 discrete isotope effects on N-demethylation and formyl oxidation reactions.

Keywords: N-alkylformamides, N-methylformamide (NMF), N,N-dimethylformamide (DMF), CYP2E1, metabolic activation.

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Abbreviations

AMCC	N-acetyl-S-(N-methylcarbamoyl)cysteine
AMPS	ammonium persulphate
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BNF	β–naphthoflavone
BSA	bovine serum albumen
CO	carbon monoxide
CYP	cytochrome P-450 isozyme
CYP2E1	ethanol-inducible cytochrome P-450 isozyme (also human, rat P-450j, rabbit P-4503a)
D	^2H
DEDTC	diethyl dithiocarbamate
DLPC	dilauroyl phosphatidyl choline
DMF	N,N-dimethylformamide
DMSO	dimethyl sulphoxide
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EMC	ethyl N-methylcarbamate
F	formamide
GC	gas chromatography
GSH	glutathione
HBSS	hanks buffered saline solution
HCHO	formaldehyde
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane-sulphonic acid
HMF	N-(hydroxymethyl)formamide
HMMF	N-(hydroxymethyl)-N-methylformamide
HPLC	high performance liquid chromatography
hr	hour
i.p.	intraperitoneal
i.v.	intravenous
KPi	potassium phosphate

LCMS	liquid chromatography mass spectrometry
LDH	lactate dehydrogenase
MIC	methyl isocyanate
min	minute
4-MP	4-methylpyrazole
MS	mass spectrometry
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	NADP reduced form
NaPi	sodium phosphate
NBT	nitro blue tetrazolium
4-NC	4-nitrocatechol
NDMA	N-nitrosodimethylamine
NEF	N-ethylformamide
NMF	N-methylformamide
NMR	nuclear magnetic resonance
P	probability
P-450	cytochrome P-450 superfamily
PBS	phosphate buffered saline
PEITC	phenethyl isothiocyanate
PMSF	phenylmethyl sulfonyl fluoride
PNP	p-nitrophenol
r.p.m.	revolutions per minute
SD	standard deviation
SDH	sorbitol dehydrogenase
SDS-PAGE	sodium dodecylsulphate-polyacrylamide gel electrophoresis
SMG	S-(N-methylcarbamoyl)glutathione
TEMED	N,N,N,N-tetramethylethylene diamine
Tris	tris(hydroxymethyl)methylamine
UV	ultra-violet

Section 1

Introduction

1.1 Foreword.

Our environment is contaminated by more than 4 million different synthetic compounds. According to estimations performed by the Environmental Protection Agency and the Food and Drug Administration in the USA more than 63,000 chemicals are in common use. If absorbed, those chemicals which are not water soluble have to be modified in the body to more polar compounds, before efficient excretion is possible. Knowledge of the ability of the body to metabolise compounds foreign to it dates back to the late 1860's. Most of the major pathways of drug metabolism were described prior to 1900. However, several important types of metabolism were not discovered until much later.

Since 1950 there have been many important contributions in the field of drug metabolism. It has increasingly been recognised that drug metabolism does not necessarily mean detoxification, but that highly toxic compounds might be formed in biological systems from compounds functioning as primary precursors. During the biotransformation process chemically very reactive intermediates are often formed, which may combine covalently with various important macromolecules such as RNA, DNA and proteins.

A major event in this area of research was the discovery of drug metabolising enzymes. In the 1950's B.B. Brodie and his colleagues described the microsomal-NADPH-O_2 system for metabolising enzymes (Brodie and Maickel, 1958). The cytochrome P-450 class of enzymes was subsequently identified as the terminal oxidase of the liver enzyme system responsible for the oxidative metabolism of many drugs (Cooper *et al*, 1965). What is now known to be the cytochrome P-450 gene superfamily encodes numerous enzymes that are remarkable in the variety of chemical reactions which they can catalyse and in the number of substrates which they accommodate. It is not an exaggeration to state that P-450 is one of the most versatile biological catalysts known.

In recent years cytochrome P-450 has become the subject of intensive research in many laboratories for two reasons:

i) to broaden the understanding of the remarkable versatility of this family of enzymes;

ii) to elucidate the relevance of P-450 catalysed reactions for the understanding of many endocrinological, pharmacological and toxicological findings.

The N-alkylformamides N-methylformamide (NMF) and N,N-dimethylformamide (DMF) exhibit hepatotoxic properties. In section 1.4 the evidence will be reviewed which suggests that the toxicity of these compounds is linked to their metabolism, via generation of a reactive intermediate. It is believed that this metabolism is catalysed by cytochrome P-450. The following chapter provides a brief review of our current understanding of formamide-induced toxicity and the role of cytochrome P-450 in drug metabolism. It provides a background for the hypotheses which have been tested in the work presented in this thesis.

1.2 The cytochrome P-450 mixed function oxidase system.

1.2.1 Diversity of cytochrome P-450.

Evolution of P-450 is believed to have begun 2-3 billion years ago with only a few genes coding for P-450 forms which were engaged in the metabolism of endogenous substrates. Early P-450s may have evolved to metabolise cholesterol and fatty acids. The reason for the increasing number of P-450 genes during the past several hundred million years is unclear. P-450 diversification may have coincided with the emergence of aquatic vertebrates onto land. As animals began to eat plants so plants, as a means of defense, countered by developing new stress metabolites (phytoalexins) to make them less palatable and/or digestible. Animals then responded with new P-450 genes to detoxify these phytoalexins (Gonzalez and Nebert, 1990). The animals responded by increasing the frequency of P-450 gene duplication and conversions, giving rise to genes coding for new P-450 forms which metabolise xenobiotics and/or endogenous substrates. A tremendous expansion in the P-450 family has occurred over the past 400 million years. Humans and rats are believed to have diverged from common ancestors some 80 million years ago and rats and mice diverged from each other less than 20 million years ago (Nebert *et al*, 1988). A consequence of human P-450 gene evolution is the polymorphism in drug metabolism leading to marked differences in response of individuals to the toxic and carcinogenic effects of drugs and other environmental chemicals (Gonzalez, 1989).

To date the cytochromes P-450 comprise a superfamily which includes 27 families, each having up to 8 subfamilies (A to H in CYP2), which may contain up to 23 individual forms (1 to 23 in CYP2C) (Nebert *et al*, 1991). The same form has been shown to contain up to 7 orthologues in the case of CYP1A1 (one for a particular animal species), and various strains of the same species may have slightly different but essentially identical forms. By definition, a family contains P-450 forms with greater than 39% sequence homology, while forms in a mammalian subfamily are more than 59% similar (Nebert *et al*, 1991). The original trivial names have been superceded by the recommended nomenclature of Nebert *et al* (1991). For example CYP11B, CYP17 and CYP21 were originally named 11β, 11α and c21 reflecting the position of the carbon in the steroid molecule which they hydroxylate.

The highest number of P-450 forms found so far are in the various strains of rats. In the search for new forms of P-450 it is important to characterise the protein and compare it with known forms. Table 1.1 summarises the more important physical and chemical properties of rat P-450 forms (Soucek and Gut, 1992). The first column gives the relative molecular weights determined by electrophoresis (SDS-PAGE) or calculations from known amino acid sequences of the protein. These values range between 48 and 56 KDa. The second column presents spectral properties, giving absorbance maxima of the absolute spectra of reduced and oxidised P-450, and differential spectra of the reduced P-450 with carbon monoxide (between 447 and 452 nm). The third column lists the forms which react with monoclonal or polyclonal antibodies against a given (different) form and serves to demonstrate similarities between epitopes or the purity of the form. The last column presents the sequence of the last 19 amino acids for the NH_2-terminal of the P-450 apoprotein.

Table 1.1

Physicochemical characteristics of rat liver microsomal P-450 proteins.

CYP	Relative molecular weight [1]	Spectral characteristics Fe^{2+}	Fe^{3+}	$Fe^{2+}CO$	Immuno cross-reactivity [2]	N-sequence
1A1	56 (59.4)	409	417	447	poly-1A2 mono-1A2	MPSVYGFPAF TSATELLLA-
1A2	53 (58.2)	410	392	447	poly-1A1 mono-1A1	MAFSQYISLA PELLLATAI-
2A1	48 (56)	411	417	452	poly-ND	MLDTGLLLVV ILASLSVML-
2A2	49 (56.3)	413	416	449	poly-ND	MLDTGLLLVV ILASLSVMF-
2B1	52 (55.9)	410	417	450	poly-2B2, 2C7/12 mono-2B2	MEPSILLLLA LLVGFLLLL-
2B2	52.5 (55.9)	413	395 415	450.6	poly-2B1, 2C7/12 mono-2B1	same as 2B1
2C6	50 (55.4)	414	417	451	poly-ND	MDLVMLLVLT LTSLILLSI-
2C7	51 (56.2)	414	394 417	447.5	poly-2B1/2 2C11/12/13 2E1	MDLVTFLVLT LSSLILISL-
2C11	51 (57.2)	415	417	450.7	poly-2C12	MDPVLVLVLT LSSLLLLSL-
2C12	50.5	414	417	449	poly-2C11	MDPFVVLVLS LSFLLLLYL-
2C13	50 (55.9)	414	417	447.5	poly-2C6/7 2C11/12	MDPVVVLLLS LFFLLF-
2D1	52	—	417	449.2	poly-ND	MELLNGTGLW SMAIFTVIF-
2E1	51.6 (56.6)	412	395	451.5	poly-ND	MAVLGITIAL LVWVATLLV-
3A1	51 (57.9)	418	420	450	poly-ND	MDLLSALTLE TWVLLAVVL-
3A2	52	410	417	449	poly-ND mono-2B1/2	MDLIFMLETS SLLLA-
3A4	51.5 (58.2)	—	396	451.8	poly-ND	MSVSALSSTR FTGSISGFL-

1 Molecular weight KDa, evaluated by SDS-PAGE (estimation according to known amino acid sequence).
2 ND-not detected.

There is now a considerable body of information on the P-450 forms in rats, and there has also been an increasing amount of data concerning the P-450 forms in humans which allows for comparison between these two species. Human forms have been less extensively studied, but there is considerable information on the polymorphic distribution of P-450 forms specifically active in the metabolism of certain drugs, for example debrisoquine (Gonzalez *et al*, 1988), phenacetin (Distlerath *et al*, 1985), mephenytoin (Srivastava, 1991) and nifedipine (Kleinbloesem *et al*, 1985). Currently available data indicate the expression of at least 16 subfamilies of microsomal P-450 in humans, four being mainly extrahepatic (Guengerich, 1992). In some cases the proteins and their genes have been characterised and in others they await further characterisation. Not much is currently known about gene structure and regulation. Despite this, the importance of individual human P-450s may be studied by specific inhibitors (eg quinidine for CYP2D1) and also under conditions of specific induction (eg smoking for CYP1A1).

Extrapolation between animals and humans is rather difficult since in contrast to experimental animals the human population is markedly outbred and there are also greater variations in diet and exposure to inducing xenobiotics. The identification of a number of human orthologous forms by use of antibodies and DNA probes has been achieved (Gonzalez *et al*, 1991). It has been found that orthologous forms from different species, coded for by closely related genes, may differ markedly in catalysis, eg human CYP2C9 has marked sequence similarity with certain rat and rabbit proteins whose catalytic activities are significantly different (Guengerich, 1989). Table 1.2 presents a survey of all rat and human forms known at the end of 1990 (Soucek and Gut, 1992) and also their sequential homologies where known. Clearly similarities are high, at around 70%, and to some extent this may be attributed to the identification of conserved regions (for P-450 reductase, haem, signal peptide) which increase the similarity. On the other hand, a change in a single amino acid may markedly or even completely alter the P-450 function, increase or reduce its activity, or even change its substrate specificity completely (Lindberg and Negishi, 1989).

Table 1.2

Sequential homology between rat and human P-450 forms.

P-450 form		sequential homology
rat	human orthologue	% cDNA similarity (amino acid)
CYP1A1	CYP1A1	80 (78)
CYP1A2	CYP1A2	75 (70)
CYP2A1	—	—
CYP2A2	—	—
CYP2A3	CYP2A6	(85)
—	CYP2A7	—
CYP2B1	CYP2B6	78 (74)
	CYP2B7	(76)
CYP2B2	—	—
CYP2B3	—	—
CYP2B18	—	—
CYP2C6	CYP2C10	(75)
CYP2C7	—	—
CYP2C11	CYP2C9	80 (77)
CYP2C12	—	—
CYP2C13	CYP2C8	74 (68)
CYP2D1	CYP2D6	(71)
CYP2D2	—	—
CYP2D3	—	—
CYP2D4	—	—
CYP2D5	—	—
—	CYP2D7	—
—	CYP2D8	—
CYP2E1	CYP2E1	75 (78)
—	CYP2F1	—
CYP2G1	—	—
CYP3A1	CYP3A3	(78)
—	CYP3A4	(73)
CYP3A2	CYP3A5	(71)
	CYP3A7	(65)
CYP3A9	—	—
CYP4A1	CYP4A9	—
CYP4A2	—	—
CYP4A3	—	—
CYP4A8	—	—
CYP4B1	CYP4B1	(>80)
CYP7	CYP7	(82)
CYP11A1	CYP11A1	79 (76)
CYP11B1	CYP11B1	(67)
CYP17	CYP17	74
CYP19	CYP19	80 (78)
—	CYP21A2	—
CYP27	CYP27	—

1.2.2 Mechanism of catalytic activity.

The cytochrome P-450 dependent mixed function oxidases catalyse a wide variety of reactions including N-dealkylations, O-dealkylations, S-oxidations and hydroxylation of aliphatic and aromatic substrates. Our knowledge of the scope of P-450 catalysed reactions is still incomplete. The cytochrome is widespread in nature and many isoforms have yet to be fully characterised or even identified. Animals, plants and micro-organisms contain P-450 and in mammals the enzyme system has been found in all tissues examined (Porter and Coon, 1991). P-450 is found predominantly in the endoplasmic reticulum and mitochondria, and in greatest abundance in the liver. As shown in Table 1.3 the substrates for P-450 encompass a host of xenobiotics and a wide variety of physiologically occurring compounds.

Table 1.3

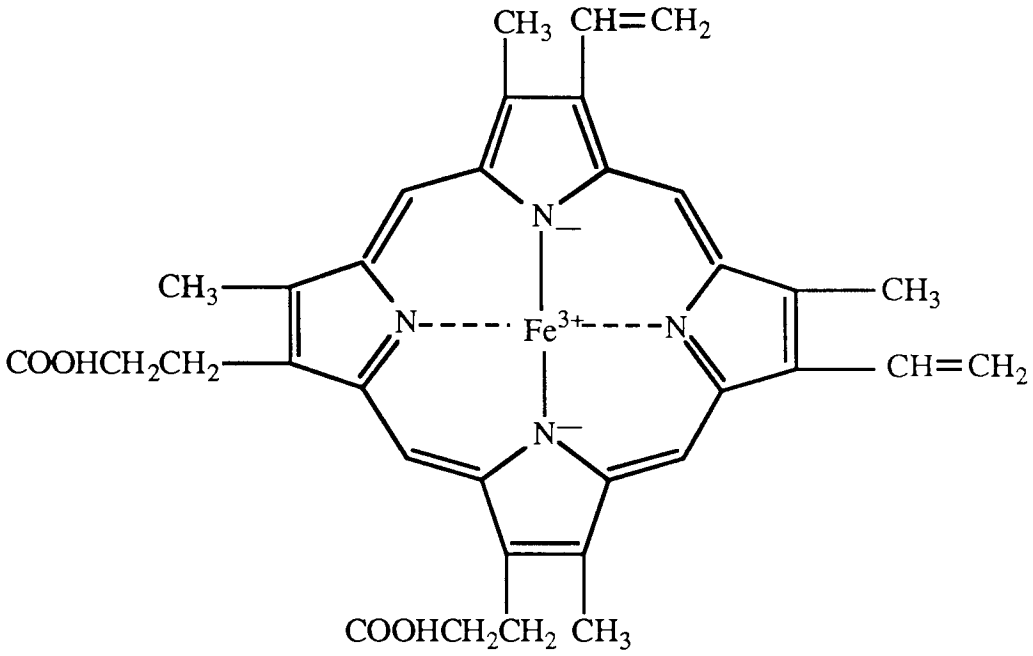
Substrates for Cytochrome P-450.

Xenobiotics	Physiologically occurring compounds
Drugs	Steroids
Carcinogens	Eicosanoids
Antioxidants	Fatty acids
Solvents	Lipid hydroperoxides
Anaesthetics	Retinoids
Dyes	Acetone, acetol
Pesticides	
Petroleum products	
Alcohols	
Odorants	

Cytochrome P-450 is the terminal oxidase component of an electron transport system present in the endoplasmic reticulum responsible for many oxidation reactions. It is classified as a haem-containing enzyme, a haemoprotein (Sato *et al*, 1965). The active site contains iron protoporphyrin IX bound in part by hydrophobic forces (Figure 1.1). The fifth ligand is a thiolate anion provided by a cysteine residue and the sixth coordinate position may be occupied by an exchangeable water molecule. Upon reduction of the iron, O_2 can be bound

Figure 1.1

Structure of ferric protoporphyrin IX.



to the sixth position (White and Coon, 1982). The haem is noncovalently bound to the apoprotein which has a different structure for each isozyme and is therefore responsible for conferring the property of different substrate specificities on individual isozymes. The name cytochrome P-450 is derived from the fact that the cytochrome exhibits spectral absorbance maxima at 450nm when reduced and complexed with carbon monoxide (Omura and Sato, 1962). The haemoprotein serves as both the oxygen- and substrate-binding locus for the mixed function oxidase reaction and undergoes cyclical oxidation/reduction of the haem iron which is mandatory for catalytic function (see Figure 1.2).

The P-450 family includes many different isozymes and more than 150 isoforms have been characterised (Porter and Coon, 1991). The purified isozymes exhibit different molecular weights, amino acid compositions, peptide maps, amino acid sequences, spectral, immunochemical and catalytic properties. They also exhibit different, but sometimes

overlapping, substrate specificities. However, the catalytic mechanisms by which the P-450's activate molecular oxygen appear to be very similar.

The diversity of the reaction catalysed by P-450 can be understood by considering that most of the reactions begin with the transfer of electrons from NADPH to NADPH-cytochrome P-450 reductase in the microsomal system. This electron transfer leads on to reductive activation of molecular oxygen followed by insertion of an oxygen atom into the substrate. Reactions which are catalysed include hydroxylations, epoxidations, deaminations, dealkylations and dehalogenations.

The overall reaction catalysed by P-450 proceeds with the stoichiometry shown below:

$$RH + O_2 + NADPH + H^+ \longrightarrow ROH + H_2O + NADP^+$$

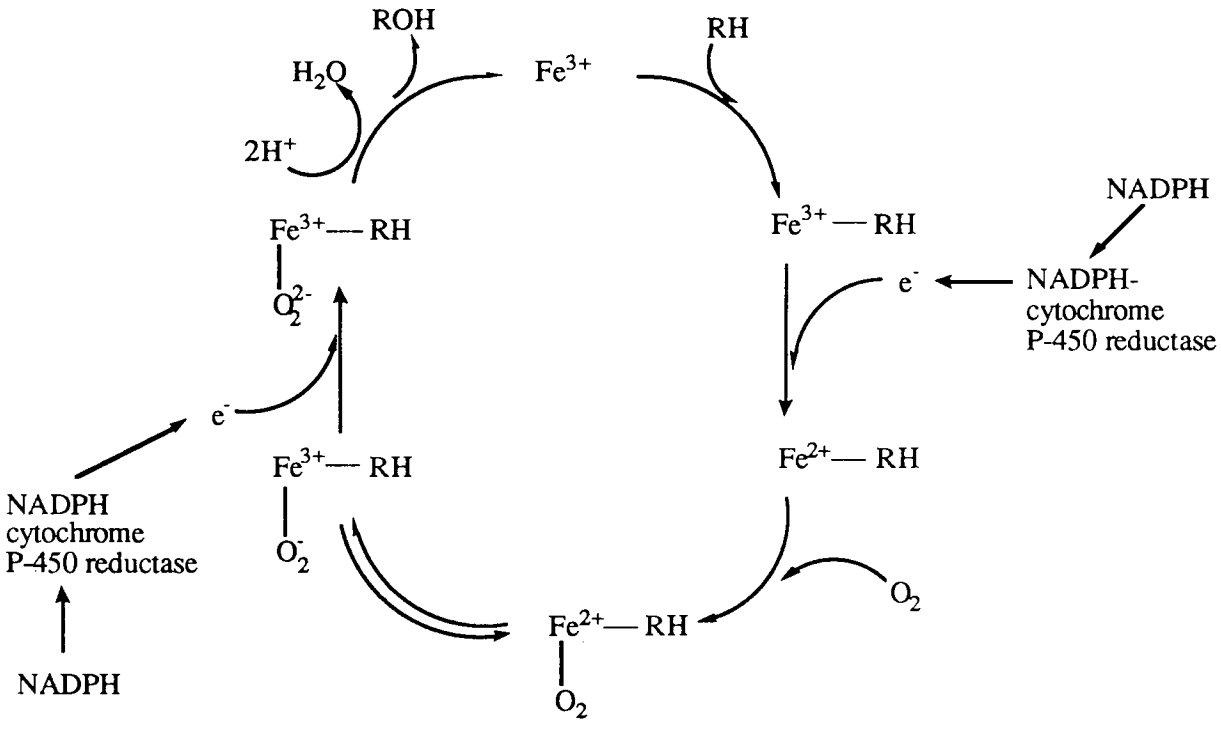
Where RH represents the substrate. The catalytic cycle for P-450 catalysed reactions is thought to consist of at least 6 discrete reactions (Gibson and Skett, 1986a) as depicted in Figure 1.2.

The first step involves binding of the substrate (R-H) to the ferric form of the enzyme. This perturbs the spin state equilibrium of the cytochrome and facilitates uptake of the first electron. The second step involves transfer of one electron from the NADPH-P-450 reductase to the iron of the ferric P-450 enzyme to give a ferrous enzyme-substrate complex. To initiate the oxidative reaction the reduced P-450-substrate complex then binds O_2 to form a ferrous enzyme-O_2-substrate ternary complex with the O_2 bound to the iron. The addition of a second electron to this ternary complex by the reductase, or possibly by involvement of cytochrome b_5 as an additional electron donor in mammalian systems, results in the formation of an iron peroxo species $Fe^{3+}O_2^{2-}$. The next step is not well understood, but involves cleavage of the oxygen-oxygen bond. One of the oxygens is released with the uptake of two protons resulting in the formation of water. The retained oxygen remains associated with the haem iron as "activated oxygen". This atom is then inserted into the substrate. This process is thought to involve hydrogen abstraction from the substrate followed by radical recombination of the resulting transient carbon and hydroxyl radicals to

form the product. This step results in the two-electron oxidation of the substrate to a hydroxy derivative. The product is then released, regenerating the native ferric P-450 that is available to begin another catalytic cycle.

Figure 1.2

Catalytic cycle of cytochrome P-450.



RH represents the drug substrate, and ROH the corresponding hydroxylated metabolite. (Adapted from Gibson and Skett, 1986)

1.2.3 Role of cytochrome P-450 in xenobiotic toxicity.

The cytochrome P-450 status of an individual can be of major significance for his/her health. Xenobiotic metabolism by P-450's convert various organic molecules to more polar metabolites which can be excreted from the organisms either directly, or following conjugation. There is an ever increasing list of chemicals which are converted by specific P-450 forms to more reactive, more electrophilic, intermediates capable of reacting covalently with biological macromolecules, proteins, nucleic acids or lipids (Kadlubar and Hammons, 1987, Parke, 1987). The binding of xenobiotic metabolites to DNA may cause modification of genetic information, somatic or germinal mutation, and the possibility of malignant growth.

A number of different consequences may result from a deficiency or unusually high level of a particular P-450 depending on the catalytic specificity. When P-450 activities are associated with critical functions such as steroid 21-hydroxylation then deficiencies can have dramatic and even fatal effects (Higashi *et al*, 1991). In other cases the presence of a P-450 may not be critical. A deficiency only becomes apparent when a substrate is encountered whose metabolism, or lack of metabolism, is detrimental. Many drugs are substrates for the polymorphic CYP2D6, and when not metabolised can produce an exaggerated response or lead to toxicity. Examples of such drugs include perhexiline and phenformin. The former causes peripheral neuropathy and the latter lacticacidosis. Both effects are due to drug accumulation as a result of a slow rate of metabolism.

Another area of interest is that of drug interactions, which are often associated with enzyme induction or inhibition by one of the drugs. CYP3A4 levels are elevated by administration of rifampicin and barbiturates resulting in more rapid elimination of 17α-ethinyloestradiol and a loss of contraceptive action (Guengerich *et al*, 1990).

A complication when studying P-450s in man is the possibility of exposure to inhibitors or inducers in the diet or in the air. There are a number of P-450 inhibitors found in the diet, for example flavenoids. Recently consumption of grapefruit juice has been shown to have a dramatic effect in decreasing the clearance of dihydropyridine drugs. This effect is probably

associated with inhibition of CYP3A4 by the flavenoid naringenin found in grapefruit juice (Guengerich and Kim, 1990). A well documented inducer of the CYP1A subfamily is cigarette smoke to which individuals may be exposed directly or as a result of "passive smoking".

Many of the P-450 substrates are procarcinogens. The activation of most of these chemicals in humans appears to be attributed to a small number of isozymes. Table 1.4 gives a list of human forms of P-450 whose ability to form mutagenic or carcinogenic metabolites has been demonstrated. From this table it can be seen that certain classes of chemicals are activated by specific cytochrome P-450 forms. Nitrosamines are activated strongly by CYP2E1 and aromatic amines by CYP1A2 and CYP2E1 (Gonzalez, 1989). Therefore it would be expected that individuals with induced levels of relevant isozymes would be at a higher risk, whilst those individuals in which the isozyme is inhibited or absent may be protected against the toxic potential of these substrates. The possibility that increased levels of these enzymes is associated with a higher cancer risk is currently under investigation.

Knowledge of the role of specific (especially human) P-450 forms in the metabolic activation of certain classes of chemicals to mutagenic and carcinogenic intermediates is steadily increasing. A knowledge of the human P-450 'profile' might therefore afford a tool with which increased risk could be predicted for exposure of a particular individual to certain chemicals. This aspect could have legal consequences with the possibility of such knowledge being used to discriminate against entry into chemical risk employment. Knowledge of a persons compliment of P-450s will also be of use to pharmacists and physicians in avoiding undesirable drug interactions. Further, the comparison of animal and human P-450s may lead to rational choices in experimental animal models in the process of safety assessment of drug candidates.

Table 1.4

Role of human P-450 forms in mutagenesis/carcinogenesis.

CYP	Substrate ----> Mutagenic metabolite suggested
1A2	aflatoxin B_1 ---> aflatoxin B_1-2,3-oxide
	2-acetylaminofluorene ---> N-hydroxyacetylaminofluorene
	2-aminoanthracene[1]
	4-aminobiphenyl ---> N-hydroxyaminobiphenyl
2A3	aflatoxin B_1 ---> aflatoxin B_1-2,3-oxide
	benzo(a)pyrene ---> benzo(a)pyrene-7,8-diol,9,10-epoxide
	N,N'-nitrosodimethylamine ---> N-demethylation
	N-nitroso-N-diethylamine ---> N-demethylation
2B7	aflatoxin B_1 ---> aflatoxin B_1-2,3-oxide
2E1	N,N'-nitrosodimethylamine ---> N-demethylation
	N-nitroso-N-benzyl-N-methylamine ---> N-demethylation
	N-nitroso-N-butyl-N-methylamine ---> N-demethylation
	N-nitroso-N-diethylamine ---> N-demethylation
3A3	aflatoxin B_1 ---> aflatoxin B1-2,3-oxide
3A4	aflatoxin B_1 ---> aflatoxin B1-2,3-oxide
	benzo(a)pyrene ---> benzo(a)pyrene-7,8-diol,9,10-epoxide
	6-aminochrysene[1]
	sterigmatocystin[1]

1 Toxic metabolite not known.

1.3 Cytochrome P-450 2E1 (CYP2E1).

1.3.1 Metabolism catalysed by CYP2E1.

Many compounds have been identified as substrates for CYP2E1. Table 1.5 provides a summary of a number of these. In general these compounds are small, relatively polar compounds and include many solvents used extensively in industry such as benzene, chloroform and trichloroethylene. The enzyme is constitutively expressed and is readily inducible in humans (Umeno *et al*, 1988a, Guengerich *et al*, 1991). There can be significant exposure to such substrates in the work place. It is therefore of great importance to determine the catalytic activity of human CYP2E1, although thus far all compounds that have been shown to be substrates in animal models are also substrates for the human form.

Guengerich *et al* (1991) have described the metabolism of a number of low molecular weight procarcinogens by human CYP2E1. The N-alkylformamides NMF and DMF are organic solvents of low molecular weight and it would therefore appear feasible that these compounds may also be metabolised by CYP2E1. As will be discussed in section 1.4.2 it has been observed that DMF inhibits the oxidative metabolism of ethanol, a substrate for CYP2E1. The toxic effects associated with combined exposure to ethanol and DMF is believed to be the result of altered enzyme activity. It is attractive to speculate that the incompatibility might be associated with both agents being substrates of CYP2E1.

The potential of common organic solvents to act as substrates for CYP2E1 has important implications. Firstly, metabolic assays for potential CYP2E1 substrates often require an organic solvent as a mobile phase, which would therefore interfere with the assay. Secondly metabolism of many of these solvents may lead to cytotoxicity or carcinogenicity. Most chemicals that cause hepatotoxicity must be bioactivated to reactive species that initiate cell damage. This requirement for bioactivation, which occurs predominantly via the liver microsomal mixed function oxidase system, leads to the speculation that CYP2E1 is involved in the bioactivation of solvent molecules. It has been demonstrated that the purified isozyme catalyses bioactivation reactions of procarcinogens. N-Nitrosodimethylamine (NDMA), for example, is hepatotoxic and carcinogenic; metabolic activation of this

compound has been shown to be catalysed by CYP2E1 (Yang *et al*, 1990).

Some substrates of CYP2E1 have been investigated as potential metabolic markers for CYP2E1. Demethylation of NDMA and hydroxylation of p-nitrophenol (PNP) and chlorzoxazone have so far been proffered as suitable metabolic markers for the presence of CYP2E1 (Koop, 1992). Metabolism of these compounds by the routes indicated have been shown to be mediated almost exclusively by CYP2E1. Of these markers, chlorzoxazone has an added advantage. It is an approved drug, and is a centrally acting muscle relaxant, which is not toxic at low doses. As such the compound has a major advantage as a potential non-invasive probe for the presence of CYP2E1 in animal models and humans, providing the *in vivo* rate of metabolism is dependent on the CYP2E1 concentration.

Table 1.5

Substrates metabolised by CYP2E1.

Aromatic compounds	**Halogenated alkanes and alkenes/alkanes**	**Alcohols/ketones/ nitriles**
pyridine	chloroform	ethanol
3-hydroxypyridine	pentane	propanol
p-nitrophenol	chloromethane	isopropanol
benzene	dibromoethane	butanol
phenol	ethyl carbamate	pentanol
acetaminophen	1,1,1-trichloroethane	glycerol
pyrazole	trichloroethylene	acetol
chlorzoxazone	ethylene dibromide	acetone
styrene	vinyl chloride	acetonitrile
aniline	vinyl carbamate	acrylonitrile
	enflurane	
	halothane	

Nitrosamines/ azo compounds	**Ethers**	**Reductive substrates**
N-nitrosodimethylamine	diethyl ether	carbon tetrachloride
azoxymethane	methyl *t*-butyl ether	chromium
methylazoxymethanol		cumyl hydroperoxide
N-nitrosopyrrolidine		*t*-butylhydroperoxide
N-nitroso-2,6-dimethyl morpholine		oxygen

1.3.2 Endogenous role of CYP2E1.

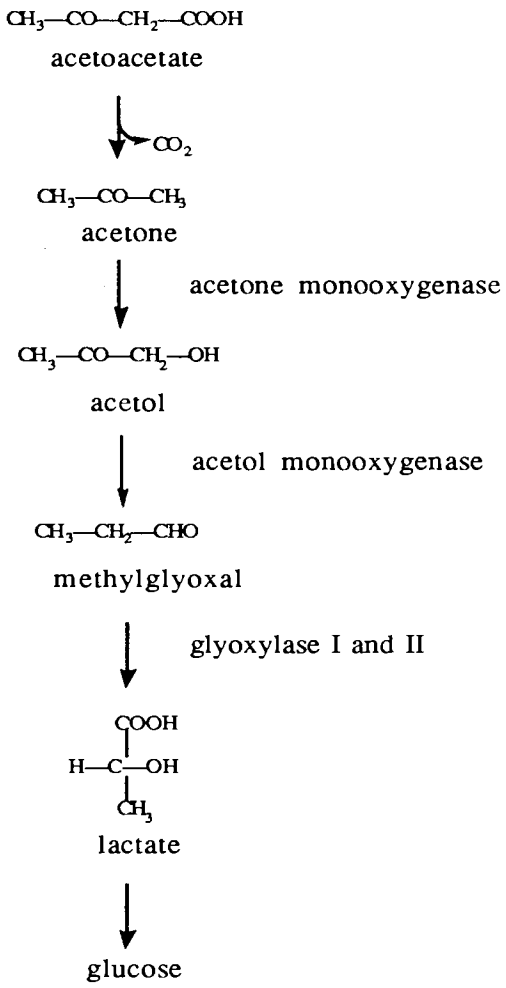
CYP2E1 is a constitutively expressed isoform of P-450 and represents approximately 5% of the total P-450 in rabbits (Koop *et al*, 1985). This isozyme was originally purified from ethanol-treated rabbits (Koop *et al*, 1982) and has subsequently been identified in all species examined, including rats, mice and humans (Ryan *et al*, 1985, Wrighton *et al*, 1987). The CYP2E1 cDNAs have been isolated from the liver of rat, mouse, rabbit and human (Song *et al*, 1986, Khani *et al*, 1988, Freeman *et al*, 1992). The deduced amino acid sequences are approximately 80% similar, containing 493 amino acids with calculated molecular masses of 56,635, 56,916 and 56,781, in rats, humans and mice, respectively (Song *et al*, 1986, Freeman *et al*, 1992). CYP2E1 is the only member of the CYP2E subfamily in rats, mice and humans (Gonzalez, 1989), whereas two highly homologous genes exist in rabbits (Khani *et al*, 1988). The second highly related form present in the rabbit, CYP2E2, differs from CYP2E1 by only 10 of 493 amino acids. Both genes are expressed in rabbit liver, however only CYP2E1 is expressed in the kidney. This fact indicates that the rabbit CYP2E gene recently duplicated, giving rise to two CYP2E subfamily members in the rabbit.

The physiological role of CYP2E1 is not clear. It has been proposed that the reason for CYP2E1 induction by acetone and other solvents, and in diabetes and starvation, relates to a constitutive role for this isozyme in gluconeogenesis. CYP2E1 is suggested to play a central role in the conversion of acetone, produced by the decarboxylation of acetoacetate, to acetol and methylglyoxal, which is then metabolised by glyoxylases I and II to produce D-lactate and finally pyruvate (Figure 1.3). CYP2E1 is thought to represent the acetone and acetol monooxygenase component in this pathway (Casazza *et al*, 1984, Koop and Casazza, 1985). This role for CYP2E1 in gluconeogenesis has important implications for the presence of the enzyme in the brain, where ketones are extensively metabolised.

Consistent with the multiplicity of P-450 cytochromes, discussed in section 1.2.1, is the considerable diversity in the mechanisms of regulation of these enzymes. This is depicted schematically in Figure 1.4, with selected examples of the isozymes influenced at each step (Porter and Coon, 1991). The most common means of regulation is transcriptional, whilst post-transcriptional mechanisms include mRNA stabilisation, and protein stabilisation. Many

Figure 1.3

Gluconeogenic pathway of acetone.

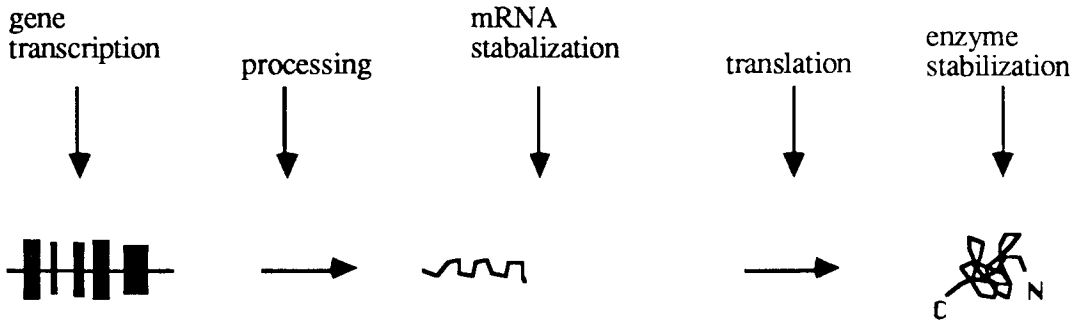


P-450s are also subject to tissue specific patterns of expression resulting in differences in isoform composition and activity in various tissues (Buhler *et al*, 1992). Data suggest three distinct patterns of constitutive P-450 expression in the liver, perivenous-restricted, perivenous-dominated and periacinar.

CYP2E1 is readily inducible by administration of a variety of structurally diverse chemicals, as well as by changes in hormonal and metabolic status of the animal. The induction of this particular gene family is distinct from other families, where the majority are induced via transcriptional activation of the gene (Gonzalez, 1989). In contrast, the induction of

Figure 1.4

Multiplicity in the regulation of cytochrome P-450 expression.



gene transcription	processing	mRNA stabalization	translation	enzyme stabilization
1A1 2C12 3A6 1A2 2D9 4A1 2B1 2E1 11A1 2B2 2H1 11B1 2C7 2H2 17 2C11 3A1/2 21A1	1A2	1A1 2H1 2B1 2H2 2B2 3A1/2 2C12 3A6 2E1 11A1	2E1	2E1 3A1/2 3A6

CYP2E1 does not involve transcriptional activation except at birth. CYP2E1 appears to be under the control of multiple regulatory mechanisms dependent on the nature of the inducing stimulus. In addition to translational regulation CYP2E1 induction is mediated via mRNA stabilisation, increased translation of existing mRNA and inhibition of protein degradation.

Regulation of the expression of constitutive forms of P-450 is somewhat different from that for inducible forms. In recent years increasing attention has been paid to discerning the molecular mechanisms involved in the physiological regulation of P-450 expression. For example, the P-450 monooxygenase system appears in the liver of some species soon after birth and expression of particular forms are coincidental with weaning.

One of the most interesting regulatory phenomena is the activation of P-450 gene transcription during development. At least 4 modes of regulation of constitutively expressed P-450 genes have been described:

i) activation of expression immediately after birth;

ii) activation of expression at the onset of puberty;

iii) activation of expression in males or females, referred to as 'sex imprinted' expression;

iv) specific suppression of gene activity in males or females at the onset of puberty.

CYP2E1 has not been detected in neonatal liver. However, significant levels of the mRNA are detected within a few hours of birth. Elevation of the mRNA, which was coincident with transcriptional activation of the gene, begins within a few hours of birth and levels peak at the age of 6 days (Umeno *et al*, 1988b). The mechanism governing gene regulation is unclear, however changes in the methylation state of the gene correlated with transcription. Umeno *et al* (1988b) found that certain cytosine residues upstream of the *CYP2E1* transcription site were demethylated within 24 hours of birth and further demethylation of cytosines downstream was detected at 1 and 10 weeks of age. However, it is not clear whether this demethylation is a result of transcriptional activation of the gene or if demethylation causes gene expression.

Transcriptional activation of *CYP2E1* at birth is in sharp contrast to the regulatory mechanisms employed in the adult, where mRNA and protein stabilisation are the most important factors in regulation of the enzyme levels. In almost all cases, elevated mRNA is observed in situations where hormonal changes are involved. Induction of CYP2E1 in chemically-induced diabetes and in the spontaneously diabetic rat was accompanied by a 10-fold elevation in mRNA, attributed to mRNA stabilisation (Song *et al*, 1987, Dong *et al*, 1988). The increase in both enzyme concentration and mRNA is reversible by administration of insulin. It has been suggested that the induction is due to an increase in the level of ketone bodies in the circulation of the diabetic rat , referred to as the 'ketone hypothesis' (Miller and Yang, 1984). A similar suggestion has been made for the CYP2E1 induction observed during fasting, however fasting does not give rise to high enough ketone levels to account for the induction observed (Dong *et al*, 1988). It has been suggested that the elevation in mRNA levels associated with fasting is as a result of increased gene transcription (Hong *et al*, 1987).

Expression of a number of P-450 isozymes are associated with sex-related differences. There have been a number of conflicting reports as to the expression of CYP2E1 in this respect. Waxman *et al* (1989) reported that CYP2E1 was expressed to a greater extent in the female rat, and differences were attributed to the growth hormone secretion pattern, whilst Yamazoe *et al* (1989) reported that CYP2E1 was more predominant in the male rat. In contrast to both these reports is the work of Thummel and Schenkman (1990), who did not observe significant differences between constitutive levels of CYP2E1 in male and female rats.

1.3.3 Xenobiotic regulation of CYP2E1 activity.

A number of diverse chemicals are known to induce CYP2E1. These include acetone, ethanol, benzene, diethyl ether, pyrazole, isoniazid and pyridine (Johansson *et al*, 1988, Arinc *et al*, 1991, Brady *et al*, 1987, Carlson and Day, 1992, Craft, 1985, Koop *et al*, 1985). The major regulatory mechanisms shown to be involved in the induction of CYP2E1 expression by xenobiotics is post-transcriptional. Administration of small molecular weight compounds such as acetone, pyrazole or ethanol to rats causes a rapid induction of CYP2E1 protein without affecting levels of the mRNA (Song *et al*, 1986). It has recently been suggested that translation does not play a role in CYP2E1 induction by either acetone or pyrazole, and that induction of the enzyme is due to protein stabilisation (Song *et al*, 1989). This conclusion was based on the observation that the rate of CYP2E1 synthesis was unchanged in rats treated with acetone for 10 days, while the rate of CYP2E1 degradation was markedly altered. Kim and Novak (1990) have suggested that induction of CYP2E1 following acute pyridine exposure involves protein synthesis, possibly through increased translational efficiency.

Support for a mechanism involving a decrease in protein degradation as a result of protein stabilisation is provided by a number of investigations. Early experiments suggested that CYP2E1 has a very rapid turnover, with induced activities returning to control levels within 12-24 hours of the cessation of treatment (Morgan *et al*, 1981, Hetu and Joly, 1985). Song *et al* (1989) measured the degradation of CYP2E1 in untreated and acetone-treated rats. They reported a biphasic degradation of CYP2E1 in untreated animals with estimated half lives of

7 and 37 hours. In the presence of acetone there was no rapid phase of degradation, only a slow phase which was approximately the same as the slow phase in the untreated animals.

Additional support for the stability of the CYP2E1 protein was provided by the studies of Eliasson *et al* (1988), who investigated the effect of ligands on the loss of the induced enzyme when hepatocytes were in primary culture. In the absence of additional ligands induced CYP2E1 was reduced by 70% within 24 hours and completely lost at 72 hours. The level of mRNA was negligible after 24 hours of culture. In contrast, when cultured in the presence of various ligands for the isozyme, e.g. ethanol, imidazole or DMSO, the loss of enzyme from the hepatocytes was not as great. After 72 hours 50% of the original CYP2E1 that was present at the time of plating still remained. However it was interesting to note that levels of mRNA were still reduced to almost zero after 1 day in culture. Quite how these ligands protect the enzyme from degradation is not known. It is possible that the presence of the ligand may maintain the enzyme in a stable conformation which prevents degradation. Eliasson *et al* (1990) reported recently that phosphorylation of CYP2E1 was correlated with enhanced degradation of the enzyme in cultured hepatocytes. When the hepatocytes were cultured in the presence of ligand the phosphorylation was inhibited as was the loss of the enzyme.

The regulation of expression of CYP2E1 is extremely complex, involving both transcriptional and post transcriptional mechanisms. In adult animals and presumably in man, control of the degradation of the enzyme is one of the principal components in the regulatory pathway and yet remains one of the least well understood.

It is pertinent to point out that of all the inducers of CYP2E1 mentioned very few can be classified as specific inducers. Johansson *et al* (1988) have demonstrated that starvation, or treatment with ethanol or acetone causes an increase in CYP2E1 protein and CYP2B1/2 protein and mRNA. Pyridine and ethanol have also been seen to induce CYP1A in addition to CYP2E1 (Carlson and Day, 1992). Pyrazole, in contrast, does appear to be a more specific inducer of CYP2E1 in the rat (Craft, 1985).

1.3.4 Inhibition of CYP2E1.

A number of compounds have been shown to inhibit CYP2E1 activity. As has been discussed earlier, one effect of CYP2E1 activity is the generation of reactive intermediates with the potential of producing toxic or carcinogenic effects. Inhibitors of CYP2E1 activity are therefore of great interest as potential chemoprotective agents. Diallyl sulphide, ellagic acid and phenethyl isothiocyanate (PEITC) are all naturally occurring in foodstuffs and are inhibitors of CYP2E1 (Brady *et al*, 1991, Wilson *et al*, 1992, Ishizaki *et al*, 1990). Diallyl sulphide is a flavour component of garlic and has been shown to protect against chemically induced toxicity and carcinogenesis in animal model systems (Wargovich *et al*, 1988, Wattenberg *et al*, 1977, Brady *et al*, 1991). Similarly, PEITC, which is present as a glucosinolate in cruciferous vegetables, lowers the NDMA-induced methylation of hepatic DNA (Chung *et al*, 1985). Ellagic acid has been shown to inhibit the mutagenicity of NDMA (Wilson *et al*, 1992)

Various mechanisms are known to underlie the action of inhibitors of xenobiotic-metabolising enzymes. Mechanism-based inhibitors act indirectly via a metabolic intermediate generated *in situ*. This mechanism generally accounts for inhibition with a relatively high degree of specificity. Depending on the nature of the intermediate, inhibition may either be reversible (e.g. ligation of the haem iron in P-450) or irreversible (covalent binding to the enzyme active site or the apoenzyme). Mechanism-based irreversible inhibitors are also known as suicide substrates. PEITC, diethyl dithiocarbamate (DEDTC) and its oxidised form disulfiram, dihydrocapsaicin and 3-amino-1,2,4-triazole have all been suggested to be mechanism-based inhibitors of CYP2E1 (Ishizaki *et al*, 1990, Guengerich *et al*, 1991, Gannet *et al*, 1990, and Koop, 1990).

One of the criteria for classification as a mechanism-based inhibitor is demonstration of NADPH dependency. Guengerich *et al* (1991) reported a time- and NADPH- dependent loss of N,N-dimethylamine demethylation and chlorzoxazone hydroxylation by DEDTC, and Ishizaki *et al* (1990) reported a similar effect of PEITC on the inactivation of certain N-demethylation reactions. Gannet *et al* (1990) described NADPH-dependent covalent binding of dihydrocapsaicin to CYP2E1. For all these inhibitors the specificity of the inhibition

requires further investigation, however as a general rule it would appear that as inhibitor concentration increases, so specificity to CYP2E1 decreases. For example, PEITC at a concentration of 5μM was a selective inhibitor of CYP2E1, whereas increasing the concentration to 21μM also inhibited the activity of the enzymes which catalyse the metabolism of benzphetamine and ethylmorphine (Ishizaki *et al*, 1990).

A further mechanism for inhibition of P-450 is that of mutual interaction of two substrates competing for the same isozyme. Morgan *et al* (1982) reported a competitive inhibition by DMSO of ethanol oxidation by rabbit CYP2E1. Yoo *et al* (1987) demonstrated competitive inhibition of NDMA demethylation by glycerol. These inhibitors are of particular interest since they are both compounds which are often used in biological studies to maintain cells in culture. If these cells are to be used in metabolic studies interpretation of results may be problematic because of interference by culture media.

1.3.5 Implications of CYP2E1 activity.

As has previously been discussed, CYP2E1 is a constitutively expressed P-450 which is one of a number of isozymes that are clearly involved in the bioactivation of compounds, thereby initiating toxicity. At present there are very few studies into the effects of human CYP2E1 and its implications in toxicity risk assessment. Guengerich *et al* (1991) have investigated the role of human CYP2E1 in the metabolism of a number of cancer suspect agents *in vitro*, however how this data correlates with a risk of toxicity *in vivo* is yet to be established.

In vitro, human CYP2E1 has been shown to be the major catalyst of the oxidation of many small molecular weight compounds, including benzene, tetrachloromethane, vinyl chloride and many other potentially toxic agents (Guengerich *et al*, 1991). The human form of the isozyme has also been implicated in the bioactivation of some N,N-dialkylnitrosamines, which are potential carcinogens.

CYP2E1 is known to be inducible by ethanol (Koop *et al*, 1985) and its activity is also known to be altered by a number of dietary constituents. Induction of CYP2E1 by broccoli has been established in the rat (Vang *et al*, 1991), whilst constituents of garlic, red peppers

and cruciferous vegetables have been shown to inhibit enzyme activity (Yang *et al*, 1992). In addition, a low fat/ high carbohydrate diet has been shown to affect hepatic microsomal CYP2E1 levels measured *in vitro* and to lower the rate of enflurane metabolism in rats (Yoo *et al*, 1991). It may therefore be suggested that individuals on a low fat diet may have, on average, lower CYP2E1 levels than those on a high fat diet. Fasting and uncontrolled diabetes have also been shown to cause an elevation in CYP2E1 levels.

As a consequence of inhibition of CYP2E1 a reduction in the toxicity of certain xenobiotics may result. However, inhibition of the formation of carcinogenic metabolites in the liver can increase the carcinogen exposure to non-hepatic organs and thus may enhance non-hepatic carcinogenesis. This effect has been demonstrated with ethanol, which has been seen to inhibit hepatocarcinogenesis of NDMA, but enhances tumour formation in the nasal cavity (Gricuite *et al*, 1981).

Knowing that many factors can modulate CYP2E1, the levels of the isozyme are likely to vary considerably between individuals. The question as to whether higher or lower levels of this isozyme are more beneficial to health should therefore be considered. A possible physiological role for CYP2E1 is in the initial step of the conversion of acetone to glucose (Koop and Casazza, 1985). However, the physiological importance of this mechanism is yet to be established. It may therefore be advantageous to possess lower levels of CYP2E1 since this is likely to result in a decrease in susceptibility towards many toxic chemicals, provided the parent compounds are not toxic and can be removed by other metabolic pathways, or by exhalation.

Therefore, *in vivo* markers such as chlorzoxazone would have a major role in helping to determine an individual's CYP2E1 status and thereby help in predicting susceptibility to toxic chemicals. CYP2E1 activity may also be of significance in a number of clinical situations where drugs are metabolised by CYP2E1. Enflurane, halothane and paracetamol are all metabolised by CYP2E1. Individuals with low CYP2E1 levels may therefore be less susceptible to paracetamol-induced toxicity, whilst alcoholics or combined administration with isoniazid, known inducers of CYP2E1, may result in increased toxicity.

1.4 Properties of N-alkylformamides.

1.4.1 N-Methylformamide (NMF).

NMF is a clear colourless liquid possessing excellent solvent properties. However, it appears to be much less important as an industrial solvent than its dimethyl analogue. NMF is also an experimental antitumour agent. The antitumour activity of NMF in mice was first shown 40 years ago in sarcoma 180 (Clarke *et al*, 1953) and the Ehrlich ascites tumour models (Furst *et al*, 1955). Although 100 formamides and related amides were tested, NMF was found to be the most potent inhibitor of Ehrlich ascites in tumour cells *in vivo*. More recently NMF and a series of derivatives were tested for activity against the M5076 ovarian sarcoma and TLX5 lymphoma, again in mice (Gate *et al*, 1986). NMF was again the most potent antitumour agent in both models. In these studies DMF, N-hydroxymethylformamide (HMF) and N-ethylformamide (NEF) were the only other compounds to show some marginal activity against certain tumours. NEF was active against TLX5 lymphoma, whilst DMF and HMF exhibited some activity against Ehrlich ascites and the M5076 tumour. The inactivity of most of the analogues indicates that there is a strong structural requirement for antitumour activity.

Surprisingly, NMF is free from the commonly observed deleterious effects of antitumour drugs on the host bone marrow (Langdon *et al*, 1985); an almost unique property for an antineoplastic agent. NMF induces terminal differentiation in certain cell lines *in vitro* (Spremulli and Dexter, 1984), but it is not clear if tumour growth is limited *in vivo* by promotion of terminal differentiation to viable non-proliferating cells, or by cytotoxicity.

On the basis of the pronounced antitumour activity and the absence of myelotoxic properties, NMF has been evaluated in phase I (McVie *et al*, 1984, Ettinger *et al*, 1985) and phase II (Tauer *et al*, 1985, Eisenhauer *et al*, 1986) clinical trials. The results have been disappointing, not least because drug administration was often limited by severe nausea, and gastro-intestinal and liver toxicity. The very first clinical evaluation was initiated in 1956 by Myers *et al*. The dose ranged from 0.1g to 0.4g per day for 2-36 days. All five patients showed symptoms of toxicity, mainly anorexia, nausea and vomiting. Hepatic damage, as

measured by liver function tests, was seen in all patients at total doses of 80-870mg/kg. The larger clinical trials of the 1980's were again hampered by toxic effects, principally general malaise, nausea, vomiting, anorexia and raised serum liver enzyme levels. Other toxic effects were skin rashes, peripheral neuropathy and alcohol intolerance in some patients. In 1986 a phase II clinical trial conducted by Eisenhauer and coworkers was terminated early because of NMF-induced hepatic and gastro-intestinal toxicity (Eisenhauer *et al*, 1986). The clinical importance of NMF-induced toxicity led to toxicological and metabolic studies in animals in order to elucidate the underlying mechanism of toxicity.

The hepatotoxic potential of NMF in mice and rats has been convincingly demonstrated. Plasma levels of the enzyme sorbitol dehydrogenase (SDH), an indicator of hepatotoxicity, were markedly increased after a single ip dose of NMF at the following threshold doses: 200mg/kg in BALB/c mice, 400mg/kg in CBA/CA mice and 800mg/kg in BDF1 mice (Pearson *et al*, 1987a). This result indicates a marked difference between mouse strains in susceptibility to NMF-induced hepatic damage. The increase in plasma enzyme levels following a dose of 200mg/kg or more in BALB/c mice was also accompanied by histological evidence of hepatic necrosis which was periacinar in type (Whitby *et al*, 1984). In Sprague Dawley rats doses of 0.2 or 0.5g/kg daily ip on 4 consecutive days were hepatotoxic as indicated by raised serum levels of SDH and glutamate dehydrogenase (Scailteur *et al*, 1981). This increase was found to be more marked in male rather than female rats. Surprisingly, the co-administration of DMF and NMF delayed onset of NMF-induced toxicity (Lundberg *et al*, 1981).

The bioavailability of NMF in mice (Brindley *et al*, 1982) and man (Brindley *et al*, 1983b) is high. In mice NMF is metabolised mainly to carbon dioxide which is exhaled in the breath, and to methylamine which is excreted in the urine (Kestell *et al*, 1985). In mouse studies, of the radioactivity injected as [^{14}C]-formyl-NMF (400mg/kg), 39% was exhaled as carbon dioxide. The amount of drug excreted unchanged in urine was only 26% (Brindley *et al*, 1982) and 15% of the dose was metabolised to methylamine (Kestell *et al*, 1985). By comparison NMF is metabolised more extensively in BALB/c mice than Sprague Dawley rats (Tulip and Timbrell, 1988).

On GLC analysis of the urine of mice which had received NMF, small amounts of formamide (F) were also detected (Brindley *et al*, 1982). This metabolite was later identified as HMF, the immediate product in the hydroxylation of NMF (Kestell *et al*, 1985). HMF is thermally labile and breaks down to give F and formaldehyde (HCHO) on the GLC column. It is however stable in aqueous solution. The extent of metabolism of NMF to HMF *in vivo* is small and constitutes less that 7% of the dose (Kestell *et al,* 1985).

A further urinary metabolite, N-acetyl-S-(N-methylcarbamoyl)cysteine (AMCC), was identified as a major metabolite of NMF in mice, rats and humans (Kestell *et al*, 1986b). This metabolite constitutes 13-20% of the administered dose (400mg/kg ip) (Kestell *et al*, 1985). Formation of this novel metabolite involves oxidation of the formyl moiety and subsequent conjugation with glutathione (Threadgill *et al*, 1987). The glutathione (GSH) conjugate S-(N-methylcarbamoyl)glutathione (SMG) has been identified as a biliary metabolite of NMF in BALB/c mice. NMF itself does not react with GSH, but requires metabolic activation.

Metabolism of NMF to urinary methylamine, AMCC and biliary SMG was found to be subject to a large primary kinetic deuterium isotope effect when the formyl hydrogen was replaced with deuterium. This indicates the existence of a common metabolic precursor for all these metabolites. Threadgill *et al* (1987) have proposed that GSH is conjugated with a reactive metabolite of NMF in the liver and this conjugate is further metabolised to AMCC. The reactive intermediate, probably MIC, has the potential to bind covalently with biological macromolecules due to its high electrophilicity.

The mechanism by which NMF causes toxicity is still under investigation. Evidence is still accumulating for the involvement of a reactive metabolite. The hypothesis is supported by the following findings:

i) NMF leads to depletion of hepatic GSH levels *in vivo* (Gescher *et al*, 1982, Pearson *et al*, 1987a), and in hepatocytes *in vitro* (Whitby *et al*, 1984);

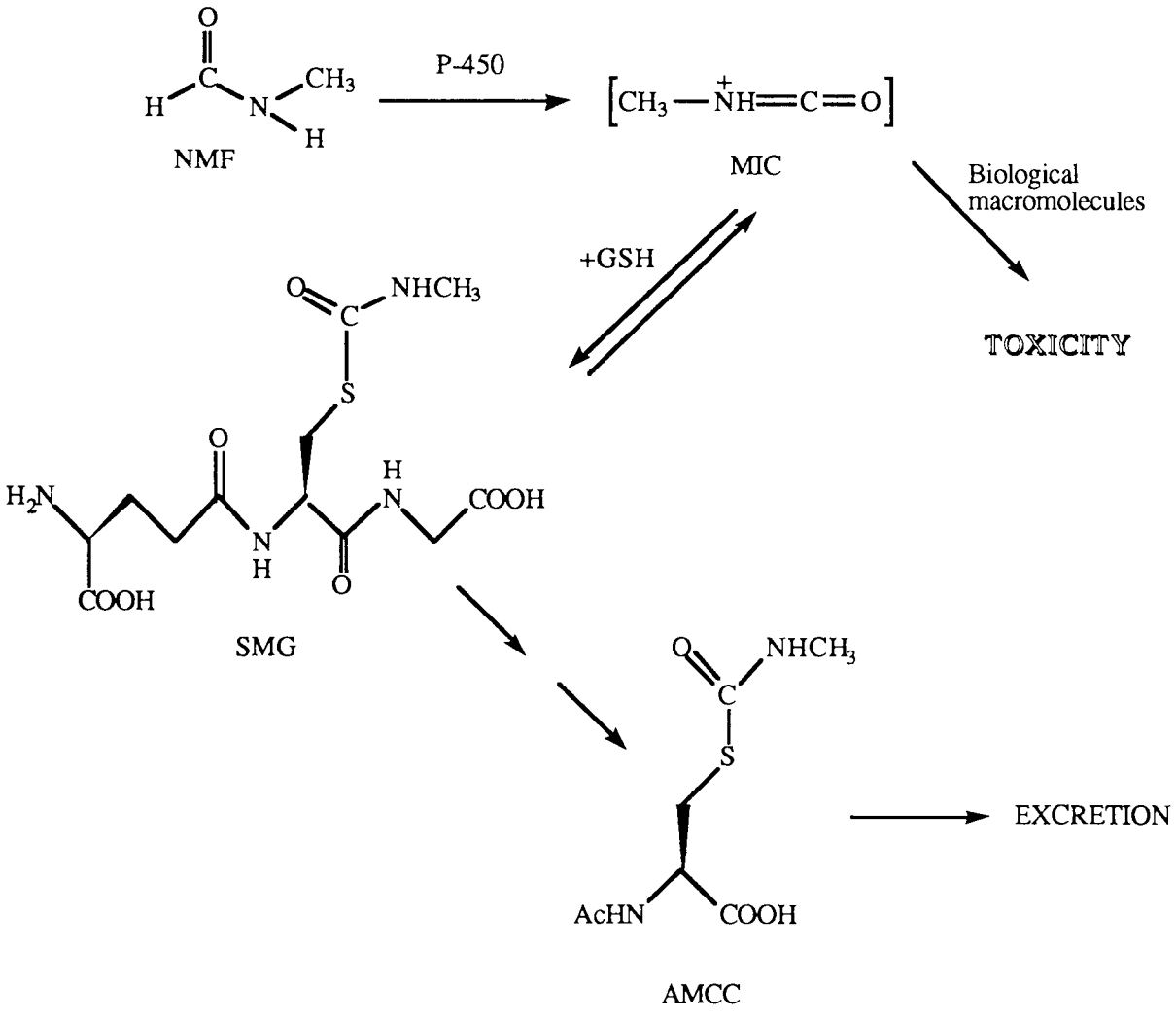
ii) an NMF metabolite or metabolites were covalently bound to liver microsomal protein (Pearson *et al*, 1987b);

iii) pretreatment of mice with cysteine or N-acetylcysteine protected against NMF-induced hepatotoxicity (Pearson *et al*, 1987a).

Figure 1.5 shows the proposed metabolic pathway of NMF which is believed to be responsible for the generation of the toxic properties of NMF. The enzymatic details of the generation of the reactive intermediate are the topic of the work described in this thesis.

Figure 1.5

Proposed scheme for the metabolic activation of NMF.



1.4.2 N,N-Dimethylformamide (DMF).

The world production capacity of DMF is approximately 225 x 10^3 tons per year and the main application of DMF is as a solvent in industrial processes. These industrial processes include the manufacture of synthetic leathers, acrylic fibres, production of surface coatings, adhesives and inks. DMF is also used to dissolve paint strippers, dyes and pigments. Outside industry DMF is used frequently as a solvent in the chemical laboratory, and as a vehicle for the administration of lipophilic chemicals in experimental biological and pharmaceutical studies. DMF has also been investigated as a potential antitumour agent due to its ability to induce differentiation in certain malignant cells (Langdon and Hickman, 1987, Spremulli and Dexter, 1984, van Dongen *et al*, 1989).

The most prominent effect of DMF in occupationally exposed humans is its ability to cause liver damage. Following acute exposure to DMF due to laboratory spillage, workers developed anorexia, nausea and vomiting, abdominal pain and raised L-alanine aminotransferase and L-aspartate aminotransferase (Potter, 1973, Chary, 1974). Liver damage associated with chronic exposure to DMF has been reported in several studies (Redlich *et al*, 1988, Tomasini *et al*, 1983, Circla *et al*, 1984). An increased incidence in hepatic disorders was found amongst employees at an Italian factory despite levels of DMF being within the recommended limits of 10ppm (30mg/m^2)(Circla *et al*, 1984). Clinical signs of liver damage were detected in 36 (62%) of a group of 58 employees in a polyurethane-coated fabric manufacturing plant (Redlich *et al*, 1988).

Other health effects associated with exposure to DMF which have been commonly reported include gastro-intestinal disorders, irritation of the eyes and upper respiratory tract, dizziness, headaches, chest tightness and alcohol intolerance. From this it can be seen that there are a wide range of health effects associated with occupational exposure to DMF which has lead to a number of metabolic and toxicological studies in animals models in order to elucidate the mechanism of its toxicity.

In general, animal studies have verified the liver as the main target organ for DMF toxicity, Elevated plasma levels of SDH were measured in Sprague Dawley rats which received either

240 or 479 mg/kg DMF ip (Lundberg *et al*, 1981). SDH increase correlated well with liver damage observed on histopathological examination. Interestingly it was also noted that the onset of hepatotoxicity was delayed in the case of the higher dose. Histological examination following DMF treatment with 769mg/kg or more ip for 21 days, showed hepatic centrilobular necrosis in mice (Dexter *et al*, 1982) and rats (Lundberg *et al*, 1981, Massmann, 1965).

The hepatic lesions produced in animals by exposure to DMF vapours seem to be less severe than from other routes of administration (Clayton *et al*, 1963). Rats exposed to 200ppm for 8 hr per day over a period of 4 weeks developed fat deposits in the liver and raised transaminase levels (Tanaka, 1971). Histopathological examination of liver tissue damaged by DMF shows affected tissue to be mainly located in the centrilobular region (Tanaka, 1971, Ungar *et al*, 1976, Itoh *et al*, 1987). The severity of damage depended on dose and ranged from mild degenerative changes with fatty deposits (Tanaka, 1971) to extensive coagulative necrosis and massive fibrosis (Itoh *et al*, 1987).

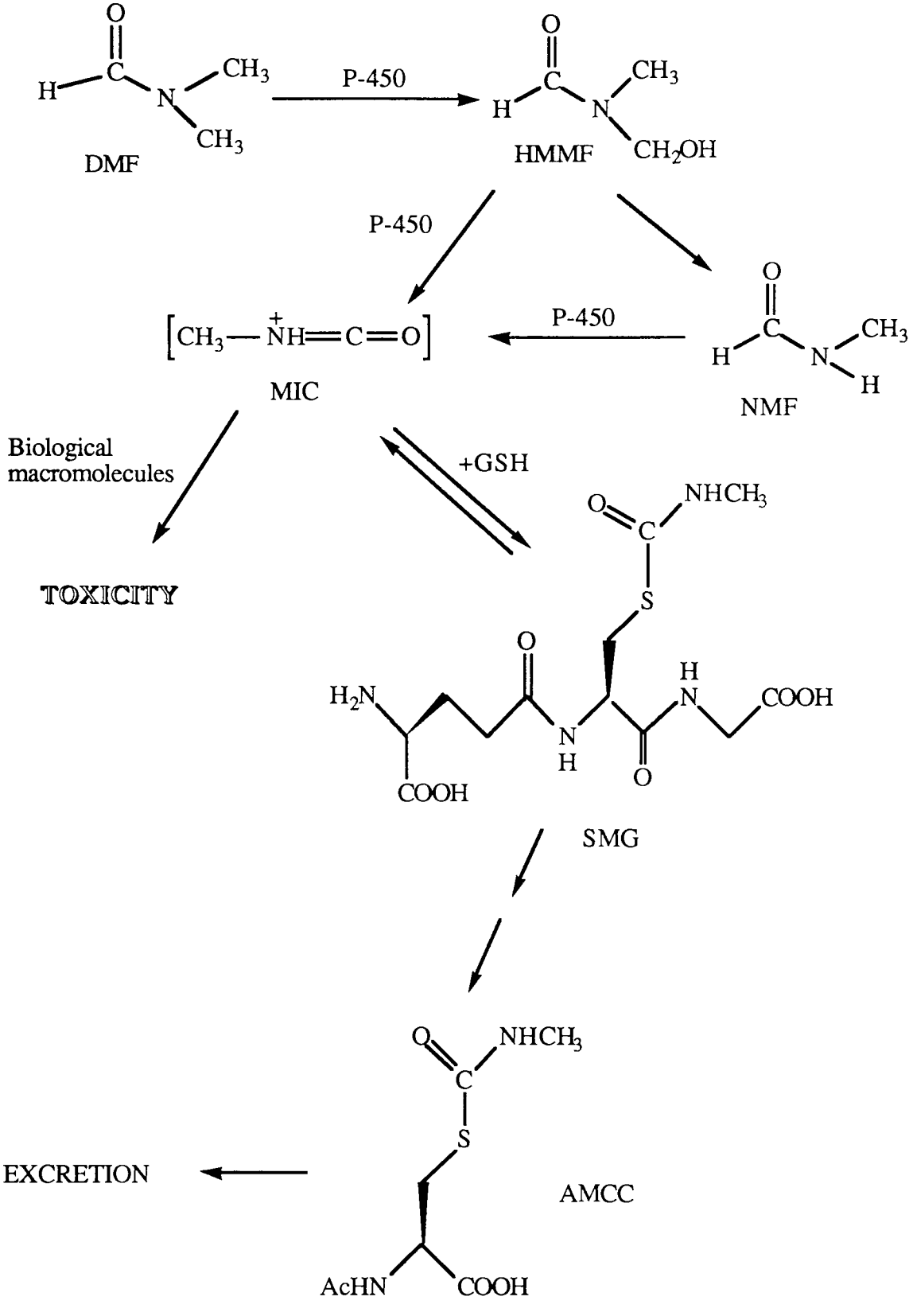
Blood and urine samples of rats (Barnes and Ranta, 1972, Kimmerle and Eben, 1975a) and dogs (Kimmerle and Eben, 1975a) which had been exposed to DMF were examined by GLC analysis, and NMF and F were detected as metabolites. Barnes and Ranta (1972) suggested that the measurement of urinary NMF in humans could provide a means for quantifying exposure to DMF. However, subsequent investigations suggest that the major metabolite of DMF which had been characterised as NMF by GLC analysis is in fact N-hydroxymethyl-N-methylformamide (HMMF) (Brindley *et al*, 1983a, Kestell *et al*, 1986a, Scailteur *et al*, 1984) and F was actually HMF. The identity of HMMF was confirmed by ^{1}H-NMR spectroscopy of urine samples of CBA/CA mice which had received DMF (Kestell *et al*, 1986a) and by chemical ionisation mass spectrometry (Scailteur *et al*, 1984). The existence of NMF as a metabolite of DMF has also been confirmed, although quantitatively it is a very minor metabolite (Scailteur and Lauwerys, 1984). Methylamine and dimethylamine have also been identified as minor metabolites of DMF, each constituting about 4% of the administered dose measured in the urine of CBA/CA mice (Kestell *et al*, 1986a).

Studies in humans exposed to DMF has led to the discovery of another urinary metabolite AMCC (Mraz and Turecek, 1987). AMCC has been confirmed as one of the major urinary metabolites of NMF in humans and rodents (Kestell *et al*, 1986b, Tulip *et al,* 1986). Studies on animals have recently also identified AMCC as a urinary metabolite of DMF, although this appears to be a much less important pathway in rodents than in humans (Mraz *et al*, 1989). Humans who were exposed to DMF in the air at 60mg/m^2, and were calculated to have absorbed between 28-60μmol/kg, excreted AMCC as between 9.7 and 22.8 % of a dose of DMF. The portion of the dose (0.1, 0.7 or 7.0 mmol/kg ip) which was metabolised in mice rats or hamsters to AMCC was only between 1.1 and 5.2%. Such results clearly suggest a quantitative difference between the metabolic pathway of DMF to AMCC in humans and rodents.

Confirmation of AMCC as a metabolite of DMF has important implications with regard to understanding the toxicity of DMF. As is the case with NMF it is thought that this metabolic pathway results in the generation of a toxic intermediate. Figure 1.6 illustrates the proposed metabolic steps in DMF metabolism. It is however unclear as to whether generation of NMF from DMF is a prerequisite for the formation of the reactive species, or whether MIC is generated directly via HMMF.

The extent of DMF metabolism in both humans and animals has been shown to be influenced by a number of factors. A high concentration of DMF appears to inhibit its own metabolism resulting in the excretion of more unchanged DMF and less HMMF (Kimmerle and Eben, 1975b, Lundberg *et al*, 1983). Delayed excretion of AMCC in the urine of rats receiving a high dose of DMF has also been observed (Mraz *et al*, 1989). These findings correlate well with the delayed onset of hepatotoxicity that was seen in rats treated with 479mg/kg DMF as compared to 240mg/kg (Lundberg *et al*, 1981). Another study reported a similar correlation between the inhibition of DMF metabolism at high doses and a reduction in toxicity (Scailteur *et al*, 1984).

Figure 1.6
Proposed scheme for the metabolic activation of DMF.



Co-administration of ethanol has been shown to inhibit the metabolism of DMF in volunteers and experimental animals (Eben and Kimmerle, 1976, Yonemoto and Suzuki, 1980). Administration of ethanol to rats and dogs prior to DMF exposure led to elevated blood levels of unchanged DMF and a delay in the appearance of metabolites (Eben and Kimmerle, 1976). The consumption of alcohol by workers exposed to DMF caused delayed excretion of HMMF (Yonemoto and Suzuki, 1980). DMF has also been observed to inhibit the metabolism of ethanol (Eben and Kimmerle, 1976). This interaction between DMF and alcohol is believed to be as a result of altered activities of the enzymes involved in the metabolism of ethanol and its metabolite acetaldehyde (Elovaara *et al*, 1983, Sharkawa, 1979). The characteristic alcohol flush is suggested to be a result of aldehyde accumulation in the blood (Hanasono *et al*, 1977). However it is pertinent to point out here that neither DMF nor NMF interfere with alcohol metabolism *in vitro* (Gescher and Tisdale, unpublished).

Several studies involving human exposure have been conducted with the aim of developing a biological monitoring assay to reflect human exposure to DMF (Kimmerle and Eben, 1975b, Yonemoto and Suzuki, 1980). As a result it has been suggested that measurement of urinary HMMF may be suitable as an indicator of DMF exposure, since this has been found to be the major metabolite. Investigation of DMF metabolism in animals (Kimmerle and Eben, 1975a) and humans (Kimmerle and Eben, 1975b) have shown that DMF is rapidly metabolised and excreted in the urine mainly as HMMF, HMF and unchanged DMF(at high doses). The discovery of AMCC as a metabolite of DMF (Mraz and Turecek, 1987) lead to further metabolic studies which have clearly suggested that the hepatotoxic properties of DMF may be associated with its metabolism to AMCC; this metabolic pathway appears to be a major one in humans (Mraz *et al*, 1989). Therefore this would suggest that analysis of urinary AMCC might be more appropriate as a suitable indicator of the hepatotoxic risk associated with exposure to DMF.

1.5 Aims of this study.

The investigations into the toxicity of the formamides NMF and DMF in experimental animals or occupationally exposed humans which are described in section 1.4, have demonstrated a wide range of effects of these compounds on health. In particular they induce hepatic damage, and it is the mechanism through which this toxicity is mediated that has been studied here.

As described previously, the hepatotoxicity of NMF and DMF is believed to be linked to their metabolism. The metabolic pathway of N-alkylformamides which involves formyl oxidation, glutathione conjugation and excretion of urinary mercapturates, is thought to be the bioactivation pathway. The toxic species is probably MIC. It was outlined in section 1.2.3 that a number of cytochrome P-450 isozymes have been associated with the generation of toxic and carcinogenic compounds through bioactivation of innocuous precursors. It has been established that the metabolic pathway associated with N-alkylformamide toxicity is catalysed by cytochrome P-450, however the isozyme(s) responsible for bioactivation were unknown prior to this work. It was one of the major aims of this work to establish whether CYP2E1 was involved with the catalysis of this metabolism.

In order to achieve this objective, the first part of this investigation concentrates on the *in vitro* metabolism of NMF. The glutathione conjugate SMG was detected in microsomal suspensions of NMF as an indicator for the bioactivation pathway. Multiple criteria are required to demonstrate a role for a particular cytochrome P-450 isozyme in the metabolism of a xenobiotic and the *in vitro* metabolism of NMF was investigated using both direct and indirect approaches, utilising the generation of SMG to quantify metabolism.

Using the indirect approach the hypothesis was tested that NMF metabolism is increased by compounds known to induce CYP2E1, such as acetone or ethanol, and that it is inhibited by selective inhibitors of CYP2E1, such as DEDTC. More direct approaches involve the use of specific inhibitory antibodies and purified enzyme preparations. Therefore the hypothesis was tested that a specific antibody against CYP2E1 inhibits NMF metabolism and that

isolated CYP2E1 generates SMG from NMF.

Rodents are commonly used to study metabolism and toxicity of chemicals, and results from such experiments can provide significant contributions to the understanding of toxic effects observed in humans and to the assessment of potential health risks for individuals. However, frequently there are differences between experimental animals and humans in the biological fate of a chemical. Therefore the aim of further studies was to assess the relevance to humans of the observations made using rodents. The degree of involvement of human CYP2E1 in NMF metabolism can be judged on the basis of the correlation between rate of this metabolism to that of another substrate for which human CYP2E1 has been shown to be a principle catalyst. This approach was also adopted here. Also, the relationship between amount of immunodetectable CYP2E1 in microsomal preparations, and the metabolic activity of interest was investigated.

The metabolic generation of HMMF from DMF, and of SMG from NMF can be conveniently studied *in vitro* in preparations of human and rodent liver microsomes. However, the metabolism of DMF *in vitro* has not yielded detectable amounts of SMG under such conditions. Also, as discussed in section 1.4, it has been observed that AMCC is excreted in the urine of humans exposed to DMF only after a considerable time delay, commencing beyond the termination of exposure, whereas urinary excretion of HMMF occurs in the first hour. A similar phenomenon was seen in rodents, in which the delay in urinary AMCC excretion following i.p. administration of DMF was increased as the dose of DMF was increased. The reason for this delay, together with the inability to detect SMG generated from DMF *in vitro,* is unclear. Another objective of this work was to test the hypothesis that these phenomena are caused by inhibition of the metabolic generation of SMG by DMF or one of its metabolites. In the second half of this study the route of DMF metabolism was investigated in order to achieve this aim.

Further insights into enzymatic details of the *in vitro* metabolism of DMF to HMMF were sought. A similar approach to that used in the investigation of NMF metabolism was taken. As previously discussed, despite HMMF being the major urinary metabolite of DMF, it is

the generation of AMCC, and not of HMMF, which is probably the more specific indicator of DMF induced-toxicity. It was therefore thought to be of interest to test the hypothesis that the formation of the reactive intermediate was analogous to that established for NMF. As is the case with NMF, generation of SMG provides a suitable end point for this particular metabolic pathway. However, SMG has thus far not been detected in incubations with DMF. Therefore the *in vivo* metabolism of DMF to SMG in bile was studied using electrospray mass spectrometry, a highly selective and sensitive analytical technique. Stable isotopes of DMF were also employed to elucidate certain mechanistic details of DMF bioactivation *in vivo*.

Overall it was hoped that the results obtained in this study would allow conclusions to be drawn as to the enzymatic details of N-alkylformamide metabolism. The information gained from these studies should contribute to the general body of knowledge in the area of xenobiotic bioactivation. More specifically, extending the knowledge of the activity of cytochrome P-450 isozymes might help to:

i) predict the relationship between new drug entities and potential P-450-catalysed metabolism;

ii) provide rational selection of animal species for toxicity testing;

iii) understand patient variability in drug response;

iv) predict drug-drug interactions;

v) predict susceptibility to disease.

Section 2

Materials

2.1 Animals.

BALB/c mice (20 to 25g) and Sprague-Dawley rats (100 to150g) were used for *in vitro* studies. These animals were purchased from Bantin and Kingman, Hull. Sprague-Dawley rats (400g) were used for *in vivo* studies. These animals were supplied by Charles River Portage, Portage, Michigan. All animals were supplied with food (rat and mouse breeding diet) and water *ad libitum*.

Rabbits (New Zealand White) were supplied by R&R Rabbitry, Stanwood, Washington.

2.2 N-Alkylformamides and their analogues.

DMF was purchased from Sigma Chemical Company Ltd., UK. and NMF was purchased from Aldrich Chemical Company Ltd., Gillingham, UK.

HMMF had previously been synthesised by Dr. A. Gledhill, Aston University. AMCC and SMG were synthesised and kindly provided by Dr. D-H Han, formerly University of Washington, Seattle, according to the method described by Han *et al* (1990).

All formamides were >99% pure.

2.3 Stable Isotopes.

$[^2H]_3$SMG was synthesised by Dr. D-H Han, formerly University of Washington, Seattle.

$[^2H]_1$DMF ($DCON(CH_3)_2$) and $[^2H]_6$DMF ($HCON(CD_3)_2$) were purchased from MSD Isotopes, USA, with an isotopic purity of 99.5 atom % excess $[^2H]$.

$[^2H]_7$DMF ($DCON(CD_3)_2$) was purchased from Aldrich Chemical Company Ltd. Milwaukee, Wi, with an isotopic purity of 99.5 atom % excess $[^2H]$.

2.4 Materials for microsomal incubations.

Chemicals used in the preparation of hepatic microsomes, in the microsomal incubations and in the assays were supplied as follows:

Phosphate Buffer.

potassium dihydrogen orthophosphate (anhydrous) and disodium hydrogen orthophosphate (BDH Chemicals Ltd., Poole, UK).

Cofactors.

The following chemicals were all purchased from Sigma Chemical Company Ltd., UK:

NADP (sodium salt)

NADPH (reduced form; tetrasodium salt)

Glucose-6-phosphate (monosodium salt)

Glucose-6-phosphate dehydrogenase (type VII, from Bakers yeast)

Glutathione (reduced form)

Magnesium chloride ($MgCl_2.6H_2O$; Fisons plc, Loughborough, UK).

Enzyme inhibitors.

The following compounds were supplied by Aldrich Chemical Company Ltd., Gillingham, UK:

Diethyl dithiocarbamate

Phenethyl isothiocyanate

Dimethyl sulphoxide

Enzyme inducers.

Acetone, Ethanol (Fisons plc., Loughborough, UK)

4-Methylpyrazole (Sigma Chemical Company Ltd., UK)

Substrates.

In addition to N-alkylformamides the following substrates were purchased from Sigma Chemical Company Ltd., UK. and used in microsomal incubations:

Aminopyrine (4-dimethylaminoantipyrine), para-nitrophenol, chlorzoxazone.

Lowry protein assay.

Bovine serum albumen (fraction V), Folin and Ciocalteau's phenol reagent (Sigma Chemial Company Ltd., UK)

Copper sulphate, potassium sodium tartrate, sodium carbonate (Fisons plc., Loughborough, UK).

Aminopyrine N-demethylation assay.

Paraformaldehyde (Hopkin and Williams, UK)

NaOH, ammonium acetate (Fisons plc, Loughborough, UK)

Trichloroacetic acid (Sigma Chemial Company Ltd., UK)

Acetyl acetone, glacial acetic acid (BDH Chemicals Ltd, Poole, UK)

p-Nitrophenol hydroxylation assay.

4-Nitrocatechol (Aldrich Chemical Company Ltd., Gillingham, UK)

Perchloric acid 70% (Fisons plc, Loughborough, UK)

Ascorbic acid (Sigma Chemial Company Ltd., UK)

Chlorzoxazone assay.

5-fluoro-2-(3H) benzoxazolone and 6-hydroxychlorzoxazone were kindly provided by Dr. R.Peter, formerly Department of Pharmacology and Toxicology, University of Erlangen-Nurnberg, Erlangen, Germany.

Propylene glycol, phosphoric acid, dichloromethane and acetonitrile were purchased from Fisons plc, Loughborough, UK)

Spectral determination of cytochrome P-450.

Carbon monoxide (Aldrich Chemical Company Ltd., Gillingham, UK)

Sodium dithionite (Fisons plc., Loughborough, UK)

2.5 Materials used for hepatocyte incubations.

Chemicals used in the isolation of mouse hepatocytes and in incubations were supplied as follows:

Sodium pentabarbitone 60mg/ml (RMB Animal Health Ltd, Dagenham, UK)

Ca^{2+} Free Hanks buffered salt solution, Horse serum (Gibco Ltd, Paisley, UK)

HEPES, NADH (disodium salt), sodium pyruvate (Sigma Chemial Company Ltd., UK)

Collagenase (extract of *Clostridium histolyticum*) (Boehringer Mannheim Ltd., Lewes, UK)

2.6 Materials for antibody isolation and purification

Chemicals used for the isolation of antibody were supplied as indicated below;

Freund's incomplete adjuvant (Sigma Chemial Company Ltd., St Louis, MO)

Ammonium sulphate and potassium phosphate, monobasic (Aldrich Chemical Company Ltd, Milwaukee, Wi.)

DEAE cellulose (DE-52 anion exchange resin) (Whatman, Maidstone, UK)

Chemicals used for the purification of antibody were supplied as follows;

Sodium phosphate, monobasic, glycine and glycerol (Aldrich Chemical Company Ltd, Milwaukee, Wi)

Sodium cholate, Dithiothreitol (DTT), EDTA, Phenylmethyl sulfonyl fluoride (PMSF) (Sigma Chemial Company Ltd., St Louis, MO)

Laurate sepharose and hydroxyapatite (prepared by the methods of Gibson and Schenkman (1978) and Levin (1962), respectively)

Emulgen 911 (a gift from Kao Corporation, Tokyo, Japan)

CM-sepharose, CNBr activated sepharose (Pharmacia, Piscataway, NJ.)

2.7 Materials for incubations with purified isozyme.

Dilauroyl phosphatidyl choline (DLPC) (Sigma Chemial Company Ltd., St Louis, MO)

Purified CYP2E1, cytochrome P-450 reductase and cytochrome b_5 were provided by Dr. K.E. Thummel, University of Washington, Seattle.

2.8 Materials used for Western blots.

Acrylamide, bis acrylamide, ammonium persulphate, TEMED, mercaptoethanol, Triton X-100 and Protein-A peroxidase conjugate (Sigma Chemical Company Ltd, UK)

SDS, glycine, bromophenol blue (BDH Chemicals Ltd, Poole, UK)

Carnation non-fat milk (Tescos, UK)

Nitro blue tetrazolium (NBT), 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Biorad, Richmond, Ca)

Goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma Chemial Company Ltd., St Louis, Mo)

2.9 Materials used for *in vivo* methods.

The following compounds were purchased from Aldrich Chemical Company Ltd, Milwaukee, Wi :

Urethane, disulfiram (tetra ethylthiuran disulfide), methanol, formic acid.

2.10 Miscellaneous

Quinoline and sodium acetate (anhydrous) (BDH Chemicals Ltd, Poole, UK)

K_2CO_3 (anhydrous) and Trizma base (Sigma Chemical Company Ltd, UK)

Ethyl acetate, chloroform, sucrose, HCl, NaOH, $NaHCO_3$, NaCl, KCl, $CaCl_2$, H_2O and $MgSO_4$, $7H_2O$ (Fisons plc., Loughborough, UK)

10% Palladium on activated charcoal, triphosgene and 4-fluoro-2-nitrophenol (Aldrich Chemical Company Ltd., Gillingham, UK)

Section 3

Methods

3.1 Metabolism of NMF *in vitro.*

3.1.1 Preparation of liver microsomes.

All procedures were performed on ice, where possible, to preserve the activity of the enzyme, and buffer solutions and equipment were pre-cooled to 4°C. The buffer solutions used were prepared as follows:

Tris Buffer (50mM) + KCl (0.154M) pH7.4

Tris base	6.055g	
KCl	11.5g	
distilled water	to 1 litre	pH adjusted to 7.4

This buffer is referred to as Tris buffer, hereafter.

Phosphate buffer (50mM) pH7.4

Solution 1

potassium dihydrogen orthophosphate, anhydrous	1.36g
distilled water	to 200ml

Solution 2

disodium hydrogen orthophosphate	3.54g
distilled water	to 500ml

Solution 1 was added to solution 2 until a pH of 7.4 was obtained.

This buffer is referred to as phosphate buffer hereafter.

Animals were killed by cervical dislocation, the liver removed and immediately washed in ice-cold Tris buffer. The liver was weighed, chopped, and transferred to a glass homogenising vessel. A homogenate comprising of liver (25%) in Tris buffer was prepared using a Camlab homogeniser. The homogenate was placed in Beckman polycarbonate centrifuge tubes and microsomes were prepared by differential centrifugation using a Beckman L8-60M Ultracentrifuge. The ultracentrifuge, rotor (type Ti70) and tubes had all previously been cooled to 4°C. Initial centrifugation was for 20 min, at 13000 rpm (9000g) to remove the large cellular fractions. The supernatant, comprising the endoplasmic

reticulum fractions and soluble proteins, was spun at 42000rpm (105000g) for 1hr, to separate the microsomal fraction from the soluble cytosolic fractions. The microsomal pellet, thus obtained, was washed in Tris buffer by gentle hand homogenisation and the homogenate respun for 1hr at 42000rpm, to remove any haemoglobin remaining. The microsomal pellet was suspended in phosphate buffer (50mM), pH7.4, to give a final concentration of 500mg wet weight of liver / ml buffer. The protein content of the final microsomal suspension, and the cytochrome P-450 concentration were determined by the methods of Lowry *et al* (1951), and Omura and Sato (1962), respectively (see 3.1.5 , 3.1.6).

3.1.2 Incubations with microsomes.

For one experiment microsomes isolated from one rat liver were used. In the case of mice microsomes were pooled from three livers. All incubations were performed in duplicate, in open vials, in a water bath at 37°C, with gentle agitation. Incubates (final volume 2ml) contained microsomes equivalent to 250mg liver, a NADPH generating system (glucose-6-phosphate (20mM), NADP (10mM), glucose-6-phosphate dehydrogenase (4 units), $MgCl_2$ (50mM), glutathione (GSH, 10mM) and N-alkylformamides (1-10mM).

Solutions were prepared as follows:

i) GSH (10mM in incubation)

GSH	0.307g	
Phosphate buffer, 50mM, pH7.4	5ml	pH adjusted to 7.4

An aliquot of 100µl was added to each incubation.

ii) NADPH generating system (1mM in incubation)

glucose-6-phosphate	56.42mg
NADP	14.85mg
magnesium chloride, 100mM	2 ml
glucose-6-phosphate dehydrogenase	40 units
phosphate buffer, pH7.4	to 10ml

An aliquot of 1ml was added to each incubation.

Samples were incubated for 5 min prior to addition of substrate to allow incubates to reach incubation temperature, and for NADPH to preform from the generating system. Incubations were initiated by addition of the substrate and allowed to proceed for up to 60 min. In control incubations N-alkylformamides were omitted. At the end of this time incubations were terminated by the addition of incubate (1.5ml) to ice-cold ethanol (3ml) containing quinoline (0.1mM) which served as internal standard for the analytical quantitation (see below).

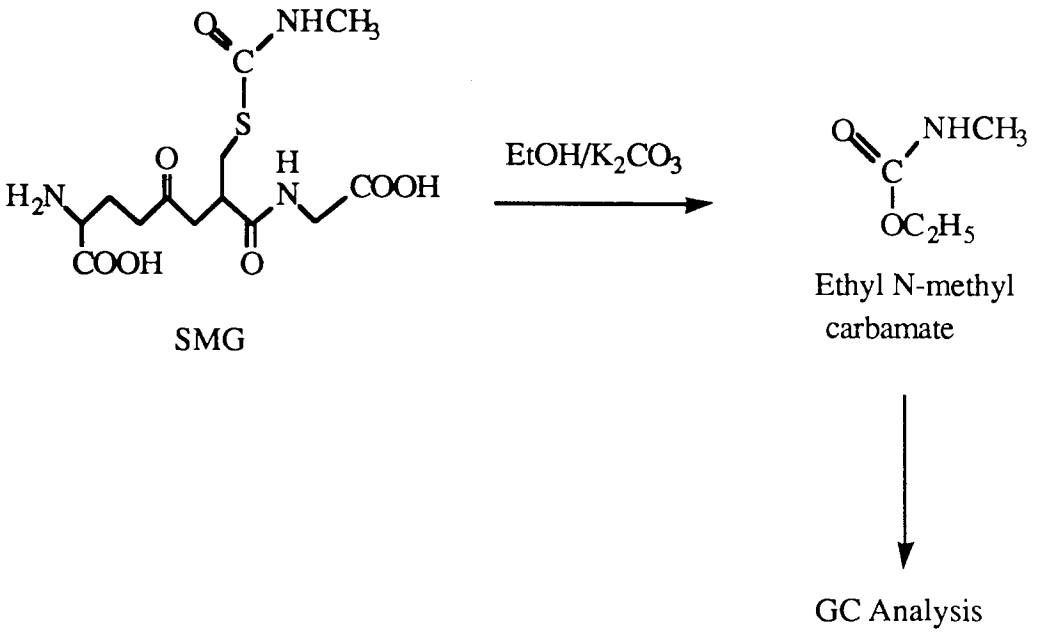
3.1.3 Derivatisation of metabolites.

Determination of SMG formation was performed using the method of Mraz (1988), with some minor modifications. The mixture of incubation sample and ethanol was vortexed and then centrifuged at 3000rpm for 10 minutes in a Heraeus Labofuge 600 centrifuge, to precipitate the protein, and the supernatant retained. Derivatisation of the SMG metabolite to ethyl N-methylcarbamate (EMC), a derivative amenable to gas chromatographic (GC) analysis, was performed by the addition of anhydrous K_2CO_3 (2.25g) to the supernatant. The mixture was mixed vigorously in a stoppered tube and centrifuged at 3000rpm for 10 min to separate the aqueous and ethanolic layers.

The top ethanol layer was retained and, for incubations being analysed for SMG, concentrated to 200µl at room temperature using a Howe gyrovap. The samples were then diluted with water (1ml) and reextracted in ethyl acetate (1ml) by vortexing. The two layers were separated by centrifugation and the top, organic layer was retained and concentrated to 200µl in the gyrovap ready for GC analysis. For samples in which quantification of only formamides was required the ethanolic layer was retained for direct analysis by GC.
Calibration curves were obtained by treating samples containing authentic SMG in the same way.

Derivatisation of SMG for GC determination.

The derivatisation of SMG with ethanol requires the presence of alkali, as indicated in the reaction scheme below.



3.1.4 Gas-Chromatographic determination of metabolites.

A Pye Unicam Series 204 gas chromatograph fitted with a nitrogen-phosphorous detector was used for all GC analysis, unless otherwise stated. A 2m glass column of 1/4 inch diameter with a chrome W-AW DMCS support, mesh range 8-100 was installed. The stationary phase was carbowax 20M containing 5% KOH, and the nitrogen carrier gas was used at a flow rate of 50ml/min. Hydrogen and air pressures were set at 0.8kg/cm^2 and 0.9 kg/cm^2, respectively. The injector, detector and column temperatures were set at 200°C, 250°C and 170°C, respectively.

The GC was connected to a Gallenkamp Euroscribe chart recorder and the attenuation varied for peak detection. Chart speed was set at 2.5cm/min for EMC, NMF or DMF detection, and then altered to 0.5cm/min for the internal standard peak. Under these conditions

retention times were EMC 1.3 min, DMF 1.5 min, NMF 2.33 min and quinoline 6.6 min. Quantification was achieved by comparing the peak height ratio of the peak of interest with that of the internal standard.

3.1.5 Lowry Protein Assay.

The protein content of microsome suspensions was determined by the method of Lowry *et al* (1951).

Standard protein concentrations ranging from 0 to 200µg protein/ml were prepared from a stock solution of bovine serum albumen (BSA) (50mg) in 0.5N NaOH (50ml). Lowry reagent was prepared by adding 1% copper sulphate (1ml) and 2% potassium sodium tartrate (1ml) to 2% sodium carbonate in 1M NaOH (100ml). Folin and Ciocalteau's phenol reagent (2.0N) was diluted with an equal volume of distilled water. All solutions were freshly prepared, with the exception of the BSA 1mg/ml stock solution. Microsomal suspensions were diluted 1 in 80 with 0.5N NaOH in preparation for protein determination.

The standard solutions (0-200µg) or suspensions of microsomes (1ml) were mixed with Lowry reagent (5ml) and allowed to stand for 10 minutes. The diluted Folin + Ciocalteau's phenol reagent (0.5ml) was added to each sample, the mixture vortexed and allowed to stand for 30 minutes. All samples were prepared in duplicate.

Absorbance was read at 750nm on a Cecil CE594 Double Beam UV Spectrophotometer, against a blank (0mg protein). A calibration curve was constructed from the BSA standards. The protein concentration of the diluted microsomal samples was determined from this and the protein content of the original samples calculated.

3.1.6 Cytochrome P-450 assay.

The cytochrome P-450 content was determined by the method of Omura and Sato (1962), as described by Gibson and Skett (1986b).

Microsomal samples were diluted 1 in 4 with phosphate buffer (50mM, pH7.4) and placed

in two quartz cuvettes. A few grains of sodium dithionite were added to each cuvette, to generate the reduced form of cytochrome P-450, and the cuvettes inverted several times. A baseline for the samples was recorded between 400 and 500nm using the Double Beam Spectrophotometer. Carbon monoxide was bubbled through the sample cuvette for 30 seconds during which time a complex forms between the reduced form of the enzyme and CO, which has a characteristic absorbance maximum at 450nm. The sample was rescanned against the reference cuvette between 400 and 500nm. The absorbance difference was measured between 450 and 490nm and the cytochrome P-450 content calculated using the following equation:

$$\text{Cytochrome P-450 concentration} = \frac{\text{Absorbance difference (450-490nm)} \times 1000}{\text{extinction coefficient (450-490nm)}}$$

Extinction coefficient $= 91 mM^{-1}cm^{-1}$

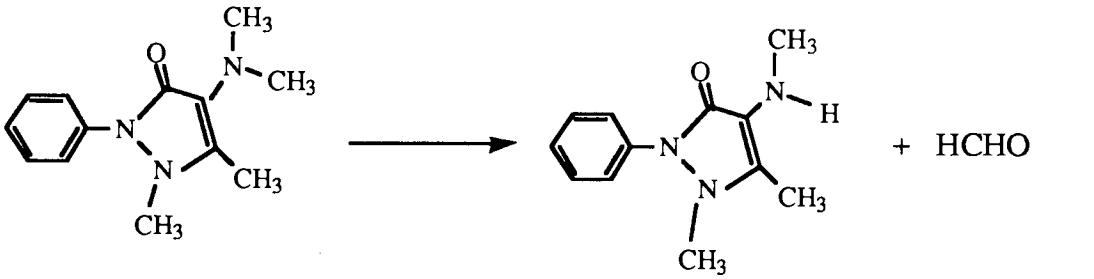
cuvette path length $= 1cm$

From this the concentration of P-450 in the diluted sample was determined.
The cytochrome P-450 content in the original microsomal suspension was calculated and expressed as nmol P-450/mg protein.

3.1.7 Aminopyrine N-demethylation assay.

Each incubation contained 2-4mg microsomal protein in the presence of 10mM aminopyrine. Metabolic generation of formaldehyde was determined using the colorimetric method of Nash(1953), which is based on the Hantzsch reaction.

Metabolic N-demethylation of aminopyrine

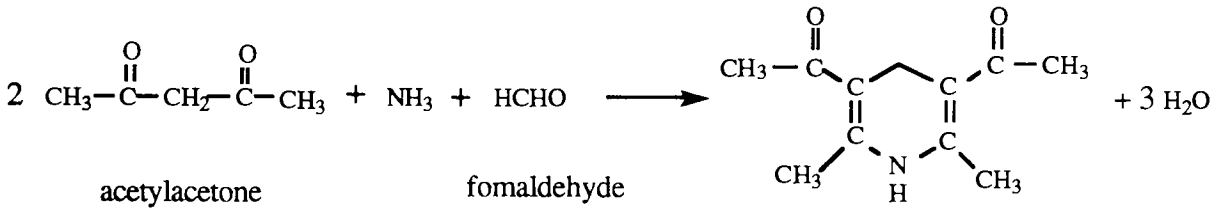


aminopyrine

monomethyl-
4-aminoantipyrine

formaldehyde

Colorimetric determination of formaldehyde (Hantzsch reaction).



acetylacetone fomaldehyde

3,5-diacetyl-1,4-dihydrolutidine

Standard formaldehyde solutions (400µl) in the range 0 to 500µM were prepared from a stock solution of 500µM.

Solutions were prepared in the following way:

i) Paraformaldehyde solution 50mM (freshly prepared)

paraformaldehyde	0.15g
NaOH (0.5M)	to 100ml

This solution was diluted 1 in 100 with distilled water to give a 500µM stock solution.

ii) Aminopyrine 200mM (freshly prepared)

aminopyrine	115.7mg
phosphate buffer (50mM, pH7.4)	5ml

An aliquot of 100µl was added to each incubation

iii) Trichloroacetic acid (TCA) 12.5%

TCA	12.5g
distilled water	100ml

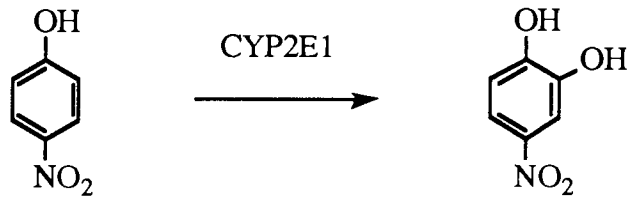
iv) Nash reagent

ammonium acetate	45g
acetic acid	0.9ml
acetyl acetone	0.6ml
distilled water	to 100ml

Open incubation vials containing the NADPH generating system (1ml), suspensions of microsomes (500µl) and phosphate buffer 50mM, pH 7.4 (400µl) were incubated at 37°C for 5 min. The incubation was initiated by addition of aminopyrine (100µl), or buffer in controls. After 30 min the reaction was terminated by the addition of 400µl incubate to 800µl TCA 12.5%. Samples were spun in a MSE Microcentaur microfuge to precipitate the protein. An aliquot of the supernatant (800µl) was added to 400µl Nash reagent and the mixture vortexed. The solution was incubated at 60°C for 30 minutes and was then allowed to cool to room temperature. The absorbance at 412nm was read against a blank (0 mmol HCHO). Values obtained for microsomal incubations with aminopyrine were corrected by subtracting the corresponding blank reading. The amount of formaldehyde formed, as determined from the calibration curve, was expressed in terms of nmol HCHO/nmol P-450.

3.1.8 Para-nitrophenol hydroxylation assay.

The enzymatic oxidation of p-nitrophenol (PNP) to 4-nitrocatechol is catalysed by CYP2E1. Determination of 4-nitrocatechol can be measured colorimetrically and may be used as an indication of CYP2E1 activity (Koop,1986).



p-nitrophenol 4-nitrocatechol

Microsomes equivalent to 1.5-2.5 mg microsomal protein were incubated in the presence of PNP (10mM) in a total incubation volume of 1ml. A standard curve was prepared in the range 0 to 100μM 4-nitrocatechol.
The following solutions were used:

i) PNP (1mM)

PNP	7mg
distilled water	50ml

An aliquot of 100μl was added to each incubation

ii) NADPH (10mM)

NADPH	8.33mg
phosphate buffer (50mM,pH6.8)	1ml

An aliquot of 100μl added to each incubation

iii) Ascorbic acid (10mM)

ascorbic acid	99mg
distilled water	50ml

An aliquot of 100μl added to each incubation.

Incubations containing microsomes (200μl), PNP (100μM), ascorbic acid (1mM) and phosphate buffer adjusted to pH6.8 (500μl), were initiated by addition of NADPH (1mM). After 30 min the reaction was terminated by the addition of 0.7N perchloric acid (500μl). The mixture was spun in a microfuge for 3 minutes to precipitate the protein. An aliquot of 1ml of the supernatant was added to 10N NaOH (100μl) and the absorbance at 546nm was read against a blank (0mmol 4-nitrocatechol).

The formation of metabolite, determined from the calibration curve, was expressed in terms of nmol 4-nitrocatechol/nmol P-450.

3.2 Role of CYP2E1 in the metabolism of NMF *in vitro*.

3.2.1 Pretreatment of animals.

Male BALB/c mice (20-25g) and male Sprague Dawley rats (100-250g) were used in this set of experiments. For induction studies animals received one of the following:

i) 200mg/kg 4-methylpyrazole in saline, i.p. for 3 days. Animals were killed 24hrs after the last dose;

ii) Acetone, 1% in drinking water for one week, for mice; 5ml/kg 50% acetone in saline, intragastrically, 24 hours prior to death, for rats;

iii) 10% ethanol in drinking water for two weeks.

In control experiments animals received drinking water *ad libitum*, and saline i.p. or intragastrically where appropriate.

3.2.2 Microsomal incubations.

Liver microsomes were prepared by differential centrifugation as described previously (3.1.1).

Microsomal incubations (2ml) were performed in the presence of 10mM NMF using the following time points, 10 mins, 30 mins and 60 mins. All incubations contained GSH (10mM) and a NADPH generating system (1mM). At the end of the incubation period reactions were terminated with ice-cold ethanol containing internal standard, and samples were derivatised as described in section 3.1.3. GC analysis on the ethyl acetate extracts was carried out using the conditions indicated in 3.1.4.

Microsomal incubations with p-nitrophenol and aminopyrine were also performed.

3.2.3 Inhibition studies.

Liver microsomes from control animals were prepared in the usual way.

Microsomal incubations were performed with a pre-incubation period of 10 minutes in the presence of inhibitor before the substrate (10mM NMF) was added.The inhibitors and alternative substrates used in this study were DEDTC (0-300μM), PNP (0-100μM) or DMSO (0-1mM). Incubations were for 10, 30 and 60 minutes following which derivatisation and GC analysis was performed.

The effect of PEITC on microsomal metabolism was investigated using the method of Ishizaki *et al* (1990). Microsomes (10ml, equivalent to 5g wet weight liver) were incubated with 5μM PEITC in a total volume of 35ml. Pre-incubation was for 20 minutes at 37°C and the reaction terminated by addition of 15ml ice-cold phosphate buffer 50mM, pH 7.4. The mixture was placed on ice for 1 hr and the microsomes were then re-isolated by centrifugation at 105,000g for 60 min. Microsomes were resuspended in 10ml phosphate buffer, 50mM, pH 7.4 and used for microsomal incubations in the usual way.

Incubations with p-nitrophenol and aminopyrine in the presence of inhibitors were also carried out.

3.3 Role of CYP2E1 in the toxicity on NMF.

3.3.1 Pretreatment of animals.

Male BALB/c mice (20-25g) received 1% acetone in their drinking water for 1 week prior to isolation of hepatocytes. Control animals were allowed drinking water *ad libitum*.

3.3.2 Preparation of hepatocytes.

Hepatocytes were prepared under the guidance of Kurt Mynett (Aston University, Birmingham). The method used for the isolation of mouse hepatocytes was based on the collagenase perfusion method of Seglen (1973), with some adaptions as described by Shaw *et al* (1988). The perfusate was pumped with a Gilson Miniplus 2 peristaltic pump through tubing attached to a bubble trap to prevent air from entering the vena cava. The perfusate was maintained at 37°C by passing it through a heating jacket. The flow rate was set a 8ml/min and the pH of the perfusate kept at 7.4.

The following solutions were prepared prior to hepatocyte isolation:

i) Krebs buffer.

A		B	
NaCl	6.87g	$NaHCO_3$	2.1g
KCl	0.4g	distilled water to 100ml	
$MgSO_4$ $7H_2O$	0.14g		
$CaCl_2$ $2H_2O$	0.38g		
NaH_2PO_4	0.18g		
glucose	2.00g		
distilled water	to 800ml		

Solution A and B were mixed, 25ml 1M HEPES added and the pH adjusted to 7.4. The buffer was made up to a final volume of 1000ml.

ii) Incubation medium.

Krebs buffer	130ml
Horse serum (10%)	20ml
BSA (0.2%)	500mg
adjusted to pH 7.4	

iii) Ca^{2+} free Hanks Buffer.

10x conc Ca^{2+} free HBSS	50ml
distilled water	440ml
1M HEPES	5ml
3.5% Na HCO_3	5ml
adjusted to pH 7.4	

iv) Collagenase solution.

Collagenase	85mg
$CaCl_2H_2O$	40ml
Incubation medium	60ml

BALB/c mice were anaesthetised with an overdose of sodium pentabarbitone (200mg/kg, ip.). The body cavity was opened via a ventral mid-line incision to expose the abdomen, and the intestines displaced to the left side of the animal to display the liver and hepatic portal vein. The diaphragm was cut and the rib cage opened to expose the heart. A thread was loosely tied around the inferior vena cava. The hepatic portal vein was cut immediately prior to cannulation of the vena cava, using a 20-gauge plastic cannula via a small incision in the right atrium of the heart. By cutting the hepatic portal vein the perfusate was able to flow freely through the liver and out of the vein so preventing the build up of pressure which could damage the hepatocytes. To secure the cannula in place the thread around the vena cava was tightened.

The liver was perfused with calcium free Hanks Buffer, for 5 minutes, to clear the liver of red blood cells and extracellular calcium. The perfusate was then changed to a collagenase solution containing calcium chloride, and was perfused with this for a further 10 minutes, to digest the liver. A honeycomb pattern appeared on the surface of the liver where the intercellular bonds were weakening and the liver was held together by the outer membrane.

The liver was dissected out into a petri dish containing ice-cold incubation medium. The gall bladder was removed and the liver was carefully dispersed by gentle teasing with a pipette tip. The suspension, thus obtained, was filtered through a nylon mesh (125μm) to separate the cells from the stringy connective tissue.The cells were allowed to sediment on ice and the supernatant was removed. The cells were washed in fresh incubation medium and this washing procedure was repeated 3 times to remove dead cells and debris. After the final wash the suspension was made up to a final volume of 6ml with incubation medium. Cell viability and yield were then determined.

3.3.3 Trypan blue exclusion assay (for determination of cell viability).

Hepatocyte suspension (100μl), trypan blue 3% (100μl) and incubation medium (800μl) were prepared as a mixture which was applied to a haemocytometer. After 2 min the cells with or without a blue stained nucleus were counted. The viable cells are seen as clear with well defined membranes, whilst the non-viable are blue. Viability was calculated as the

percentage of trypan blue-negative cells in the sample.

$$\% \text{ viability} = \frac{\text{number viable cells} \times 100}{\text{total number of cells}}$$

Cell suspensions with viabilities of >80% were used in incubations.

Cell yield (cells/ml) was calculated by the average number of cells per grid, x 10^5.

Incubations contained $1x10^6$ cells/ml in a total incubation volume of 3.5 ml. The following equation was used to calculate the volume of a cell suspension required to achieve this.

$$\frac{\text{number of cells in 3.5ml (ie } 3.5x10^6\text{)}}{\text{number of cells/ml in cell suspension}}$$

3.3.4 Hepatocyte incubations.

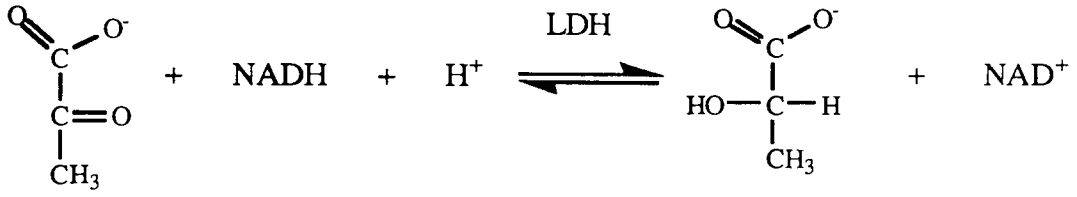
Suspensions of hepatocytes were incubated for up to 6 hr in silanised conical flasks (25ml) and gassed with 95% O_2 : 5% CO_2. The flasks were maintained at 37°C in a gently shaking water bath. Incubations contained hepatocyte suspension (cell density $1x10^6$ cells/ml), incubation medium, substrate and enzyme inhibitor where necessary, to give a final volume of 3.5ml. Isolated hepatocytes were incubated for up to 6 hours in the presence of 0, 5 and 10mM NMF. For inhibition studies 10mM DMSO was also added to incubations. In control experiments hepatocytes were incubated in the absence of substrate and also in the presence of DMSO (10mM) alone.

Aliquots of incubates (100µl) were removed at 0, 2, 4 and 6 hr time intervals after the addition of NMF, for the determination of extracellular lactate dehydrogenase (LDH) activity. Maximal LDH release was initiated by the addition of Triton-X100 (213µl, 16% v/v) to each incubation flask at the end of the experiment, and a 100µl aliquot taken after a further 10 min incubation. All aliquots were centrifuged at 3000rpm for 2 min in a MSE Micro Centaur centrifuge and the supernatant retained and stored frozen (-4°C) for up to 1

week, before being analysed for LDH release.

3.3.5 Lactate dehydrogenase assay.

Release of LDH was recorded by measuring the rate of formation of lactate from pyruvate , as indicated in the equation below.



Pyruvate Lactate

The supernatant obtained in hepatocyte incubations was thawed and stored on ice. An aliquot of 20µl was taken and added to a cuvette kept at 37°C containing phosphate buffer (100mM), pH7.4 and an NADH solution (100µl, 3.5mM) in a final volume of 2.5ml. After 2 min the LDH reaction was initiated by the addition of sodium pyruvate (100µl, 32mM) to the cuvette. The rate of reaction was monitored against a blank in which the incubation supernatant had been omitted, using a Cecil CE594 spectrophotometer. The change in absorbance at 340nm over 5 min was recorded. Cytotoxicity was expressed as a percentage of maximal release of LDH.

3.4 Isolation of anti-rat CYP2E1 IgG.

The techniques described in this section were performed under the guidance of Dr. Ken Thummel in the Department of Pharmaceutics, University of Washington, Seattle, WA.

3.4.1 Animal treatment.

A female New Zealand white rabbit (from R&R Rabbitry, Stanwood, WA.) in which antibody against cytochrome P-4502E1 had previously been raised was again used for

collection of IgG. Purified rat cytochrome P-4502E1 (100μg), isolated and purified by Dr. K.E. Thummel, was made up to 200μl using phosphate buffer, pH7.4 and this was then diluted further with 200μl Freund's adjuvant to form an emulsion of a suitable injection volume.

A pre-boost blood sample, of 10ml, was collected through the marginal ear vein of the rabbit and allowed to stand for 60 min at room temperature. The clot which formed was broken up using a glass rod and the blood sample centrifuged at 2000rpm for 10 minutes. The serum was pipetted off and stored at -70°C .

Following a sub-cutaneous injection of 100μg rat CYP2E1, 30ml of blood was collected from the rabbit every 2 weeks, for 8 weeks. For each sample the serum was separated by centrifugation, as described above, and stored at -70°C.

3.4.2 Test for antibody titre.

Following the 4 week post-boost blood collection the serum samples so far obtained were tested for IgG to determine whether the boost injection had been successful. The sera samples tested were pre-boost serum, 2 week , and 4 week post boost and a serum sample known to contain antibody against CYP2E1. Four antigens were chosen against which the sera samples were tested. These were purified rat CYP2E1(~1pmole), purified human CYP2E1(~1pmole), human liver microsomes (~5pmole P-450) and bovine serum albumin as a non specific marker.

Each of the four antigens (1 to 2.5μl) was slowly spotted onto four squares of nitrocellulose paper using a micropipette and the spots (2mm diameter) allowed to dry. Once completely dry the nitrocellulose papers were soaked in a blocking solution (see section 3.4.7) for 60 min, with continuous shaking at room temperature. The blocking solution was discarded and each piece of nitrocellulose placed in a separate tray ready for exposure to a serum sample. To each tray 30ml blocking solution was added and 30 μl of one of the four serum samples. The nitrocellulose papers were left to shake gently at room temperature, for 90 min, following which the blocking solution was discarded. The nitrocellulose was

washed three times by shaking with 30ml blocking solution for 10 min each wash. The sheets were then incubated together for 45 min at room temperature with a 1 in 1000 dilution of goat anti-rabbit IgG alkaline phosphatase conjugate, diluted in blocking solution (30μg conjugate in 30ml buffer).

At the end of this time the papers were washed three times in blocking solution, twice with PBS, pH7.4 and finally with 0.1M Tris pH7.5. The antigen components were then visualised by incubation in a developing solution of Tris 0.1M, pH 9.5 (10ml), NBT (2ml), BCIP (2ml), by gentle agitation for approximately 5 min, or until a suitable colour intensity had been achieved. Once spots had developed the papers were washed thoroughly in deionised water and dried flat between tissue paper.

3.4.3 Isolation of anti-rat CYP2E1 IgG.

The following solutions were required for the isolation of IgG from rabbit serum:

i) Saturated ammonium sulphate (4.1M)

ammonium sulphate	776g
deionised water	1 litre
pH adjusted to 7.4 with 1M HCl	

ii) 20mM potassium phosphate (KPi)

potassium phosphate monobasic	21.76g
deionised water	8 litres
pH adjusted to 7.6	

iii) 200mM potassium phosphate (KPi)

potassium phosphate, monobasic	27.2g
deionised water	1 litre
pH adjusted to 7.9	

All solutions were pre-cooled to 4°C before use.

The four post-boost sera samples, which were known to contain anti-CYP2E1 as indicated from the previous dot blots, were thawed and the samples pooled. The pH of the sera was adjusted to 7.2-7.4, using 1M HCl, with stirring at 4°C. While continuing to stir ammonium sulphate was added dropwise, in a ratio of 40ml ammonium sulphate to 60ml serum, over a period of 5 minutes. A white precipitate formed after approximately 2/3 of the total ammonium sulphate volume had been added. After all the ammonium sulphate had been added the solution was stirred slowly for 1 hr at 4°C.

The precipitated protein was pelleted by centrifugation at 10,000g for 20min at 4°C. The supernatant was discarded and the milky white pellet was resuspended in a volume of 20mM KPi, pH 7.6 that was approximately 50% of the original serum volume by breaking it up with a spatula and vortexing gently. The redissolved protein was dialysed at 4°C against 2L of 20mM KPi, pH 7.6 for 6 hr, followed by dialysis overnight, to remove excess ammonium sulphate.

Whilst the protein was dialysing two anion exchange columns (3.5cm x 30cm) were prepared. Two columns were required due to the volume of protein to be run. DE-52 anion exchange resin (120g) was swollen in 200mM KPi, pH 7.9 (500ml) and washed in the same buffer 3 times, by allowing the resin to settle, decanting off the supernatant, adding fresh buffer and stirring with a glass rod. The resin was then washed, in the same way, with 20mM KPi, pH7.6, twice. The resin swells to approximately 240ml. Two columns were poured and the columns were equilibrated with 20mM KPi, pH 7.6 by allowing 500-1000ml to be pumped through each overnight. The pH of the eluent was tested the following morning to determine whether equilibration had been achieved.

The dialysed protein was centrifuged at 10,000g for 10min to pellet debris and the supernatant retained for loading onto the columns. The protein was carefully pipetted onto the top of the columns and loaded at a rate of 40-50ml/hr by pumping air through the system. Once all the protein had been loaded the sides of the columns were rinsed with buffer which was allowed to flow through by gravity. The wash was repeated to ensure all

the protein was loaded. The upper 3-4cm of the columns could be seen to turn milky white where the albumen was beginning to accumulate.

The pump was connected to a 20mM KPi, pH7.6 reservoir and set at 30ml/hr. Approximately 200ml of buffer was passed through to elute the IgG, and 10 minute fractions of the eluent were collected using a fraction collector. The absorbance at 280nm was measured for all fractions, referencing against 20mM KPi, pH7.6, to determine the peak elution of IgG. Those samples in which the majority of IgG were eluted were retained and pooled. The expected IgG could be calculated from the following equation.

$$\text{expected IgG (mg)} = \frac{\text{absorbance 280nm x volume eluent}}{\text{Extinction coefficient}}$$

IgG has an extinction coefficient of 1.3 $mg.ml^{-1}.cm^{-1}$.

The specificity of the anti-rat CYP2E1 was tested by Western blot analysis against samples of liver microsomes, as described in sections 3.4.5 to 3.4.7.

3.4.4. Isolation of pre-immune IgG.

In order to prepare a non-specific IgG a female New Zealand rabbit which had not previously been treated was used. The animal was anaesthetised and a maximum bleed taken by means of cardiac puncture. The blood was allowed to stand at room temperature for 30 minutes and then centrifuged at 2000 rpm as described previously, to obtain a serum sample. The pre-immune IgG was isolated by the method described above (section 3.4.3) and the samples stored at -70°C for 4-6 weeks.

3.4.5 Gel Electrophoresis.

The following solutions were prepared:

Acrylamide Gel Buffers

Separating buffer:	Tris base	0.75M	45.41g
	100% SDS	0.2%	1.0g

made up to 500ml with deionised water and adjusted to pH 8.8 with HCl.

Stacking buffer:	Tris base	0.25M	15.14g
	100% SDS	0.2%	1.0g

made up to 500ml with deionised water and adjusted to pH 6.8 with HCl.

30% Acrylamide:	Acrylamide/bis acrylamide (37.5:1)		30.8g

made up to 100ml with deionised water.

Ammonium persulphate:	AMPS	5%w/v	0.1g

made up to 2ml with deionised water.

Gel Formulae

Separating gel 7.5%		Stacking gel 3%	
30% acrylamide	5.0ml		0.5ml
2x sep. buffer	10ml	2x stack buffer	2.5ml
D.I. water	4.9ml		1.9ml
TEMED	20µl		20µl
5% AMPS	120µl		120µl

Electrode buffer

Tris base	6.05g
glycine	28.82g
SDS 100%	2g

made up to 2 L with deionised water.

SDS gel-loading buffer

0.5M Tris pH 6.8	5ml
SDS 10%w/v	10ml
mercaptoethanol	0.5ml
glycerol	5ml
bromophenol blue	0.4ml
deionised water	10ml

Glass plates (15x12cm and 15x11cm) and spacers were prepared ready for pouring a SDS-polyacrylamide gel. Glass plates were cleared of grease by washing with ethanol, to prevent air bubbles when pouring the gel.The larger plate was placed flat on the bench and spacers placed on either side parallel to the two edges. The smaller plate was placed in position, resting on the two spacer bars. The plates were then carefully placed in a support stand, ensuring at each stage that the bottom edges of plates and spacers were even so as to achieve a leak proof system.

The required quantity of acrylamide solution was placed in a sidearm flask and the solution deaerated by applying a vacuum. TEMED and ammonium persulphate were added to the acrylamide solution immediately prior to pouring since polymerisation begins as soon as the TEMED has been added. The solution was introduced into the space between the two glass plates using a disposable pasteur pipette. The gel was poured leaving sufficient room for the stacking gel (the length of the teeth of the comb to be inserted, plus 1cm). The surface was carefully overlayed with isobutanol to prevent oxygen diffusing into the gel and inhibiting polymerisation. The acrylamide was allowed to polymerise for 30 mins at room temperature.

Once the separating gel had polymerised, the isobutanol was poured away and the top of the gel washed several times with deionised water to remove any unpolymerised polyacrylamide. As much fluid as possible was drained from the top of the gel and the remainder was removed using filter paper. The stacking gel was prepared in the same way as the separating gel and then added to the top of the separating gel. A teflon comb was immediately inserted into the stacking solution and more solution added to fill the spaces in the comb completely. The gel was left for 30 minutes at room temperature. Once polymerisation was complete the comb was carefully removed and the wells rinsed out with deionised water to remove any unpolymerised acrylamide. The gel was inserted into the electrophoresis tank which was filled with electrode buffer. Any bubbles trapped at the bottom of the gel were displaced.

Samples (1pmole human CYP2E1, 20μg human liver microsomes) were prepared by diluting to 100μl in buffer and heating to 100°C for 3 min with SDS gel-loading buffer (100μl), to denature the proteins. Each sample (20μl) was loaded into a well using a Hamilton microlitre syringe. An equal volume of loading buffer was loaded into wells that were not used for samples.

The electrophoresis apparatus was connected to an electrical power supply and run at 40 mA. Once the dye front had moved into the separating gel the current was increased to 80mA and the gel run until the bromophenol blue reached the bottom of the separating gel. The power supply was then turned off and the glass plates removed from the apparatus. The glass plates were placed on the bench and using a thin spatula the corner of the upper plate lifted. Ensuring that the gel remained attached to the lower plate, the upper plate was pulled away. The stacking gel was cut away and a small slice taken from the top right hand corner of the stacking gel for orientation purposes.

3.4.6 Transfer of proteins to solid support.

The following procedures were all performed whilst wearing gloves since oils and secretions from the skin will prevent the transfer of the proteins from the gel to the filter.

Two pieces of Whatman 3MM paper and one piece of nitrocellulose paper (BA-83, 0.2μm pore size, Schleicher & Schuell) were cut to the size of the gel. These were allowed to soak in transfer buffer (Tris base 12.11g, glycine 57.65g, deionised water to 4 litres. The pH should be approximately 8.3). Two porous pads were also fully submerged in transfer buffer and any trapped air bubbles displaced.

A sandwich was prepared in the following way once everything had been thoroughly soaked in transfer buffer. The bottom half of a plastic cassette was placed flat in a tray containing transfer buffer. One porous pad was placed on top of this followed by a sheet of Whatman 3MM paper. The nitrocellulose paper was aligned on top and the SDS-polyacrylamide gel orientated on this with the cut corner at the top right. The second piece of Whatman paper was added and then the second porous pad. The other half of the cassette was locked into place.

The transfer chamber was filled with transfer buffer and the cassette placed in the transfer apparatus with the gel side facing towards the cathode and the nitrocellulose towards the anode. The unit was attached to an electrical power supply and the proteins transferred from cathode to anode under a 100V field for 60 minutes. During this time the apparatus was kept cool by means of an internal coil through which cold water was passed. After 60 minutes the power supply was switched off and the transfer apparatus disassembled. The nitrocellulose filter was removed and placed in a tray containing deionised water. The top right corner was cut for orientation.

3.4.7 Immunological probing.

Phosphate Buffered Saline (PBS)

NaCl (0.79%) 31.4g

Na_2HPO_4 2.6g

NaH_2PO_4 0.6g

made up to 4 litres with deionised water; pH is approximately 7.4.

Blocking buffer

carnation non-fat milk (2.0%) 20g

Triton X-100 (0.2%) 2g

made up to 1 litre with PBS and adjusted to pH 7.4 with NaOH.

The nitrocellulose filter was placed in a tray containing 30ml blocking buffer and left to shake at room temperature for 2 hr to block any non-specific binding sites. The blocking buffer was discarded and 30ml fresh buffer was added. The primary antibody (ie.that isolated from the sera samples) was added at a dilution of 1:1000 (30µg/30ml blocking buffer). The paper was incubated with the primary antibody for 2 hr at room temperature with gentle shaking.

The blocking solution with antibody was discarded and the nitrocellulose washed three times (10min each time) with blocking solution. Fresh blocking buffer (30ml) was added and the nitrocellulose incubated for 1 hr with a 1:1500 dilution of anti-IgG conjugate (goat anti-rabbit IgG alkaline phosphatase conjugate). This solution was discarded and the paper washed three times with blocking solution (10 mins each time) twice with PBS and twice with 0.1M Tris, pH7.5.

The washed nitrocellulose paper was placed in the colour development solution (0.1M tris, pH 9.5, 15ml; NBT, 1.5ml; BCIP, 1.5ml) and mixed with gentle agitation. The progress of the reaction was monitored carefully and when the bands had reached a suitable intensity

the developing solution was discarded, and the nitrocellulose washed with copious amounts of deionised water to remove any traces of developing solution. The filter was allowed to dry between tissue paper under a flat heavy object.

3.4.8 Purification of anti-rat CYP2E1 IgG.

The following buffer solutions were prepared and all were cooled to 4°C before use.

Buffer 1 (2 L)
50mM NaPi, pH 7.25
20% glycerol
0.8%(w/v)Emulgen 911
1mM DTT
1mM EDTA

Buffer 2 (4 L)
50mM NaPi, pH 7.25
20% glycerol
1mM DTT
1mM EDTA

Buffer 3 (2 L)
50mM NaPi, pH 7.25
20% glycerol
0.8%(w/v) Emulgen 911
0.4%(w/v) sodium cholate
1mM DTT
1mM EDTA

Buffer 4 (2 L)
Buffer 3 + 1M Na Cl

Laurate sepharose (100g) was swollen to 200ml in buffer 1 and a column (3.5 x 30 cm) was packed with this material. The column was equilibrated with the high salt buffer (4) using approximately 1 litre, in order to clean the column, and then equilibrated with 1 litre of the emulgen free buffer (2).

Human liver microsomes were prepared in the following way:
50g of human liver known to have a relatively high P-4502E1 phenotype was carefully thawed by stirring slowly with 1 litre 0.9% NaCl, at 4°C. The NaCl was decanted off and 200ml of 10mM KPi, pH7.4 containing 0.25M sucrose was added. The large chunks were broken up by placing this mixture in a blender and treated to 4 x 5 second bursts. The liver was then homogenised in the conventional way. The homogenate was centrifuged initially at 4,000rpm for 5 min, to bring down cell fragments slowly, and the speed was then increased to 13,000rpm for 20 min. The supernatant was filtered through gauze to ensure that most of the fat was removed, and then spun in an ultracentrifuge at 37,000rpm for 60min. The microsomal pellet was resuspended in 10mM KPi, pH7.4 + 1.15%KCl, homogenised and respun at 37,000rpm for 60min.

The final microsomal pellet was resuspended in buffer 2 to give a final volume of 120ml. The cytochrome P-450 content was calculated. An aliquot of microsomal suspension (250µl) was added to 750µl of a buffer solution containing detergent (50mM NaPi, pH7.25, 20% glycerol, 1mM EDTA, 1mM DTT, 0.2% emulgen 911). A few grains of sodium dithionite were added and a baseline between 400-500nm recorded, using a single beam UV spectrophotometer. Carbon monoxide was slowly bubbled through the solution for 30 seconds, and the absorbance spectrum recorded against the baseline, between 400-500nm. The absorbance difference between 450 and 490nm was recorded and the P-450 content calculate using the equation in section 3.1.5. The P-450 content was calculated to be 500mg in the 120ml. To obtain a concentration of 2mg/ml the 120ml microsomal suspension was diluted to 250ml with 10% emulgen 911 (5ml), 100mM PMSF in ethanol (0.25ml) and 125ml buffer 2. The suspension was mixed by passing once through the homogeniser by hand, and then stirring for 30min at 4°C.

To remove glycogen the sample was centrifuged at 37,000rpm for 45 min and the supernatant decanted off and retained. A small translucent glycogen pellet remained in the centrifuge tube. The supernatant was loaded onto the laurate sepharose column at a rate of 15ml/hr.

Once loaded the pump was connected to a reservoir containing buffer 1, and set at a flow rate of 20ml/hr. After the first P-450 fraction had eluted, visible as a faint yellow colour in those fractions, the reservoir was changed to buffer 3 to elute the second P-450 peak. This was again visible. The cytochrome b_5 and any debris was eluted to waste with the high salt buffer(4). All the fractions containing P-450 were pooled and a P-450 determination carried out.

The following dialysis buffer was prepared
5mM NaPi, pH6.5
20% glycerol
0.1mM DTT
0.1mM EDTA

The pooled P-450 samples were dialysed against 2 x 4L of this buffer, for 18hr each time.

CM-sepharose gel (50ml) was washed with 4 x 500ml 200mM NaPi, pH6.5 and once with dialysis buffer. A column was packed and the sepharose allowed to equilibrate with 500ml of dialysis buffer. The dialysed P-450 was loaded onto the column and the eluent collected until it appeared colourless. The pooled CM sepharose flow through was retained and the absorbance at 418nm recorded as an indicator of P-450 content. This sample contains most of the P-450 isozymes, but not CYP2E1. This isoenzyme is retained by the column under these conditions due to its high isoelectric value as compared to other isozymes.

In order to wash off the CYP2E1 the following buffers were prepared.

40mM NaPi pH 6.8
20%glycerol
0.5% Emulgen 911
0.1mM DTT
0.1mM EDTA

120mM NaPi pH 6.8
20% glycerol
0.5% Emulgen 911
0.1 mM DTT
0.1mM EDTA

The 40mM buffer (200ml) was used to elute the column overnight and the 120mM buffer was then used to elute the remainder. The CYP2E1 fractions were retained and frozen at -70°C for later purification.

An AMICON system was used to concentrate the CM Sepharose flow through from 400ml down to 100ml. This system involves the application of pressure (via nitrogen) onto the liquid thus forcing buffer to pass through a filter at the base of the chamber, whilst the proteins are too large to pass. The concentrated sample was then dialysed against 5mM NaPi pH7.25 containing 20% glycerol to remove the DTT which may bind to the activated sepharose used in the next purification step.

To check that the CM sepharose flow through sample contained no CYP2E1 dot blots were performed as in section 3.4.2.
Two pieces of nitrocellulose were taken and on each was spotted 10µl CM sepharose flow through, and 1µl 120mM NaPi eluent (ie containing CYP2E1 as positive control). Once blocked these were incubated with either anti-CYP2E1 or anti-CYP3A4 (expected to be present in the CM flow through) at a concentration of 1µg/ml, for 90 min. After washing the

papers in blocking buffer they were incubated with a 1 in 1000 dilution of the secondary antibody (goat anti-rabbit IgG, alkaline phosphatase conjugate), for 45 min. The blots were developed in the same way as described previously.

To remove the emulgen from the P-450 sample the following buffers were prepared at 4°C.

Buffer 1	Buffer 2	Buffer 3
5mM KPi pH 7.25	200mM KPi pH 7.25	50mM KPi pH7.25
20% glycerol	20% glycerol	20% glycerol
0.4 % cholate	0.4% cholate	

Hydroxyapatite (25ml) was packed into a column, 1cm x 6cm, using buffer 1. The P-450 solution was loaded overnight (15ml/hr) and buffer 1 was then run through until the absorbance at 280nm was < 0.02, indicating all the emulgen had been removed. The 200mM buffer was passed through the column and the P-450 band was collected as it was visible as a brown band on the column. This sample was then dialysed against buffer 3 overnight.

A CNBr-activated sepharose was washed and prepared for the coupling of the P-450 sample containing no CYP2E1. CNBr-activated sepharose (15g) was taken and suspended in 100ml 1mM HCl. The gel was washed with 2L of 1mM HCl and then with 500ml $NaHCO_3$ (0.1M), pH8.3 containing NaCl (0.5M) using a sintered glass filter. The activated gel was divided between 4 x 50ml capped tubes and the P-450 solution shared evenly between them. The tubes were shaken gently at room temperature for 2 hr, and left overnight at 4°C. After centrifugation at 1000rpm for 5min the supernatant was decanted off, leaving P-450 coupled to the gel. This gel was shaken gently with glycine (1M) for 2 hr at room temperature and then washed in PBS by gentle agitation. A column was packed with the gel and equilibrated with PBS (15ml/hr) overnight.

The isolated IgG which had been stored in 20mM KPi, pH 7.4 was thawed and dialysed against PBS (2 x 4L). The IgG solution was run through the P-450-bound column at

20ml/hr and PBS washed through following this. The eluent fractions were collected and the absorbance at 280nm was measured. Those fractions containing IgG were pooled ready for the testing of specificity (section 3.4.9).

The purified IgG was concentrated from 77ml to approximately 8ml using an AMICON system. The concentrated IgG was dialysed against 2 x 1L 50mM KPi, pH7.4 and frozen at -70°C in 1ml vials. The frozen samples were lyophilised to dryness and stored at -70°C until required. The pre-immune IgG obtained in 3.4.4. was also concentrated to approximately 10ml, frozen in 1ml aliquots and lyophilised.

3.4.9 Specificity of purified antibody.

SDS-PAGE was performed as in sections 3.4.5 and 3.4.6

The same samples were run on the gel and transferred onto the nitrocellulose in the same way. Immunological probing was carried out as described in section 3.4.7. The primary antibody used was 30µg of the freshly purified anti-CYP2E1 diluted in 30ml blocking buffer. After developing the blot the nitrocellulose paper was dried flat between tissue paper and a photograph taken.

3.5 Inhibition of *in vitro* metabolism of NMF by anti-rat CYP2E1, and its metabolism by purified CYP2E1.

3.5.1 Microsomal incubations with anti-rat CYP2E1.

Rat liver microsomes were prepared in the usual way by differential centrifugation. Cytochrome P-450 assays were performed to determine the amount of antibody that would be required on a mg IgG/nmolP-450 basis, assuming a 200µl volume of microsomes per incubation. The lyophilised IgG was reconstituted by the addition of 1ml deionised water to each vial. The IgG concentration was determined by diluting a small sample 1 in 50 using phosphate buffer, 50mM, pH7.4 and reading the absorbance at 280nm against a phosphate buffer blank.

The IgG concentration was calculated using the following equation.

$$\text{mg IgG/ml} = \frac{\text{Abs 280nm x dilution factor (50)}}{\text{extinction coefficient}}$$

IgG has an extinction coefficient of $1.3 mgml^{-1}cm^{-1}$.

Incubations (1ml) contained microsomes (200µl), anti-rat CYP2E1 IgG or pre-immune IgG (0-15mg IgG/nmol P-450), NADPH generating system (1mM), and GSH (10mM). Pre-incubation was for 5 min at 37°C and incubations were initiated by the addition of NMF (10mM). After a 30 min incubation period reactions were terminated by the addition of ice-cold ethanol (2ml) containing internal standard. Samples were centrifuged at 3000rpm to precipitate the protein and the supernatant retained for derivatisation of SMG. Anhydrous K_2CO_3 (1.5g) was mixed vigorously with the supernatant and the mixture centrifuged at 3000rpm to separate the aqueous and ethanolic layers. The ethanol layer was concentrated in the gyrovap and reextracted into ethyl acetate as described previously. The formation of SMG from NMF was determined by GC analysis of the samples using the conditions indicated in section 3.1.4.

3.5.2 Reconstitution of rat CYP2E1.

Reconstitution and incubations were performed by Dr. Ken Thummel (Department of Pharmaceutics, University of Washington, Seattle, WA.). Purified CYP2E1 (0.25 nmoles, 75.8µl of a 3.3mM suspension), or buffer for controls, was added to each vial in which incubations were to be performed. DLPC (30µg, 10µl of a 3mg/ml suspension) was added and mixed by sonication for 3 min to form vesicles. The solution was left for 15 min at room temperature. Cytochrome P-450 reductase (1.0 nmole, 73µl of a 13.7mM suspension) was added and left to stand for 5 min at room temperature. After addition of 0.25 nmole cytochrome b_5 (12.8µl of a 19.6mM suspension) the mixture was incubated for 5 min at room temperature. At this stage the vials containing reconstituted enzyme were stored on ice in preparation for incubation with substrate.

3.5.3 Metabolism of NMF by reconstituted rat CYP2E1.

All incubations were performed in glass vials, at 37°C, in a shaking water bath for 1 hr. Incubations contained 0.25nmole reconstituted CYP2E1, with a negative control consisting of only cytochrome P-450 reductase and b_5. All vials contained 10mM NADPH, 10mM GSH and 10mM NMF in a total volume of 1ml. Additional incubations containing microsomes isolated from diabetic rats (equivalent to 0.5nmol P-450), both in the presence and absence of NADPH, were performed for comparative purposes. After 60 min the reaction was terminated by the addition of 2ml ethanol containing 25mM quinoline. Samples were derivatised as described in 3.1.3 using anhydrous K_2CO_3. Samples were packed under nitrogen into glass vials and sent to Aston University for analysis.

The samples were extracted into ethyl acetate and analysed on a Hewlett Packard 5890 Series II Gas Chromatograph. The GC was equipped with a HP-20M (carbowax 20M) capillary column, 25m x 0.32mm x 0.3μm film thickness, which was connected to a nitrogen-phosphorous detector. Helium was used as carrier gas, with nitrogen as the make-up gas (total flow 30ml/min). The hydrogen and air flow rates were set at 3ml/min and 100-120ml/min respectively. The injector and detector were preheated to 200°C and 220°C respectively. The GC was run using the following temperature programme ; 170°C for 3.5 mins, increasing to 200°C at a rate of 15°/min and maintaining at 200°C for 3 mins. Under these conditions compounds had the following retention times; DMF 2.7 min, EMC 2.9 min, NMF 3.7 min and quinoline 6.5 min.

The GC was connected to a HP 3396A integrator and quantification of peaks was achieved using peak height ratios of the peak of interest to the internal standard peak.

3.5.4 Metabolism of p-nitrophenol by purified rat CYP2E1.

The ability of purified rat CYP2E1 to metabolise PNP was determined by Dr. Ken Thummel (Department of Pharmaceutics, University of Washington, Seattle.). Rat CYP2E1 was reconstituted as described in section 3.5.2. Incubations were performed using 0.1 nmole CYP2E1 and this was reconstituted with 0.4 nmole P-450 reductase and 0.1 nmole

cytochrome b_5. Incubations with PNP (2mM) were initiated by the addition of 1mM NADPH and terminated after 10 min by the addition of perchloric acid as described in section 3.1.8. Incubations with untreated rat liver microsomes and liver microsomes from diabetic rats (1.0 nmole P-450) were also performed.

3.6 Metabolism of NMF by human CYP2E1.

3.6.1 Preparation of human liver microsomes.

Excess samples of healthy human liver were obtained after graft reduction of donor liver from the Liver Transplant Unit at the Queen Elizabeth Hospital (Birmingham, UK). Tissues originated from three females, aged 10, 28 and 34 years, and three males, aged 12, 45 and 55 years. Liver samples had been stored at -70°C for 3 weeks to 8 months before use.

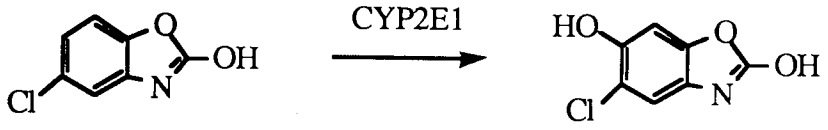
A liver homogenate was prepared in Tris-buffered (50mM, pH7.4) KCl solution (0.154M). Microsomes were prepared in the usual way by differential centrifugation as described in 3.1.1. Extra care was taken when handling human liver. Double gloves were worn and all waste material was disinfected in bleach before being discarded. Suspensions of liver microsomes were prepared at a concentration of 500mg wet weight liver / ml phosphate buffer (50mM, pH7.4). Lowry protein assays and cytochrome P-450 assays were performed as outlined in sections 3.1.5 and 3.1.6 respectively.

3.6.2 Microsomal incubations with NMF.

Microsomal incubations were performed in duplicate for each liver sample. Incubations were for 30min, at 37°C, in a shaking water bath. Each vial contained microsomes (250µl), NADPH generating system (1mM), GSH (10mM) and NMF (10mM) in a total volume of 1ml. Reactions were terminated by the addition on 750µl incubate to 1.5ml ice-cold ethanol containing internal standard. After centrifugation the supernatant was treated with 1.13g K_2CO_3 to derivatise SMG as described in 3.1.3. Samples were analysed by GC using the conditions indicated in 3.1.4.

3.6.3 Metabolism of chlorzoxazone.

The ability of the six human liver microsome samples to metabolise chlorzoxazone to its 6-hydroxy metabolite was investigated, with the assistance of Dr. C Schiller and P. Jheeta (Aston University, Birmingham).

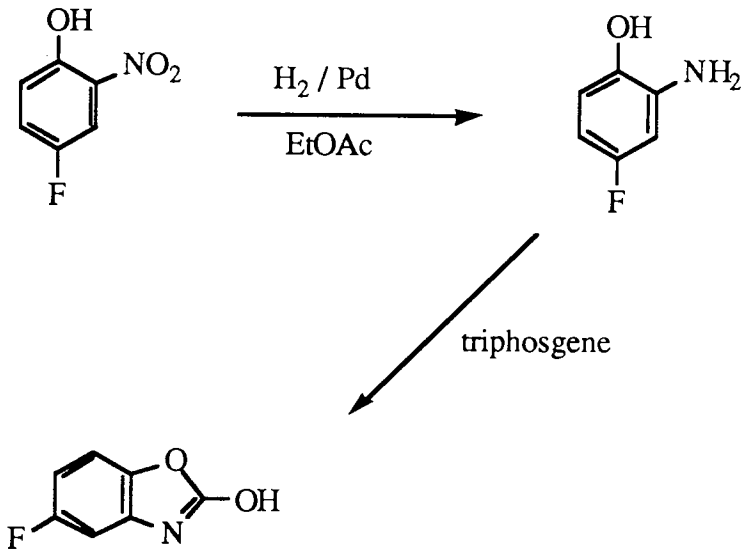


chlorzoxazone 6-hydroxychlorzoxazone

Metabolite (6-hydroxychlorzoxazone) and internal standard 5-fluoro-2(3H)-benzoxazolone were kindly donated by Dr. Raimund Peter (formerly Department of Pharmacology and Toxicology, University of Erlangen-Nurnberg, Erlangen, Germany.))

Additional 5-fluoro-2(3H)-benzoxazolone was prepared with the assistance of Dr. Alan Clark using the following method.

3.6.4. Synthesis of 5-fluoro-2-(3H)-benzoxazolone.



4-Fluoro-2-nitrophenol (40.00g; 0.065M) was dissolved in ethyl acetate (100ml) containing 10% Pd/C (700mg) and hydrogenated at room temperature and pressure until there was no further uptake of H_2 (theoretical volume 4.28litres). The reaction mixture was transferred under argon, filtered through celite under argon directly into the reaction vessel which contained anhydrous sodium acetate (15.66g; 0.19M), i.e. 3 equivalents since triphosgene gives off 3 equivalents of HCl in reactions with amines. The suspension was vigorously stirred and treated dropwise with a solution of triphosgene (6.85g; 0.023M) in ethyl acetate and refluxed for 2 1/2 hours. The cooled solution was washed with water (200ml) and 5% HCl (200ml). The ethyl acetate layer was filtered through celite to give a red solution, dried over $MgSO_4$ and filtered. Solvent was removed in a vacuum oven to give a brown residue. On attempted recrystallisation from water some dark oil was present, and the solution was treated with charcoal and filtered to give an orange brown solution, which on cooling yielded orange needles.

To purify, the sample was loaded onto a silica column and eluted using chloroform:ethyl acetate (70:30). Those aliquots collected which contained pure sample were pooled and the solvent evaporated. White crystals were obtained which gave one spot on t.l.c. analysis. To confirm the identity of the compound NMR and mass spectrometry were used.

3.6.5 Chlorzoxazone assay.

Chlorzoxazone (400µM in 0.05M potassium phosphate buffer, pH 7.4) was incubated with human liver microsomes (containing between 8.2 and 14.3 mg protein/ml) and a NADPH generating system (consisting of 20mM glucose-6-phosphate, 10mM NADP and 4IU of glucose-6-phosphate dehydrogenase) in a total volume of 1ml. After 3 min preincubation at 37°C incubations were initiated by adding the NADPH generating system. After 20 min the reactions were quenched by the addition of 50µl of H_3PO_4 (43%w/v) and 100µl of 5-fluoro-2(3H)-benzoxazolone solution (5µg/100µl 0.02% propylene glycol in water) as an internal standard.

The reaction mixtures were extracted twice with 1ml dichloromethane. The layers were separated by centrifugation at 6000g for 10 min and the combined organic layers evaporated

to dryness in a gyrovap at room temperature. Residues were dissolved in 100µl of CH_3CN-H_2O mixture (4 : 6 v/v) and analysed (20µl aliquots) by HPLC. Calibration curves were established using authentic 6-hydroxychlorzoxazone.

HPLC analysis was performed on a Waters system comprising of two Waters 510 pumps, a WISP 710 autoinjector attached to Shimadzu SPD-6A UV detector set at 287nm and connected to a Waters Maxima 820 workstation. Separation was achieved on a 50 x 4.6 mm Supelcosil LC-8-DB (5mm) column in series with a 20 x 2 mm guard column filled with LiChrosorb RP-8 (5mm) packing material. The mobile phase consisted of a mixture of CH_3CN and 0.5% H_3PO_4 26 :74 v/v at a flow rate of 1.8ml/min. The effluent was monitored at 287nm. Retention times of 6-hydroxychlorzoxazone, 5-fluoro-2(3H)-benzoxazolone and chlorzoxazone were 0.9min, 1.5min and 2.6min, respectively.

3.6.6 Immunodetection of CYP2E1.

Samples of human liver microsomes (2mg/ml) were prepared in a final volume of 100µl. This solution was diluted with 100µl loading buffer and then boiled for 10 min to denature the proteins.

Gels for SDS-PAGE were prepared as in 3.4.4 using the following formulae.

Stock I

44% w/v acrylamide

0.8% w/v bis acrylamide

stock II

30% w/v acrylamide

0.8% w/v bis acrylamide

Separating gel 7.6%	
Stock I	4ml
1.5M Tris pH8.8	7.5ml
Deionised water	11.5ml
SDS 10% w/v	0.6ml
TEMED	56µl
AMPS 10% w/v	80µl

Stacking Gel	
Stock II	2ml
0.5M Tris pH6.8	3ml
deionised water	6.4ml
SDS 10%w/v	120µl
TEMED	32µl
AMPS 10%w/v	40µl

A 20µl sample was loaded into the wells or 5µl prestained low molecular weight markers obtained from Sigma). Any wells not containing a sample were loaded with loading buffer alone. SDS-polyacrylamide gels were exposed to 200V for approximately 1 hr, or until the dye had almost reached the bottom of the gel. Proteins were transferred onto nitrocellulose as described in section 3.4.6. After 1 hr the nitrocellulose was removed and all the non-specific sites blocked by shaking with blocking buffer (as in section 3.4.7) for 2 hr. A 1µg/ml solution of anti-rat CYP2E1 in blocking buffer was prepared and the sheet of nitrocellulose incubated with this for 60 min at room temperature and then overnight at 4°C. As a negative control a sheet of nitrocellulose onto which proteins had been transferred was incubated overnight in blocking buffer alone.

The papers were incubated with a secondary protein (Protein-A peroxidase conjugate) at a concentration of 0.25µg/ml blocking buffer for 2 hr. The nitrocellulose was then washed in blocking buffer, followed by PBS. A colouring solution was prepared by the addition of a

spatula tip of chloronaphthol in 1ml methanol diluted to 50ml in PBS. This solution was heated gently until warm and immediately prior to use 40µl H_2O_2 was added. This solution was left in contact with the nitrocellulose papers until a stain of desired intensity had developed.

The nitrocellulose paper was dried between tissue paper and the intensity of each band measured using a LKB Bromma 2202 Laser Densitometer. Each band was scanned in three different places and the intensity recorded as a mean of these readings. The intensity per mg microsomal protein was calculated and these values correlated with the ability of human microsome samples to metabolise NMF to SMG or chlorzoxazone to 6-hydroxychlorzoxazone.

3.6.7 Influence of anti-rat CYP2E1 on human liver microsomal metabolism of NMF.

Microsomes isolated from a human liver sample were co-incubated with NMF (10mM) and anti-rat CYP2E1 or pre-immune IgG (0-10mg IgG/nmolP-450) in the same way as for rat liver microsomes (section 3.5.1). Incubations (1ml) were for 30 minutes at 37°C in a shaking waterbath. Reactions were terminated with 2ml ethanol containing internal standard, and derivatisation was performed using 1.5g anhydrous K_2CO_3. Following reextraction into ethyl acetate samples were analysed in the usual way, by GC.

3.7 Metabolism of DMF *in vitro.*

3.7.1 Pretreatment of animals.

For induction studies male Sprague-Dawley rats (100-150g) were given acetone 1% in drinking water for one week prior to experiments. Control animals received drinking water *ad libitum.*

3.7.2 Microsomal incubations.

Rats liver microsomes were prepared in the usual way by differential centrifugation of a liver homogenate. Incubations consisting of microsomes (equivalent to 4-6 mg microsomal

protein), an NADPH generating system (1mM), GSH (10mM), DMF (10mM) and the CYP2E1 inhibitor DEDTC (0-100μM) in a total volume of 2ml were carried out as described previously for up to 60 min. For derivatisation of samples and GC analysis see 3.7.3.

In a preliminary experiment the effect of coincubation with DMF on the metabolism of NMF was determined. Suspensions of rat liver microsomes (500μl) were incubated for 60 mins with NMF (1mM), a NADPH generating system (1mM) and GSH (10mM), in the presence of varying concentrations of DMF (0.2 to 10mM).

3.7.3 Sample preparation for GC analysis of DMF metabolites.

Incubates were treated with ethanol and anhydrous K_2CO_3 to derivatise any SMG that may have been present. A 200μl sample of the ethanol layer was retained for the GC analysis of HMMF. The remainder was concentrated and extracted into ethyl acetate for the analysis of EMC, as described in 3.1.3.

3.7.4 Effect of coincubation with anti-rat CYP2E1.

Incubations with 10mM DMF were performed with human liver microsomes in the presence of anti-rat CYP2E1 or non-specific IgG in the concentration range 0-10mg IgG/nmolP-450, for 30 mins, as described in 3.5.1. Samples were prepared for GC analysis in the usual way. For the determination of HMMF formation the ethanolic layer was injected directly onto the GC.

3.7.5 Metabolism of DMF by purified rat CYP2E1.

Purified rat CYP2E1 was reconstituted as in section 3.5.2.
Incubations were performed using 0.2nmoles CYP2E1 and this was reconstituted with 0.8 nmoles P-450 reductase and 0.2 nmoles cytochrome b_5.

Incubations for 60 min, in the presence of 10mM DMF, were performed as indicated in 3.5.3, including a negative control. Microsomes from diabetic rats were again used in incubations. Incubates were treated as before, packed under nitrogen into glass vials and sent to Aston University. Samples were analysed by GC using a Hewlett Packard capillary

GC under the conditions stated in section 3.5.3.

3.8 Metabolism of DMF *in vivo*.

3.8.1 Animal pretreatments.

Male Sprague-Dawley rats 400g were pretreated with either;

i) 1% acetone in drinking water for 1 week, or

ii) disulfiram 1g/kg dispersed in corn oil, intragastrically 24 hours prior to receiving a dose of DMF

Control animals received drinking water *ad libitum* and were given corn oil intragastrically when appropriate.

Animals received DMF i.p. (500mg/kg in saline) either immediately prior to bile collection, or 26 hours before bile collection.

3.8.2 Collection of bile samples

Male Sprague-Dawley rats (400g) were anaesthetised with urethane (500mg/kg ip.). Once fully anaesthetised a midline incision was made in the abdomen and the central bile duct located. The bile duct appeared as an almost translucent vessel running in the mesenteries close to the duodenum. The bile duct was cleared of connective tissue and cannulated via a small incision. The cannula was inserted up the bile duct until the liver was reached. At this point the cannula was slightly pulled back to improve the bile flow. Two sutures were used to hold the cannula in place, and the abdomen was closed with 4 sutures. Bile was collected over ascorbic acid (1ml, 50mM) for either the 4 hour period immediately following an ip. dose of DMF (500mg/kg), or between 24 and 28 hours following the dose. During collection of bile the animals remained anaesthetised.

3.8.3 Preparation of samples

Bile was diluted 1:1 with ascorbic acid (50mM) and the mixture was filtered through a 0.2μm filter. To 20μl of the filtered bile/ascorbic acid, 2μg $[^2H]_3$ SMG was added (200μl of a 10μg/ml solution). The mixture was diluted to a final volume of 1ml with methanol/ 1% aqueous formic acid (1:1). Samples were stored at -70°C until analysis.

3.8.4 Mass spectrometric analysis.

The analysis of samples by MS was performed by M.R. Davis (Department of Medicinal Chemistry, University of Washington, Seattle).

Samples were introduced into the ion spray source of a Sciex API 111 triple quadrupole mass spectrometer by means of flow injection at a rate of 40µl/min (methanol/1% formic acid as carrier). The first mass analyser was set to transmit into the collision cell the MH^+ ions of unlabelled and $[^2H]_3$ SMG (at *m/z* 365 and 368, respectively), while the second mass analyser was adjusted to detect the resulting $[MH-129]^+$ ions (at *m/z* 236 and 239) corresponding to loss of a glutamic acid moiety. The ratio of the latter ion currents was employed to determine the concentration of unlabelled SMG in bile, by reference to a standard curve which was prepared by addition of differing amounts of SMG with a fixed amount of $[^2H]_3$ SMG, to drug free bile. This selected reaction monitoring approach gave a linear standard curve over a range of SMG concentrations from 0 to 1200µg/ml.

3.8.5 Deuterium isotope effects on DMF metabolism to SMG.

It was desirable to study deuterium isotope effects at both the methyl and formyl positions of DMF. To this end two approaches were used, one utilising $[^2H]_6$ DMF and the other $[^2H]_7$ DMF as indicated below.

Results of experiments described in section 3.8.4 indicated that only very small amounts of SMG are excreted in the bile of DMF treated animals. It was decided to perform all experiments with animals induced with 1% acetone in drinking water for 1 week in order to maximise the amounts of SMG formed. For each study equimolar concentrations of the relevant isotopic mixture were injected i.p. at a dose of 500mg DMF / kg, and pooled bile over 24-28 hr post-dose was collected.

The following equimolar isotopic mixtures were administered at a total dose of 500mg/kg;

Experiment 1: $[^2H]_6$ DMF : DMF compared with $[^2H]_6$ DMF : $[^2H]_1$ DMF.

Experiment 2: $[^2H]_7$ DMF : DMF compared with $[^2H]_7$ DMF : $[^2H]_1$ DMF.

Despite using this induction procedure the levels of SMG were too small to measure accurately. Therefore to improve the detection a new analytical protocol was developed by M.R. Davis (Department of Medicinal Chemistry, University of Washington, Seattle) which involved the derivatisation of the conjugate in bile, followed by extraction and on-line LC-MS/MS. A 2ml aliquot of bile was converted to the N-benzyloxycarbonyl methyl ester derivative by the method of Hoffmann and Baillie (1988). The product was extracted on a C18 Sep Pak cartridge (Baillie *et al*, 1989). The resultant extract was analysed by LC-MS/MS on a Sciex API 111 triple quadrupole mass spectrometer. This was achieved by coupling a narrow bore reverse-phase column (15cm x 2mm i.d.) to the API system via a splitting tee (1:3 split ratio, MS:UV detector). The mobile phase was acetonitrile/ water (1:3) containing trifluoroacetic acid (0.6%) and elution was performed isocratically at a flow rate of 200µl/min, of which 50µl/ml entered the ion source. The mass spectrometer was operated in the ion-spray mode, and quantitative analysis was carried out by injecting one half of the bile extract and performing selected reaction monitoring for the transitions *m/z* 527----> 438 (unlabelled SMG) and *m/z* 530----> 441 ($[^2H]_3$ SMG). The ratio of responses in the resulting ion current profiles were then used to calculate the apparent isotope effects in the conversion of DMF to SMG.

Section 4

Results and Discussion

4.1 Metabolism of NMF by rodent liver microsomes *in vitro*.

4.1.1 Introduction.

The hepatotoxicity of NMF in humans has previously been discussed in section 1.4.1. NMF-induced hepatotoxicity can be reproduced in rodents (Whitby *et al*, 1984) implicating them as a suitable model for investigation of the mechanism of NMF toxicity.

It has been described in section 1.4.1 that the mechanism by which N-alkylformamides cause liver damage in mice is intrinsically linked to their metabolism. As indicated earlier the metabolic pathway of NMF which appears to be associated with the generation of hepatotoxicity leads via the oxidation at the formyl moiety to a reactive intermediate, probably methyl isocyanate. This intermediary reacts with GSH to yield the conjugate SMG, which is further metabolised to the mercapturate AMCC, as a urinary metabolite.

Details of the enzymes which catalyse the metabolic oxidation of N-alkylformamides are virtually unknown, except that the hepatic microsomal cytochrome P-450 appears to be involved (Cross *et al*, 1990). It has been demonstrated previously that an *in vitro* system comprising liver microsomes, NADPH as co-factor and GSH, may be used as an experimental model for the study of the metabolic route believed to be responsible for the toxicity of NMF (Cross *et al*, 1990). The requirement of NADPH for the microsomal formation of the N-methylcarbamoylating metabolite of NMF indicates that the reaction is probably catalysed by cytochrome P-450. Pretreatment of mice with phenobarbitone or β-naphthoflavone however, did not have any influence on this microsomal oxidation. These results are consistent with the suggestion that the metabolism of NMF is catalysed by cytochrome P-450, but not by the phenobarbital- or β-naphthoflavone- inducible isozymes.

During a visit to the department Dr. A. Boobis suggested that the isozyme CYP2E1 should be considered as a possible mediator of the reaction. This suggestion is based on the similarity of formamides with certain other substrates of CYP2E1, some of which have been listed previously in table 1.5. To test the hypothesis that the N-formyl oxidation of NMF is catalysed by CYP2E1 the influence of a number of inducers and inhibitors of CYP2E1 were

investigated. Rodents received either 1% acetone or 10% ethanol in their drinking water, or 4-methylpyrazole (200mg/kg for 3 days) via the i.p. route, in order to induce CYP2E1. To determine the extent of CYP2E1 induction the influence of these treatments on PNP hydroxylation was established since this reaction is known to be catalysed specifically by CYP2E1 (Koop, 1986). For comparison aminopyrine N-demethylation was also studied. This reaction is catalysed by a number of isozymes including CYP2B1/2, however CYP2E1 has not been shown to be involved (Reinke and Moyer, 1985). This study was designed to ascertain specificity of induction under the conditions used here.

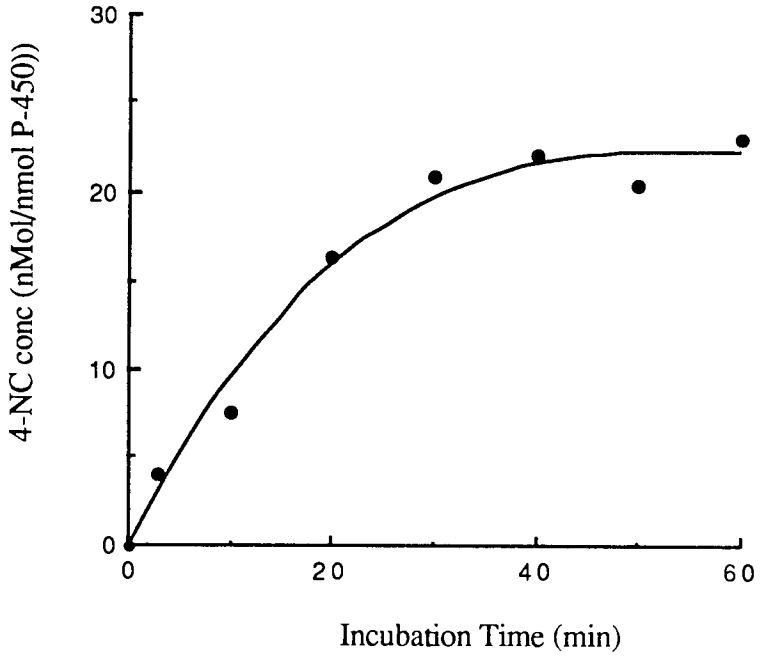
The inhibitors of CYP2E1 which were chosen can be classified into two categories. Those regarded as specific inhibitors of CYP2E1 at the concentrations used e.g. DEDTC, PEITC, and those which were alternative substrates for CYP2E1 e.g. DMSO, and as such would compete for the active site of the enzyme when co-incubated with the substrate of interest. The influence of these compounds on the metabolism of NMF, PNP and aminopyrine was also investigated.

4.1.2 Results

The metabolic conversion of PNP to 4-NC was described by Reinke and Moyer (1985). Under conditions identical to those used in the original work, the 3 min incubation time generated an amount of 4-NC which was too small to be quantified accurately. It was therefore proposed to increase the incubation period. A time course study was performed to investigate the linearity of the reaction (Figure 4.1.1). The formation of 4-NC was linear for up to 30 min under the conditions used, and it was calculated to be 0.72 nmoles 4-NC formed/nmol P-450/min. Therefore, in further experiments a 15 min incubation period was chosen for investigation with enzyme inducers, and 30 min incubation in the case of enzyme inhibition. These incubation times allowed for a measurable amount of 4-NC to be formed, whilst still remaining in the linear region of the plot of metabolite formation against time.

Figure 4.1.1

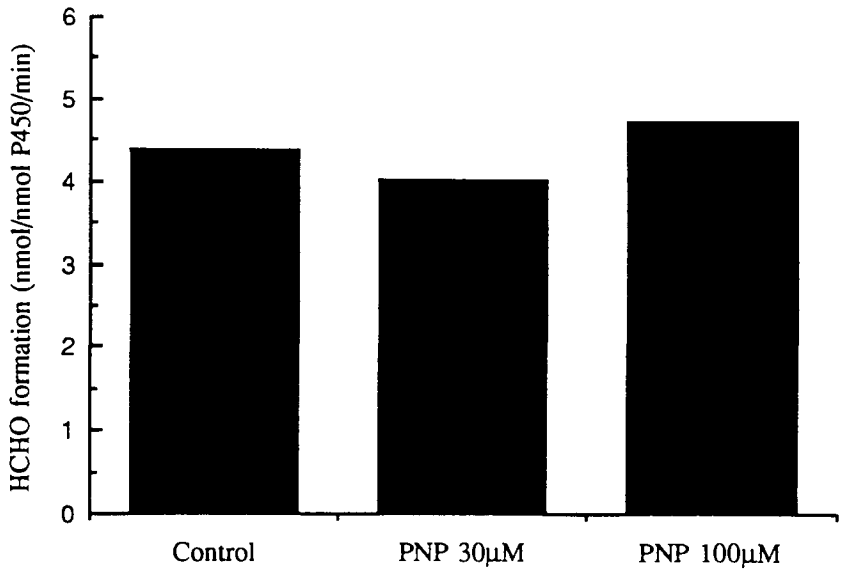
Effect of incubation time on the metabolic generation of 4-NC from PNP (10mM) in suspensions of rat liver microsomes.



Values are the mean of 2 separate experiments.

Figure 4.1.2

Effect of PNP (30μM or 100μM) on the metabolism of aminopyrine (10mM) in suspensions of rat liver microsomes.



Values are the mean of 2 separate experiments.

To confirm that CYP2E1 is not implicated in the N-demethylation of aminopyrine coincubations of aminopyrine and PNP were performed (Figure 4.1.2). If the formation of HCHO from aminopyrine was under the influence of CYP2E1, coincubation with PNP, a second substrate of CYP2E1, would decrease the generation of HCHO from aminopyrine. The rate of formation of HCHO from aminopyrine alone in suspensions of rat liver microsomes was 4.39nmol HCHO/nmol P-450/min. Coincubation with PNP (100μM and 30μM) gave a rate of formation of 4.7nmol HCHO/nmol P-450/min and 4.03nmol HCHO/nmol P-450/min, respectively (mean of 2). These results indicated that CYP2E1 was unlikely to be involved in the N-demethylation of aminopyrine.

Pretreatment of rats or mice with inducers of CYP2E1 did not influence aminopyrine N-demethylation significantly (Figures 4.1.3 & 4.1.4). In control rats (Figure 4.1.3) the extent of aminopyrine metabolism was 4.69 ± 1.59 nmol HCHO/nmol P-450/min (n=6), whilst pretreatment with 4-methylpyrazole (n=3), acetone (n=5) or ethanol (n=3) gave values of 3.63 ± 1.06, 5.92 ± 1.31 or 4.69 ± 0.45 nmol HCHO/nmol P-450/min, respectively. Similar results were seen in incubations with suspensions of mouse liver microsomes (Figure 4.1.4). Here the control value was 4.48 ± 1.53 nmol HCHO/nmol P-450/min (n=9), compared to 4.09 ± 1.31 (4-methylpyrazole, n=4), 5.06 ± 0.82 (acetone, n=8), and 4.47 ± 1.44 (ethanol, n=9) nmol HCHO/nmol P-450/min.

In incubations of rat liver microsomes with PNP (Figure 4.1.5) a turnover of 0.68 ± 0.14 nmol 4-NC/nmolP-450/min was observed in control animals (n=6). This rate increased 2-3 fold in microsomes isolated from animals which had been treated with inducers. The effect was most significant with acetone where the rate of metabolism increased 2.5 fold to 2.00 ± 0.22 nmol 4-NC/nmol P-450/min (n=5), whilst 4-methylpyrazole and ethanol treatments increased metabolism to 1.42 ± 0.10 (n=3) and 1.73 nmol 4-NC/nmol P-450/min (n=2), respectively. In incubations with mouse liver microsomes the rate of metabolism of PNP was increased after pretreatment of animals with acetone and ethanol, whilst 4-methylpyrazole appeared to have no effects (Figure 4.1.6). In control experiments (n=9) PNP was converted to 4-NC at a rate of 0.85 ± 0.33 nmol 4-NC/nmol P-450/min. Ethanol (n=7) and acetone (n=3) increased formation of 4-NC to 1.23 ± 0.6 and 1.79 ± 0.42 nmol

4-NC/nmol P-450/min, respectively. Pretreatment with 4-methylpyrazole (n=3) gave a turnover of 0.97 ± 0.03 essentially the same as control values.

The time course for the metabolic formation of SMG from NMF (10mM) was also measured in these microsomal suspensions (Figures 4.1.7 & 4.1.8). Pretreatment with acetone or ethanol induced the formation of SMG 2 fold in mice and 3-4 fold with rats. Figure 4.1.9 summarises these results. In control rats (n=3) NMF was metabolised to SMG at a rate of 0.31 ± 0.08 nmol SMG/nmol P-450/min, whilst acetone (n=3) increased this biotransformation rate to 1.05 ± 0.36 and ethanol (n=2) to 1.04 nmol SMG/nmol P-450/min. A similar result was observed in mice where the control value of 0.54 ± 0.18 (n=6) was increased to 0.99 ± 0.36 and 1.06 ± 0.61 with acetone (n=6) and ethanol (n=6) respectively. In the case of animals which had received 4-methylpyrazole the rate at which NMF was metabolised was elevated in microsomes isolated from rats, but not in microsomes from mice. The rate of metabolism was increased to 0.86 ± 0.35 (n=3) in rats, representing a 3 fold increase, whilst in mice the rate was 0.47 ± 0.16 (n=4), slightly decreased in comparison to control animals.

Figure 4.1.3

Effect of inducers of CYP2E1 on the rate of metabolism of aminopyrine (10mM) in suspensions of rat liver microsomes.

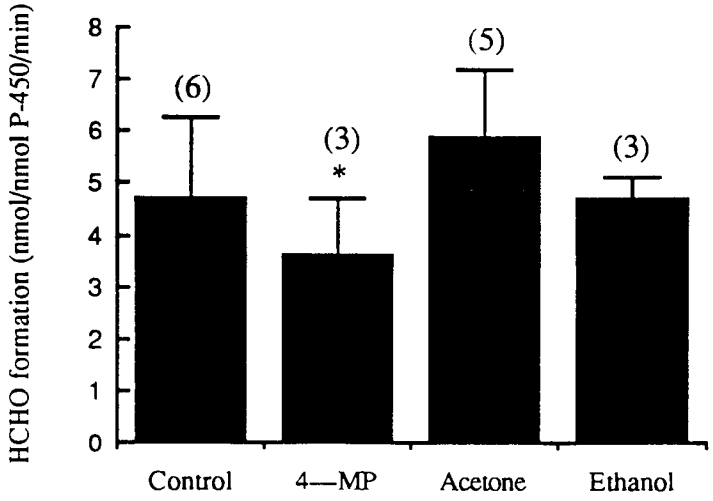
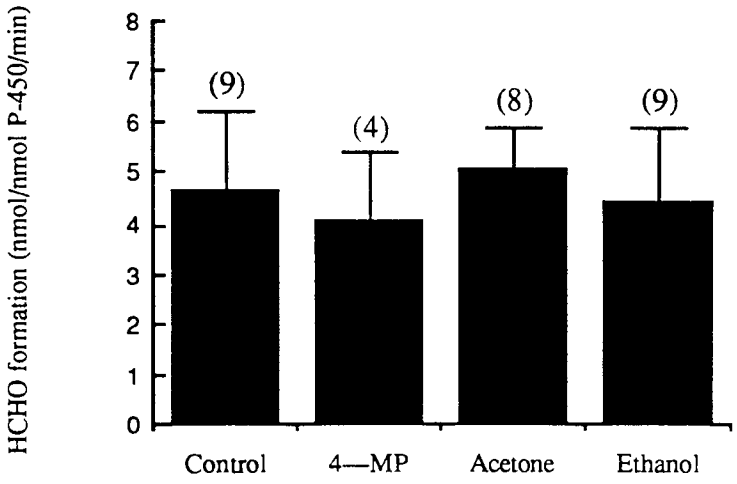


Figure 4.1.4

Effect of inducers of CYP2E1 on the rate of metabolism of aminopyrine (10mM) in suspensions of mouse liver microsomes.



Pretreatments were as described in section 3.2.1. Values are the mean ± SD of 3-9 experiments, number of experiments in brackets.

Stars indicate significant difference from control, * $P < 0.1$.

Figure 4.1.5

Effect of CYP2E1 inducers on the rate of metabolism of PNP (10mM) in suspensions of rat liver microsomes.

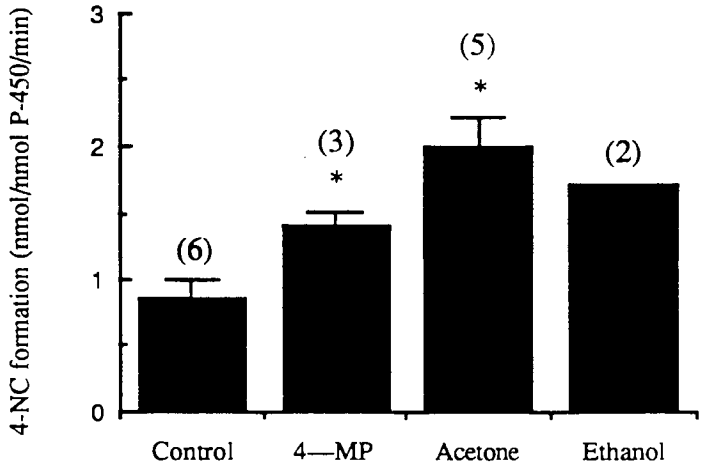
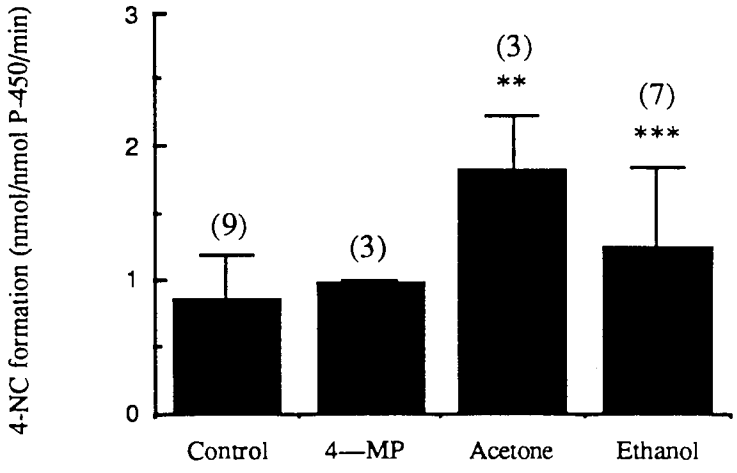


Figure 4.1.6

Effect of CYP2E1 inducers on the rate of metabolism of PNP (10mM) in suspensions of mouse liver microsomes.

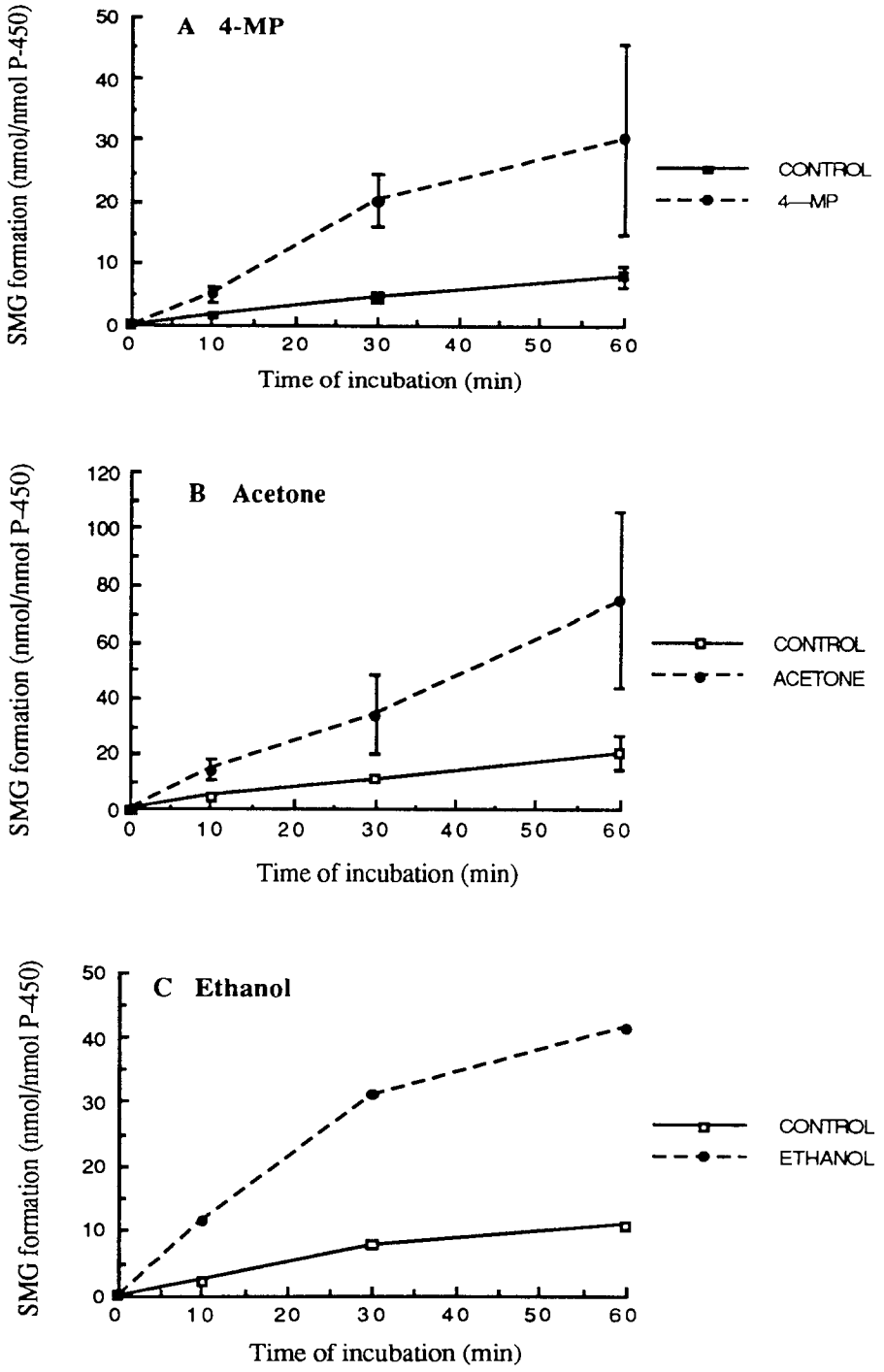


Pretreatments were as described in section 3.2.1. Values are the mean of two or the mean ± SD of 3-9 experiments, number of experiments in brackets.

Stars indicate significant difference from control, *P < 0.001, **P < 0.005, ***P < 0.1.

Figure 4.1.7

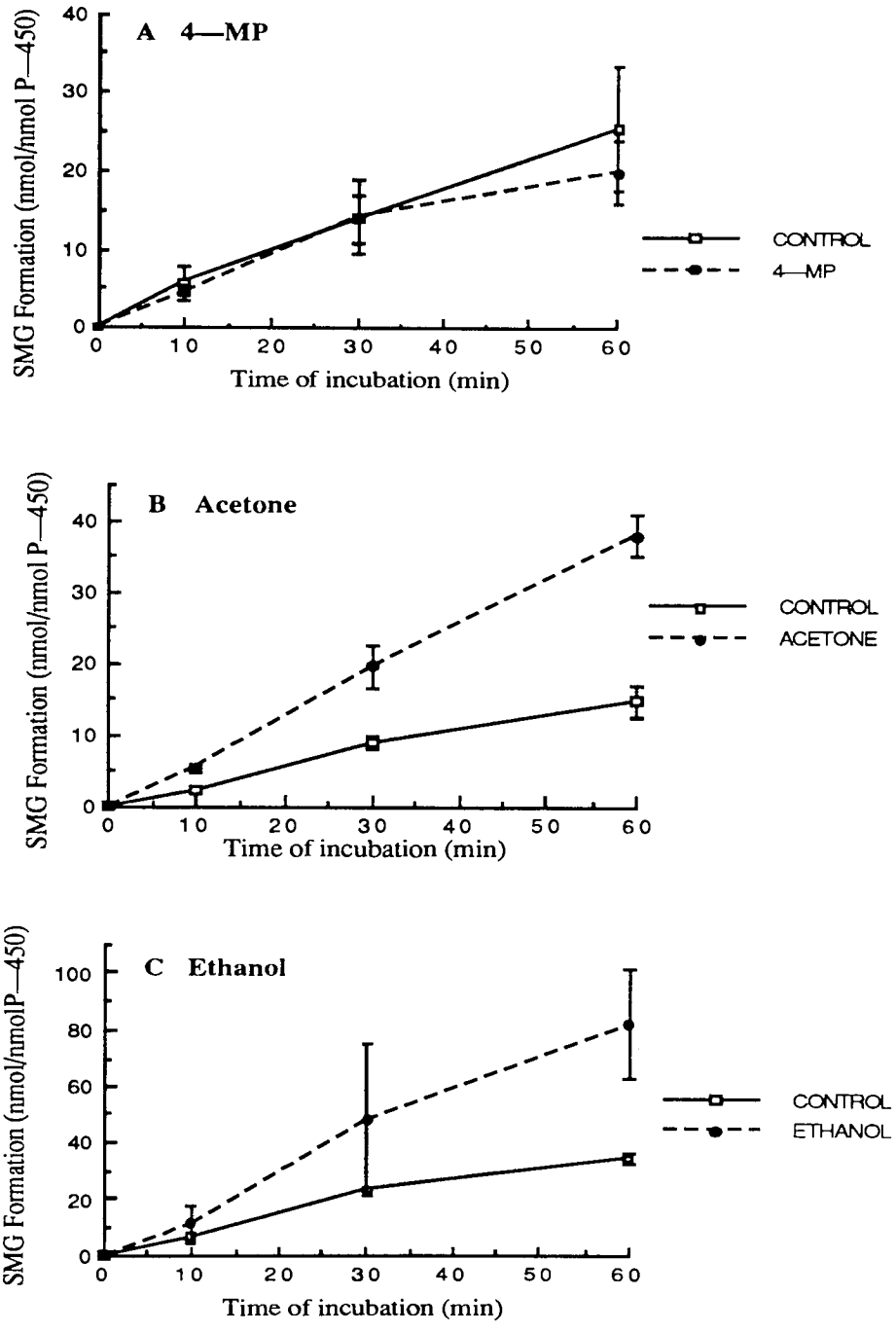
Effect of inducers of CYP2E1 on the metabolism of NMF (10mM) in suspensions of rat liver microsomes.



Pretreatments were as described in section 3.2.1. Values are the mean of two or the mean ± SD of 3 experiments.

Figure 4.1.8

Effect of inducers of CYP2E1 on the metabolism of NMF (10mM) in suspensions of mouse liver microsomes.

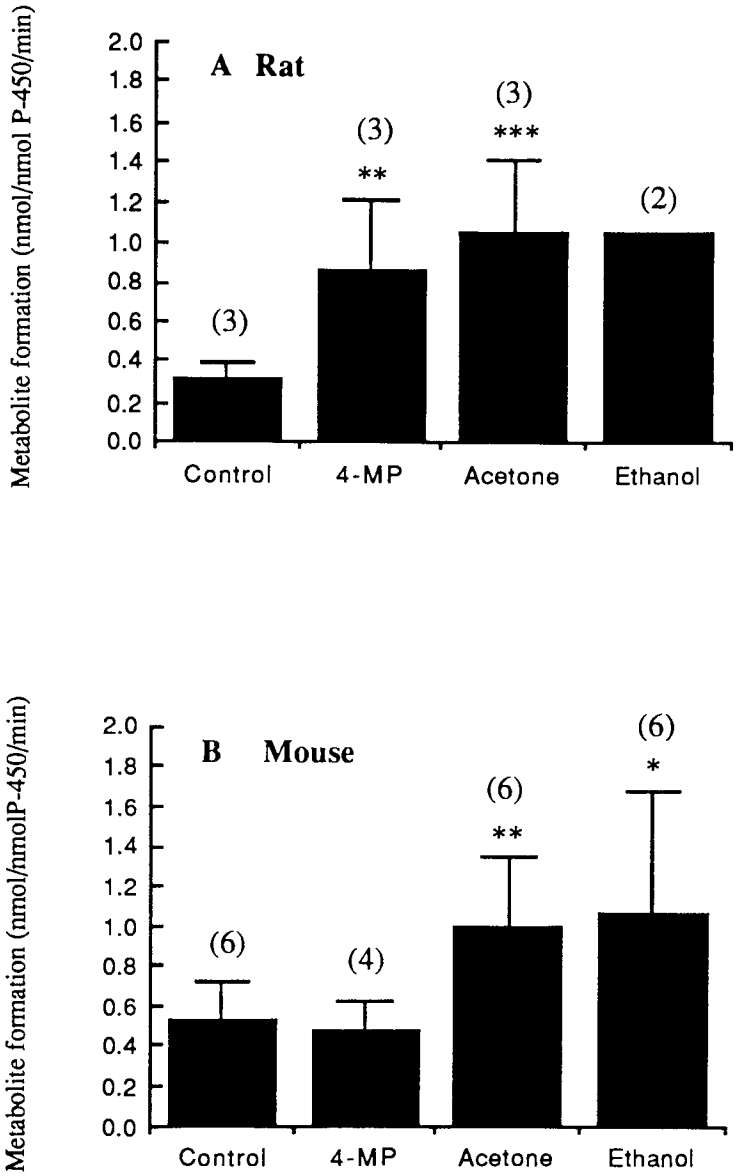


Pretreatments were as described in section 3.2.1.

Values are the mean ± SD of 6 experiments (4-MP values are mean ±SD of 4 experiments.)

Figure 4.1.9

Effect of inducers of CYP2E1 on the rate of NMF (10mM) metabolism in suspensions of rat (A) and mouse (B) liver microsomes.



Pretreatments were as described in section 3.2.1. Values are the mean of two or the mean ± SD of 3-6 experiments, number of experiments in brackets.

Stars indicate significant difference from control, * $P < 0.1$, ** $P < 0.005$, *** $P < 0.001$

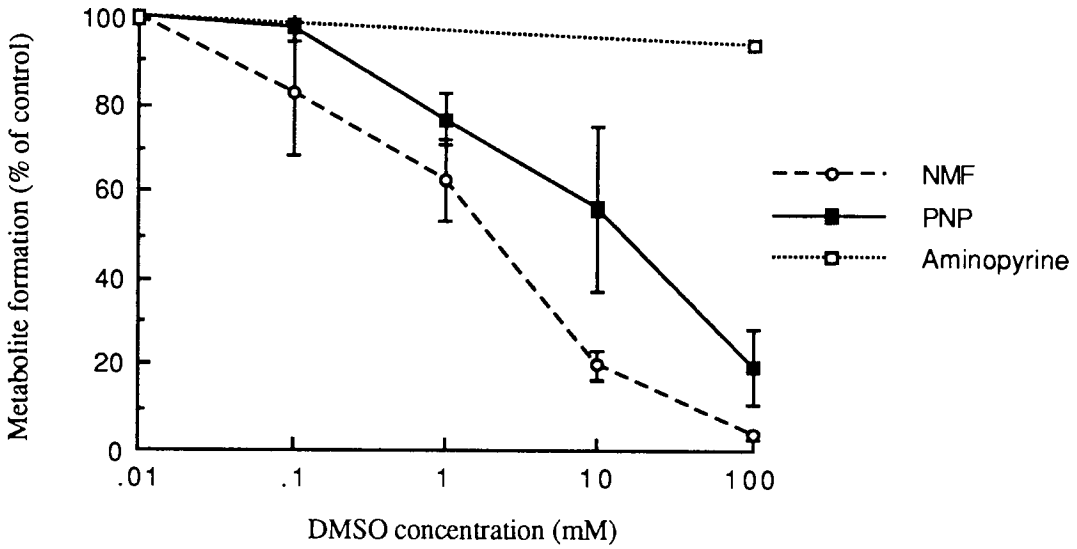
The effect of inhibition of CYP2E1 was also investigated using rat liver microsomes. DMSO and PNP were shown to have little effect on aminopyrine N-demethylation at the concentrations used. Figure 4.1.10 illustrates the effect of DMSO, up to 100mM, on the microsomal metabolism of NMF, PNP and aminopyrine. Over a 30 min incubation period the rates of metabolism of NMF, PNP and aminopyrine in control microsomes were 0.18 ± 0.02 (n=3), 0.59 ± 0.10 (n=3) and 2.9 (n=2) nmol/nmol P-450/min, respectively. At the maximum DMSO concentration used (100mM), the metabolism of NMF and PNP was impeded to the following percentages of control values 3.8 ± 0.9 (n=3) and 19.4 ± 8.6 (n=3) %, whilst aminopyrine N-demethylation was unaffected. PNP similarly inhibited NMF metabolism (Figure 4.1.11). At 100μM PNP, SMG formation was reduced by 60%, whilst aminopyrine metabolism was unaltered.

DEDTC, a relatively specific inhibitor of CYP2E1 (Guengerich *et al*, 1991), was co-incubated with NMF in incubations with suspensions of rat liver microsomes. Figure 4.1.12 summarises these results. At the concentration of DEDTC investigated (100μM) there was no effect on the N-demethylation of aminopyrine. Formation of HCHO was calculated to be 94.92 ± 2.3% of control values (n=5). However, the oxidation of PNP to 4-NC was inhibited to 29.22 ± 4.23 % (n=5) of control values. The most dramatic inhibitory effect of DEDTC was on the metabolism of NMF. The rate of formation of SMG was inhibited by 95.0 ± 1.0% (n=4) compared to controls.

Results from incubations of suspensions of liver microsomes which had been pretreated with another inhibitor, PEITC, were less clear (Figure 4.1.13). Aminopyrine N-demethylation appeared to be inhibited from 6.29 ± 0.34 nmol HCHO/nmol P-450/min in control incubations to 5.31 ± 0.86 nmol HCHO/nmol P-450/min (n=3). However, there was little effect on PNP hydroxylation. Here, with microsomes from controls the rate of 4-NC formation was 0.3 ± 0.04 nmol/nmol P-450/min, compared to 0.31 ± 0.1 (n=3) in PEITC treated microsomes. The metabolic generation of SMG from NMF was inhibited to approximately 50% by PEITC. The rate of formation of SMG was 0.31 ± 0.1 nmol/nmol P-450/min in controls and this was impeded to 0.18 ± 0.12 in microsomes preincubated with PEITC.

Figure 4.1.10

Effect of DMSO on the metabolism of aminopyrine (10mM), PNP (10mM) and NMF (10mM) in suspensions of rat liver microsomes.



Values are the mean of two or the mean ± SD of 3 experiments.

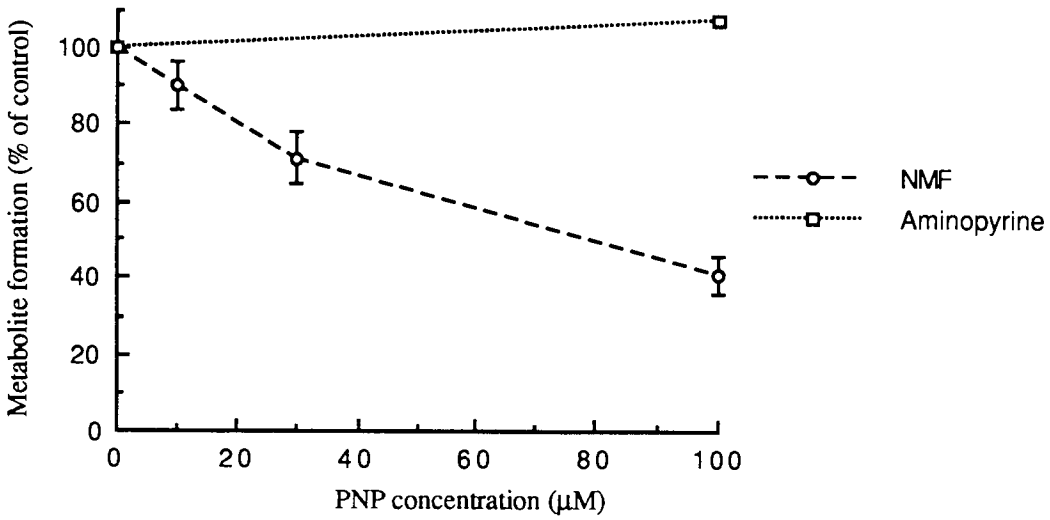
100% values represent : NMF, 0.23 ± 0.08 nmol SMG/nmol P-450/min,

PNP, 0.53 ± 0.1 nmol 4-NC/nmol P-450/min,

Aminopyrine, 2.9 nmol HCHO/nmol P-450/min.

Figure 4.1.11

Effect of PNP on the metabolism of aminopyrine (10mM) and NMF (10mM) in suspensions of rat liver microsomes.



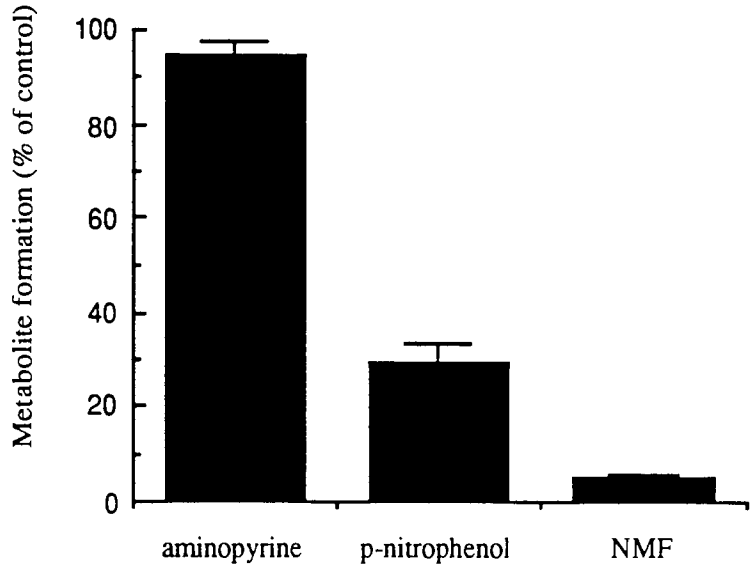
Values are the mean of two or the mean ± SD of 3 experiments.

100% values represent : NMF, 0.23 ± 0.08 nmol SMG/nmol P-450/min,

Aminopyrine, 4.39 nmol HCHO/nmol P-450/min.

Figure 4.1.12

Effect of DEDTC (100μM) on the metabolism of aminopyrine (10mM), PNP (10mM) and NMF (10mM) in suspensions of rat liver microsomes.



Values are the mean ± SD of 4 experiments.

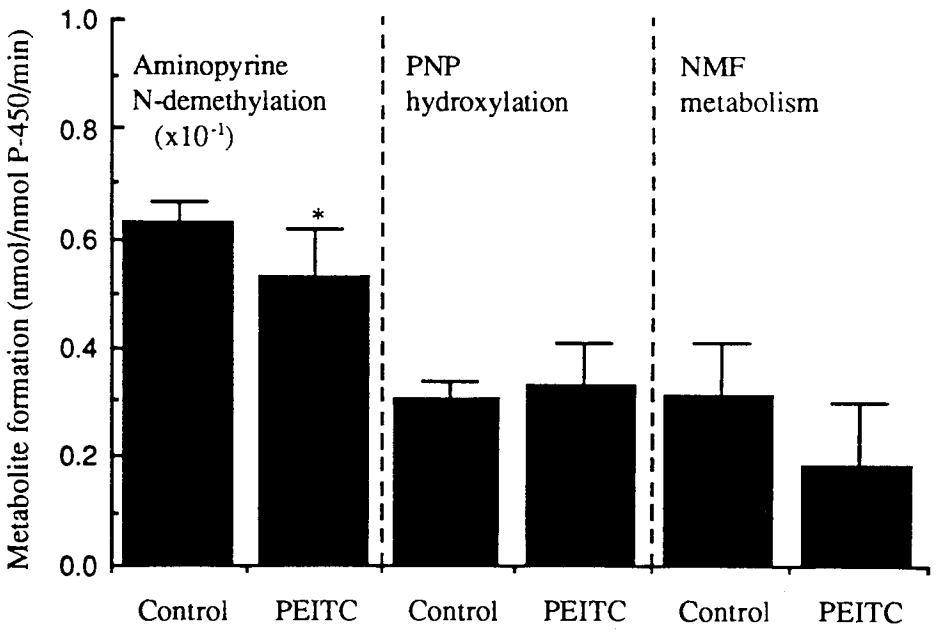
100% values represent : NMF, 0.34 ± 0.12 nmol SMG/nmol P-450/min,

PNP, 1.10 ± 0.28 nmol 4-NC/nmol P-450/min,

Aminopyrine, 5.46 ± 1.16 nmol HCHO/nmol P-450/min.

Figure 4.1.13

Effect of PEITC (5μM) on the rate of metabolism of aminopyrine (10mM), PNP (10mM) and NMF (10mM) in suspensions of rat liver microsomes.



Values are the mean ± SD of 3 experiments.

Star indicates significant difference from control, * $P < 0.1$.

4.1.3 Discussion

The enzymatic details of the metabolic conversion of NMF to SMG in suspensions of rodent liver microsomes have been investigated, with specific reference to the cytochrome P-450 isozyme CYP2E1.

Induction of CYP2E1, as indicated by an increase in the hydroxylation of PNP, was accompanied by an induction in the rate of NMF metabolism. Formation of the SMG metabolite in suspensions of liver microsomes isolated from induced animals was raised 2-4 fold over control values.

Perhaps the most interesting of the inducers chosen was 4-methylpyrazole. Pretreatment with this compound, a known inducer of CYP2E1 in the rat, did not induce PNP metabolism in the mouse. This is consistent with the findings of Honkakoski *et al* (1988) who investigated the difference between pyrazole, acetone and ethanol as inducers of the monooxygenase system in the mouse. They concluded that whilst pyrazole, acetone and ethanol belong to the same class of inducers in the rat and rabbit, this is not the case in the mouse. In the mouse strains studied, including BALB/c which was used in the work described in this thesis, acetone and ethanol induced CYP2E1 whilst pyrazole strongly induced a different P-450 isozyme, known as $P\text{-}450_{coh}$. These observations compare favourably with the induction experiments performed here. NMF metabolism was inducible by acetone and ethanol pretreatment in the BALB/c mouse, but was unaffected by 4-methylpyrazole.

It is also interesting to note that whilst 4-methylpyrazole treatment in mice was not accompanied by an increase in PNP or NMF metabolism, there was a reduction in the extent of aminopyrine N-demethylation in both rats and mice. This suggests that 4-methylpyrazole, in addition to being an inducer of CYP2E1 in rats, may also be detrimental in that it might destroy the monooxygenase system. Craft (1985) demonstrated that in microsomes obtained from rats receiving pyrazole (200mg i.p.) daily for 4 days, aniline hydroxylase activity (CYP2E1 catalysed) was induced, but ethoxycoumarin O-deethylase and aminopyrine

demethylase activities were significantly reduced. Benzphetamine demethylase (Tu *et al*, 1981) and aryl hydrocarbon hydroxylase (Evarts *et al*, 1983) activity also decline on treatment with pyrazole. Craft (1985) provided evidence that pyrazole is a suicide inactivator of cytochrome P-450 by demonstrating that incubations of microsomes with pyrazole resulted in a reduction in cytochrome P-450 by a mechanism dependent on the presence of NADPH.

Inconsistent with a destructive effect of 4-methylpyrazole on cytochrome P-450 is the observation that total P-450 was not decreased, but raised 1.5 to 2 fold (results not shown) in microsomes isolated from 4-methylpyrazole treated rats in this study. Craft (1985) also demonstrated that successive pyrazole treatment increased total P-450 content to the same extent as was recorded in the experiments performed here. However, when animals received pyrazole as a single dose 4 days prior to sacrifice in that study the microsomal content was reduced to approximately 50%. This provided further evidence for the toxic effects of pyrazole.

Of the remaining two inducers investigated both have been demonstrated to induce CYP2B1 in addition to CYP2E1 (Johansson *et al*, 1988, Ronis and Ingelman-Sundberg, 1989). The induction of NMF metabolism by these compounds cannot therefore be assumed to be solely as a result of CYP2E1 without further evidence. Earlier work performed in our laboratory has demonstrated that phenobarbitone, a strong inducer of CYP2B1/2 (Gonzalez, 1989), does not induce NMF metabolism in the mouse (Cross *et al*, 1990). This result implies that the induction observed with the inducers in this study is indeed the result of the activity of CYP2E1 and not of CYP2B1.

In contrast, pyrazole has not been reported to induce more than one cytochrome P-450 isoenzyme (Honkakoski *et al*, 1988) and may therefore tentatively be described as a specific inducer of CYP2E1 in the rat. Assuming that this specificity is the same for the structural analogue 4-methylpyrazole our result would then provide strong evidence for the involvement of CYP2E1 in NMF metabolism.

Inhibition studies have also been used to demonstrate the role of CYP2E1 in the metabolism of NMF. The mechanism-based inhibitor DEDTC was used to show a concentration dependent inhibition in the formation of SMG. At 100μM DEDTC there was a 95% inhibition of the metabolism of NMF, and a 70% inhibition in PNP hydroxylation, but no effect on aminopyrine N-demethylation. This finding compares favourably with the results by Guengerich *et al* (1991). These workers found that at high concentrations of DEDTC (1mM) inhibition of a variety of enzymatic reactions were observed. However, in the range 100-300μM inhibition with DEDTC was much more specific. The metabolism of chlorzoxazone and NDMA, known to be relatively specific markers of CYP2E1 activity, were inhibited considerably and of the remaining enzyme reactions studied only mephenytoin 4-hydroxylation was inhibited, but this was not extensive.

PEITC has been reported to be a potent and selective inhibitor of CYP2E1 at 5μM (Ishizaki *et al*, 1990). Interestingly, in experiments performed here there was some loss of aminopyrine N-demethylase activity (approximately 15%), suggesting a lack of specificity of the inhibitor using this protocol. NMF metabolism itself was inhibited by 42%. This additional inhibition over that observed with aminopyrine can tentatively be attributed to CYP2E1 inhibition. Surprisingly however, on incubation with PNP these microsomes were no less efficient at converting PNP to 4-NC than control microsomes. This discrepancy between PNP and NMF metabolism is difficult to rationalise if both are substrates for CYP2E1. Ishizaki *et al* (1990) demonstrated CYP2E1 inhibition as measured by a 70% reduction in NDMA demethylation. PNP metabolism would have been expected to show a similar result.

DMSO and PNP are competitive inhibitors of CYP2E1 by virtue of being alternative substrates for the enzyme and as such competing for the active site of the enzyme. Both DMSO and PNP in coincubation with NMF reduced the generation of SMG. DMSO is an hydroxyl radical scavenger (Cederbaum *et al*, 1977). It could therefore be speculated that DMSO inhibits cytochrome P-450 by scavenging hydroxyl radicals generated by the microsomes during the oxidation of NADPH. However this mechanism is not believed to be applicable to the inhibition of CYP2E1 by DMSO. This activity is attributed to competition

at the active site of the enzyme. Morgan *et al* (1982) have demonstrated competitive inhibition by DMSO of the oxidative metabolism of ethanol (k_i = 17mM). It would therefore appear that the inhibitory action of DMSO on CYP2E1-catalysed oxidations is that of a classical enzyme inhibitor and not that of a scavenger of cytochrome P-450-generated hydroxyl radicals in solution. In the light of this observation Morgan *et al* (1982) went on to look at the spectral binding characteristics which were indicative of a high to low spin transition. Such a shift is typically manifested by N- or O- containing compounds which bind to heme ligands. This result provided further evidence for involvement of the enzyme active site.

Overall, the effects observed in microsomal incubations with either induced or inhibited CYP2E1 activity show that the metabolic generation of SMG from NMF is probably calalysed either exclusively or in part by CYP2E1.

4.2 Toxicity of NMF *in vitro*.

4.2.1 Introduction

Chemicals may exert their toxic effects either directly, or through formation of one or more active metabolites. *In vitro* studies of the mechanisms by which NMF causes hepatoxicity suggest that it is metabolised to a chemically reactive, potentially toxic species, which is bound covalently to hepatic macromolecules (Pearson *et al*,1987b) A major obstacle to a detailed elucidation of this link between metabolism and toxicity is that until relatively recently it has not been possible to detect metabolites of formamides in incubations *in vitro* (Brindly *et al*, 1982). Shaw *et al* (1988) described the use of suspensions of isolated mouse hepatocytes as a suitable model system for the study of formamide cytotoxicity and metabolism. They concluded that there was excellent agreement between observations recorded *in vivo* (Kestell *et al*, 1987, Threadgill *et al*, 1987), and those recorded in hepatocyte suspensions, concerning the structure toxicity relationship, GSH depletion and metabolism of formamides.

Kestell and co-workers have found NMF and NEF to be hepatotoxic *in vivo*, whilst F, DMF and N-methylacetamide were not (Kestell *et al*, 1987). *In vitro*, NMF and NEF were cytotoxic towards hepatocytes and were capable of depleting GSH and generating N-alkylcarbamoylating species (Shaw *et al*, 1988). The NMF analogs F, DMF and N-methylacetamide, on the other hand, were devoid of cytotoxic potential in hepatocytes and did not deplete GSH or generate N-alkylcarbamoylating species. From these observations the authors concluded that suspensions of isolated mouse hepatocytes are a suitable model system for further investigations of the metabolism and biochemical changes associated with the hepatotoxicity of these compounds.

Isolated and cultured hepatocytes have been used increasingly over the past 20 years for metabolic and toxicological studies. Isolated hepatocytes have an advantage over microsomal preparations in that the metabolising enzymes are maintained in their physiological concentrations and spatial relationships, and remain viable for longer than subcellular fractions. They express most of the functional activities of the intact liver and are

therefore a representative model for the situation *in vivo*. An added advantage is that unlike microsomal preparations, studies with hepatocytes give additional information about the effect these compounds have on cell viability, as well as metabolic data. Cultured hepatocytes allow longer term studies, but the rapid loss of cytochrome P-450 and other drug metabolising enzymes present severe short-comings. Conditions of culture allowing long term maintenance of all cytochrome P-450 isozymes have not as yet been achieved. It is however possible to induce the synthesis of isozymes in hepatocytes in culture by coincubation with substrates for particular isozymes (Schuetz *et al*, 1988). For example, coincubation with ethanol in cultured hepatocytes results in return of CYP2E1 levels to those measured in freshly isolated hepatocytes. However this phenomenon is accompanied by the expression of other isozymes (eg CYP3A) which are not normally influenced by ethanol treatment *in vivo* (Sinclair *et al*, 1991).

The role for CYP2E1 in the metabolism of NMF has been described in section 4.1.3. Since the toxicity of NMF is linked to metabolism it would seem probable that CYP2E1 catalyses the metabolic generation of the toxic species. In order to test this hypothesis the cytotoxicity of NMF was investigated in suspensions of mouse hepatocyte prepared by the method of Seglen (1973) with modifications by Shaw *et al* (1988).

Cytotoxicity is usually evaluated by the measurement of trypan blue exclusion, morphological changes and/or cytosolic leakage of LDH. Shaw (1988) compared the measurement of both release of LDH and uptake of trypan blue by BALB/c hepatocytes. Almost identical cytotoxicity values were recorded with each technique, therefore only LDH release was measured in experiments performed in this study. The cytotoxicity of NMF was measured in hepatocytes in which the CYP2E1 activity had been modulated by a suitable inducer or inhibitor.

4.2.2 Results

The role CYP2E1 plays in the metabolic toxification of NMF was investigated. The cytotoxic potential of NMF was assessed by measurement of the release of LDH from hepatocytes into the medium. Mice had received either 1% acetone in their drinking water

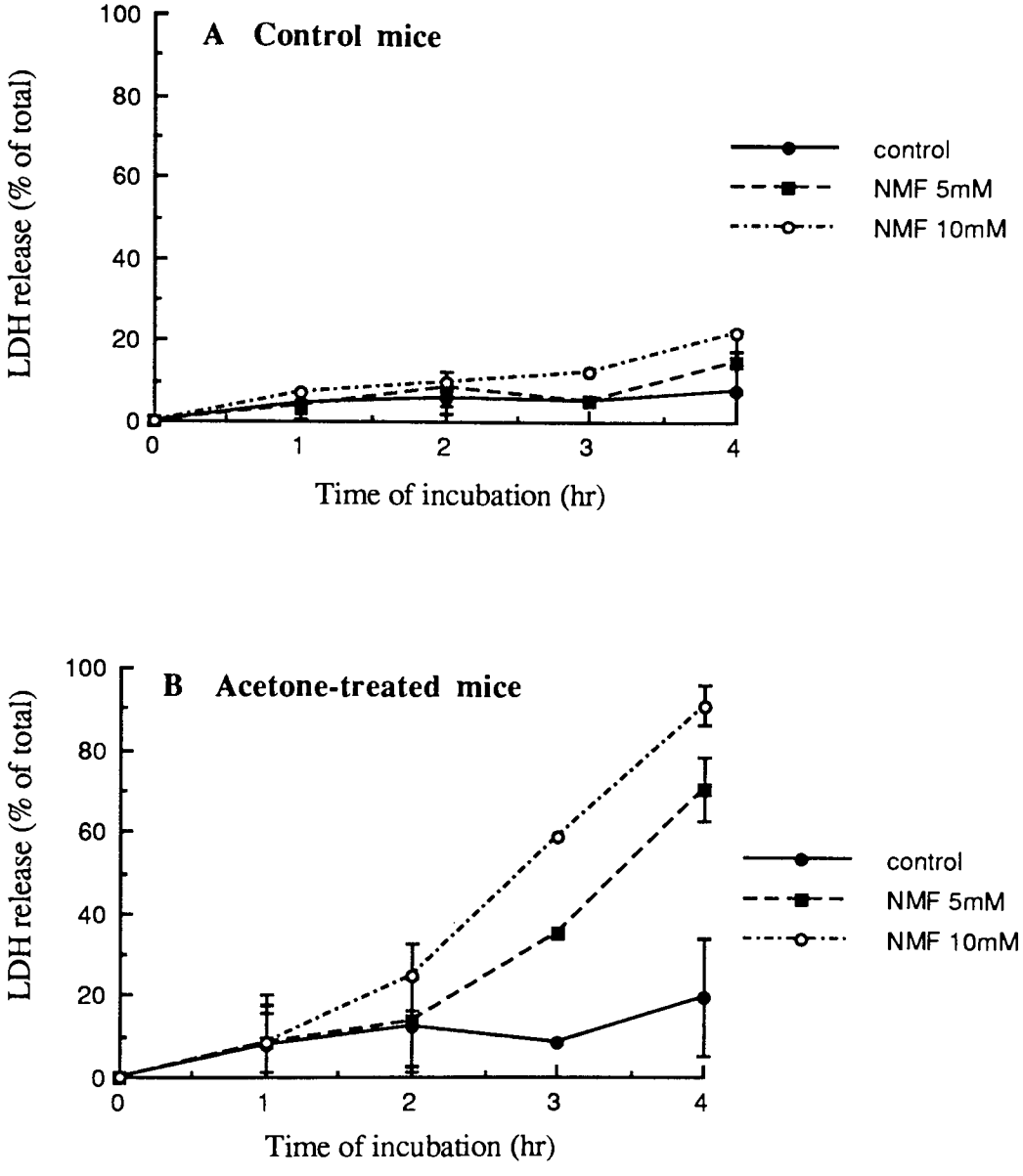
for 1 week whilst control animals were allowed drinking water *ad libitum*. This pretreatment schedule had previously been shown to be effective in inducing CYP2E1 as indicated by the microsomal metabolism of PNP (section 4.1). The metabolic conversion of PNP to 4-NC in suspensions of hepatocytes was also increased 2.8 fold (mean of 2), indicating that the treatment schedule was effective at inducing CYP2E1 activity in hepatocyte suspensions.

Figure 4.2.1 shows that hepatocytes isolated from acetone-pretreated animals were dramatically more susceptible to NMF-induced cytotoxicity, as measured by LDH release. Isolated hepatocytes incubated for 4 hr in suspensions from which NMF was omitted released <10% LDH (n=3). Introduction of NMF, 5 or 10mM, to incubations increased LDH release only slightly, to 15.0 ± 7.6% and 22.0 ± 8.7% (n=3), respectively (Figure 4.2.1 A). However pretreatment of animals with acetone increased the cytotoxicity of NMF. In control incubations, with hepatocytes isolated from acetone pretreated animals incubated in the absence of NMF, LDH release was <20% (n=3), whilst there was a 4-5 fold increase in toxicity with NMF 5 and 10mM. At 4hr LDH release was 70.4 ± 7.9% and 91.1 ± 5.0% (n=3), respectively (Figure 4.2.1 B).

The role of DMSO as a substrate-inhibitor of CYP2E1 had been outlined in section 4.1.3. DMSO, at 10mM was incubated with NMF and hepatocytes isolated from untreated animals (Figure 4.2.2). DMSO alone did not affect cell survival over a 6 hr incubation period, as compared to control incubations. Incubation with NMF (10mM) alone lead to a LDH release of 58.8 ± 19.0% in 6 hr. However, on coincubation of isolated hepatocytes with NMF and DMSO, the NMF-induced cytotoxicity was significantly diminished, to 19.7 ± 11.8%. This value for LDH release was similar to that observed in incubations omitting NMF.

Figure 4.2.1

Cytotoxicity of NMF (5mM or 10mM) on suspensions of hepatocytes isolated from control and acetone-treated mice.

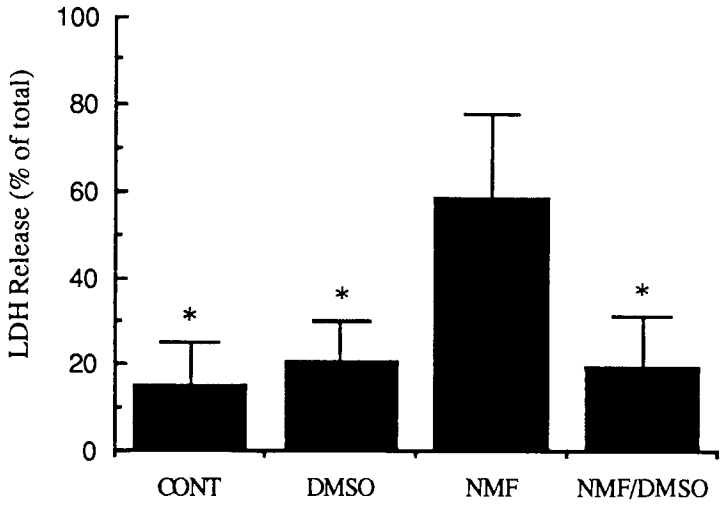


Pretreatments were as described in section 3.3.1.

Values are the mean of two or mean ± SD of 3 experiments.

Figure 4.2.2

Effect of DMSO (10mM) on NMF (10mM) induced cytotoxicity in suspensions of uninduced mouse hepatocytes.



Values are the mean ± SD of 3 experiments.

Star indicates significant difference from NMF toxicity value, * $P < 0.05$.

4.2.3 Discussion

Suspensions of mouse hepatocytes were used to investigate the role of CYP2E1 in the toxicity of NMF, measured by LDH release from cells. Acetone (1%) in the drinking water induced the metabolism of PNP by hepatocytes to a similar extent to that observed in microsomal incubations. As previously discussed acetone also induces CYP2B1. Since it is believed that the toxicity of NMF requires metabolic activation (Threadgill *et al*, 1987) and it has been shown in microsomal suspensions that CYP2B1 activity does not play a role in NMF metabolism (Cross *et al*, 1990) it may be assumed that any toxic effects mediated via acetone treatment are indeed as a result of CYP2E1 induction and not CYP2B1.

Over a 4 hr incubation period the mean viability of hepatocytes isolated from acetone-induced animals was lower than in those isolated from control animals. This was also accompanied by a greater variation in viability between isolations as indicated by wider standard errors. It would therefore appear that acetone pretreatment resulted in less stable hepatocytes when maintained in suspension. This could be attributed to a toxic effect of acetone on the hepatocytes following exposure to this solvent.

The toxic effects of NMF were increased dramatically in hepatocytes in which CYP2E1 activity had been induced. In comparison, within a 4 hr time period, very little toxicity with 5 or 10mM NMF was seen. In order to determine effects of inhibitors of CYP2E1 on NMF toxicity it was necessary to increase the incubation period to 6 hr to achieve some demonstrable toxicity with 10mM NMF. A 6 hr incubation period is probably the maximum time over which studies with isolated hepatocyte suspensions can be performed. Isolated hepatocytes show degenerative changes within a few hours and for long term *in vitro* maintenance hepatocytes must be attached to a sub-stratum support (Guillouzo, 1986). After 6 hr incubation toxicity to NMF had increased sufficiently to allow determination of inhibitory effects with DMSO.

DMSO was observed to have a profound effect on the protection of hepatocytes to NMF induced toxicity. At 10mM DMSO it appeared that toxicity of NMF was completely inhibited. At this concentration in microsomal studies there was an 80% inhibition in NMF

metabolism (section 4.1.2). If a similar inhibition in metabolic activity is achieved in the isolated hepatocytes then it would be reasonable to expect that only low levels of the toxic species were generated. Hence within the 6 hr incubation period no toxicity was seen.

The limitations of long term hepatocyte studies have been discussed briefly in section 4.2.1. Freshly isolated or short term cultured hepatocytes appear to be suitable for studying acute toxicity of chemicals, whilst long term studies are not possible due to rapid degeneration in P-450 levels. *In vitro* studies on chronic exposure of NMF are therefore, at present, not possible. However it has been demonstrated that the cytochrome P-450 content in human hepatocytes is much more stable than rodent hepatocytes in culture (Guillouzo, 1986). Initial results in this area suggest that the system will become a suitable model for predicting hepatic metabolic pathways and hepatotoxicity of new drugs in man. It could partly replace and reduce the use of animals for pharmacological and toxicological studies.

The results from these experiments further confirmed a role for metabolic activation of NMF to a toxic intermediate. Induction of the metabolising enzyme CYP2E1 resulted in an increase in toxicity, whilst inhibition of this enzyme lead to decreased toxicity.

4.3 Metabolism of NMF by purified rat-CYP2E1 and the effect of anti-rat CYP2E1 IgG.

4.3.1 Introduction

A role for CYP2E1 in the metabolic toxification of NMF has already been implicated from results of studies in microsomes and hepatocytes (sections 4.1 and 4.2). Inducers of CYP2E1 were shown to increase the metabolic formation of SMG from NMF in incubations with rat and liver microsomes, and coincubation with CYP2E1 inhibitors resulted in a reduction in metabolite formation in incubations with rat microsomes. In studies using isolated hepatocytes CYP2E1 induction increased the cytotoxicity of NMF, whilst the CYP2E1 substrate DMSO reduced NMF-induced cytotoxicity to control values.

The limitation of using enzyme inducers and inhibitors has previously been discussed (sections 1.3.3 and 1.3.4). The degree of specificity that can be achieved is never absolute and therefore unequivocal interpretation of this type of data cannot be obtained. A role for CYP2E1 in the metabolism of NMF can be clearly inferred from the induction and inhibition experiments described in section 4.1. To further confirm the involvement of this isozyme studies using purified CYP2E1 and antibody raised against CYP2E1 were considered.

The use of specific inhibitory antibodies against P-450 isozymes is an effective and direct approach to identifying a role of a specific enzyme in catalysis. This strategy has been used with both polyclonal and monoclonal antibody. However, the most direct experimental approach to identifying CYP2E1 as a metabolising enzyme of a specific substrate involves measuring activity with the purified enzyme in a reconstituted system containing NADPH, cytochrome P-450 reductase and cytochrome b_5. Nevertheless, in some instances results from such experiments can be misleading since with some isoforms the purified enzyme is not always catalytically competent. However, there are no reports of the purified CYP2E1 from rats, rabbits, hamsters or humans being inactive.

The conditions for reconstitution of the enzyme are very important. It has been shown that the inclusion of cytochrome b_5 results in k_m values similar to those of microsomal

incubations. Cytochrome b_5 has been reported to affect a variety of cytochrome P-450 catalysed monooxygenase reactions. Depending on the isozyme and substrates, cytochrome b_5 may be stimulatory, inhibitory or have no effect. In the CYP2E1-catalysed oxidation of NDMA it has been speculated that interactions between cytochromes b_5 and P-450 may cause conformational changes which facilitate the binding of NDMA to the active enzyme complex (Patten *et al*, 1986). However the mechanism of action of either cytochrome b_5 or P-450 in such interactions is not clearly understood .

In experiments performed in this study purified rat CYP2E1, which had been isolated from livers of streptozotocin-treated rats was used for metabolism studies, and a sample was also used for immunisation of a rabbit in order to raise polyclonal antibody. In order to obtain an optimal immune response it is generally advisable to use an animal that is phytogenetically removed from the source of the immunogen. In this case the antigen used was CYP2E1 isolated from rats, and hence a rabbit was chosen for immunisation. The antibody thus obtained was then used in microsomal incubations with NMF.

4.3.2 Results

IgG was isolated from the serum collected from a rabbit which had been immunised with purified CYP2E1. In dot blots to determine that antibody had indeed been raised, serum samples were tested against the following antigens, purified rat CYP2E1, human liver microsomes, purified human CYP2E1 and BSA. The serum samples tested were pre-boost serum, a sample known to contain anti-CYP2E1 IgG, and the samples collected 2 and 4 weeks following the boost injection of purified CYP2E1.

Figure 4.3.1 shows the experimental design of these blots, and a photograph of the blots obtained. Each blot contained almost identical information. BSA gave negative results in all blots, whilst all other antigens were positive with each serum sample tested. This clearly indicates that even in the pre-boost sample anti-CYP2E1 IgG was present, and thus the rabbit must still have had an antibody titre from the previous time it had been treated. Therefore all the sera samples collected could be used for the isolation of IgG. The IgG

isolated from the pooled serum samples was estimated to be 250mg, as calculated from the absorbance at 280nm.

The specificity of the isolated IgG was determined by immunoblotting with proteins from human liver microsomes. Although results indicated a band corresponding to the same molecular weight as the band observed with purified CYP2E1 (~53KDa), a number of other bands could be observed. This indicated that there was cross reaction of the antibody with other proteins in the microsomal sample. Further purification of the antibody was therefore necessary.

In order to purify the anti-CYP2E1 IgG it was necessary to prepare a CNBr-activated sepharose column to which cytochrome P-450 isozymes other than CYP2E1 were bound. To separate CYP2E1 from a microsomal preparation the human liver microsomes were passed down a CM-sepharose column. This cation exchange column contains negatively charged groups at a neutral pH and hence by controlling the salt concentration it is possible to selectively elute proteins with a different isoelectric point. CYP2E1 has a high isoelectric point and at low salt concentrations, where proteins with a lower isoelectric point are eluted, it remains strongly bound to the cation exchange column. It was therefore possible to separate CYP2E1 from other isoforms using this ion exchange method. To confirm that CYP2E1 was not present in the eluent dot blots were performed and samples were incubated with anti-CYP3A4 (positive control) or anti-CYP2E1. Once it had been confirmed that the eluent did not contain CYP2E1 the proteins were bound to the CNBr-activated sepharose.

The isolated IgG for purification was then passed down this column. IgG against isozymes other than CYP2E1 were bound to the proteins on the column whilst any anti-CYP2E1 flowed through in the eluent. The isolated IgG was calculated to be 192mg after purification, compared to the original 250mg.

The specificity of the purified IgG was again tested using human liver microsomes. Figure 4.3.2 shows the Western blot obtained. In the lanes corresponding to liver microsomes there was a band at the same molecular weight as that for purified CYP2E1. Since no other band

could be seen this indicated that the antibody was no longer cross reactive and recognised CYP2E1 alone.

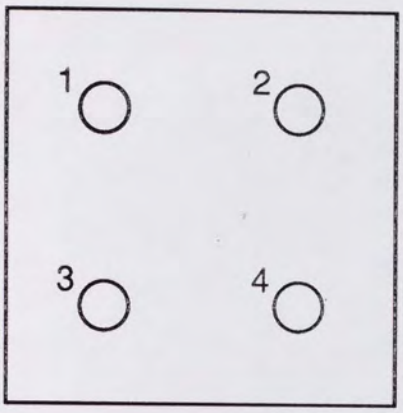
This monospecific antibody against CYP2E1 was added to incubations of NMF (10mM) in suspensions of rat liver microsomes. Figure 4.3.3 shows that the antibody inhibited NMF metabolism substantially. SMG formation was inhibited by 75.0 ± 5.0% of control values (n=3). Clearly a non-specific IgG, isolated from pre-immune rabbits, did not have an inhibitory effect.

The influence of this antibody on PNP metabolism was also investigated (Figure 4.3.4). A concentration of 10mg anti-rat CYP2E1 IgG/nmol P-450 inhibited the formation of 4-nitrocatechol to 31.5 ± 13.6% of control values (n=4) (100% metabolism represented by 1.62 ± 0.42 nmol 4-NC/nmol P-450/min), whilst pre-immune IgG (10mg/nmol P-450) only reduced metabolism weakly.

SMG was detected in incubations of NMF (10mM) and GSH, with purified rat liver CYP2E1 after reconstitution with cytochrome P-450 reductase and cytochrome b_5. Over a 1hr incubation period 27nmol SMG/ nmol P-450 was generated (n=2). This value was 5 times the amount obtained under identical incubation conditions in microsomes from livers isolated from streptozotocin-treated rats, which served as the source of purified enzyme. Here 5nmol SMG/nmol P-450 was generated over the 1 hr incubation period (n=2). The microsomal metabolism of PNP by microsomes isolated from diabetic rats was 2.46 nmol 4-NC/nmol P-450/min (n=2) and with the purified CYP2E1 this increased to 4.42 nmol 4-NC/nmol P-450/min (n=2).

Figure 4.3.1

Design and photograph of dot blots for determination of the presence of anti-CYP2E1 IgG in sera samples collected from a rabbit following a boost injection of CYP2E1.



1 purified rat CYP2E1
2 human liver microsomes
3 purified human CYP2E1
4 BSA

A Pre boost serum
B Positive control ie known to contain CYP2E1 IgG
C 2 week post-boost serum
D 4 week post-boost serum

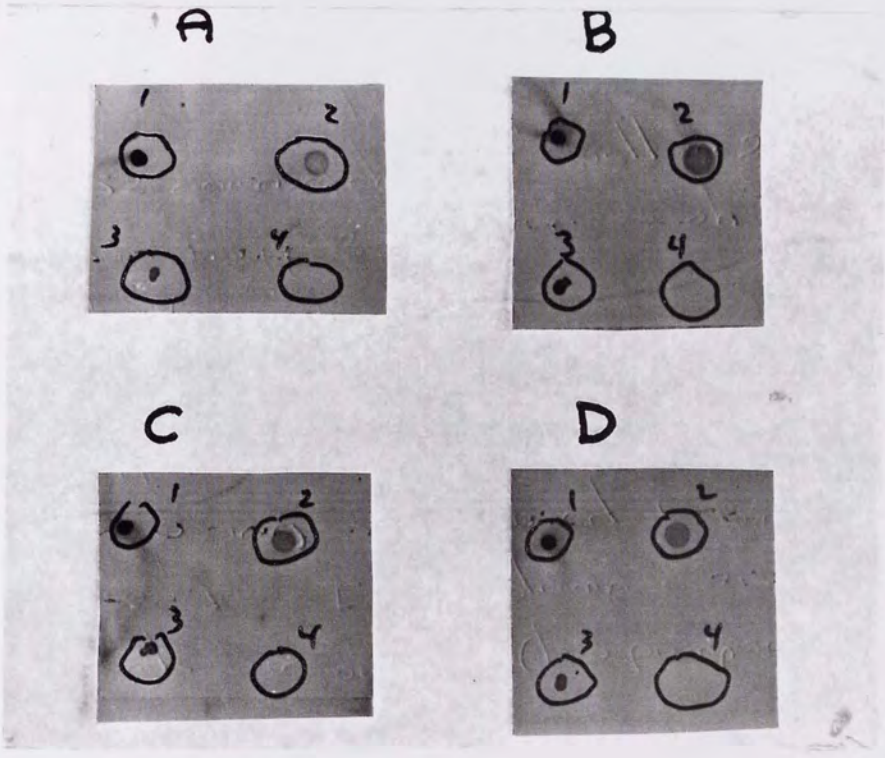
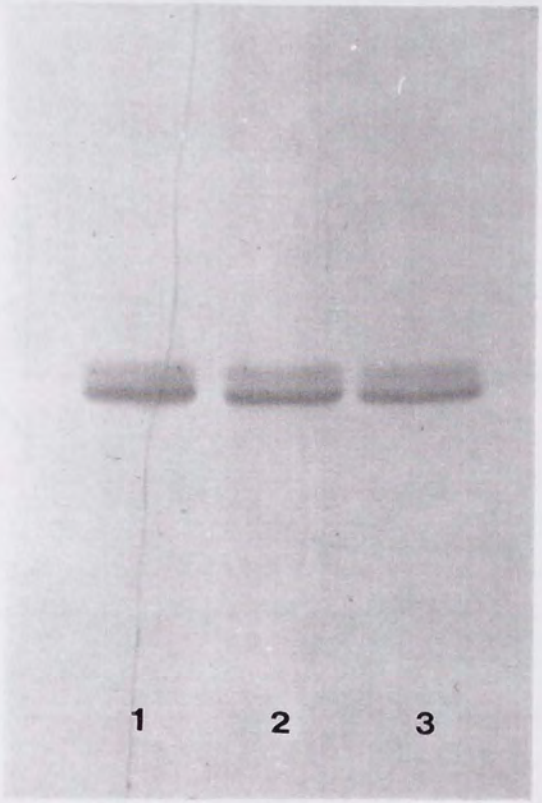


Figure 4.3.2

Western blot of human liver microsomes and purified human CYP2E1, probed with freshly purified anti-rat CYP2E1 IgG.

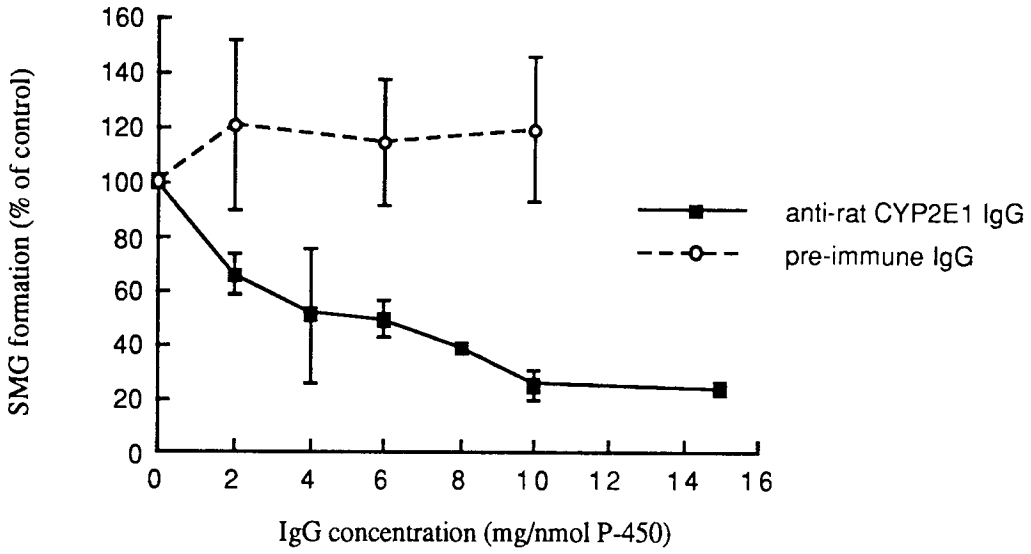


Lane 1 Purified human CYP2E1 (1pmole protein applied).

Lanes 2 and 3 Human liver microsomes (20µg protein applied).

Figure 4.3.3

Effect of coincubation with anti-rat CYP2E1 IgG or pre-immune IgG on the metabolism of NMF (10mM) in suspensions of rat liver microsomes.

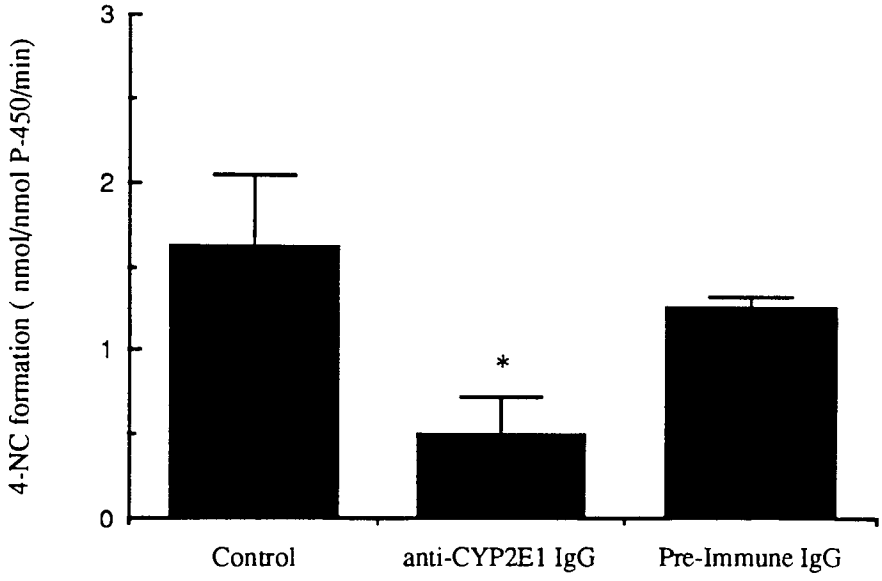


Values are the mean of two or mean ± SD of 3 experiments.

100% value represents 0.52 ± 0.11 nmol SMG/nmol P-450/min.

Figure 4.3.4

Effect of coincubation with anti-rat CYP2E1 IgG or pre-immune IgG on the rate of metabolism of PNP (10mM) in suspensions of rat liver microsomes.



Values are the mean ± SD of 4 experiments.

Star indicates significant difference from control, * P > 0.001.

4.3.3 Discussion

Anti-rat CYP2E1 IgG was isolated and purified from the serum of a rabbit inoculated with purified rat CYP2E1. After purification by immunoabsorbative techniques, this polyclonal antibody recognised a liver microsomal protein band which co-migrated on SDS-PAGE with purified rat CYP2E1. However, the blot comprised of a double band of the CYP2E1 protein. It is possible that this effect is merely an artifact of the method and apparatus used, since Dr. Ken Thummel, in whose laboratories this technique was performed, had recently noticed a similar double banding in other blots which had not been present in earlier experiments. Another possible explanation for these bands is that of glycosolation of the protein. If the enzyme has been glycosolated to different extents then as a result of an increase in sugar molecules attached to the protein the molecular weight of the enzyme will be slightly higher. In immunoblotting techniques this would be reflected by bands corresponding to slightly different molecular weights as were observed here.

The antibody did not affect aminopyrine N-demethylation, whilst SMG formation from NMF was inhibited to 25% of control. This effect was similar to the inhibition observed in the case of PNP where 31% of PNP activity was retained. The use of antibodies has been employed by a number of investigators for studying CYP2E1-mediated metabolism. Terelius *et al* (1991) looked at the effect of a rabbit antibody raised against rat CYP2E1 to demonstrate that acetaldehyde is a substrate for CYP2E1. They too observed a maximal inhibition of metabolism by 81%. Benzene has also been shown to be a substrate for CYP2E1 (Johansson and Ingelman-Sundberg, 1988). Again inhibition of metabolite formation by anti-CYP2E1 IgG was a factor in demonstrating this. A maximal inhibition of 80% was observed. The polyclonal antibody raised in this study had similar maximal inhibitory effects on metabolism of NMF.

To demonstrate that CYP2E1 does indeed catalyse NMF metabolism incubations with purified CYP2E1 were performed. The purified enzyme metabolised both NMF to SMG and catalysed the formation of 4-NC from PNP. The rate of NMF metabolism was 5 times greater than that from microsomes isolated from streptozotocin-treated rats. There was also a similar difference with the metabolism of PNP, however it was not quite as pronounced.

The results from incubations with anti-rat CYP2E1 IgG and purified rat CYP2E1 clearly show that this isozyme catalyses the metabolism of NMF in rodents. It has so far been found that there appears to be a conservation of catalytic specificity of CYP2E1 among humans and animal orthologues (Gonzalez, 1989, Guengerich and Shimada, 1991). To determine whether this holds true for NMF studies using human tissue are required. Section 4.4 discusses the metabolism of NMF in suspensions of human liver microsomes and activity of the human CYP2E1.

4.4 Metabolism of NMF by human CYP2E1 *in vitro*.

4.4.1 Introduction.

Rodents are widely used as a model for studying metabolism and toxicity of chemicals. However, extrapolation of data from animal experiments to humans is often problematic due to species variation in absorption, distribution, metabolism and elimination of compounds. Access to human metabolic data is therefore useful, although due to ethical considerations it is generally not possible to perform *in vivo* studies. Despite this, *in vitro* studies using human tissue can be invaluable in providing important information which may then be correlated with *in vitro* animal data, or with results from *in vivo* studies.

Qualitative investigations as to the metabolites of NMF have identified AMCC as a urinary metabolite in patients exposed to NMF in clinical trials (Kestell *et al*, 1986b). Quantitative data could not be obtained as the identification was performed on urine samples which had been stored in the freezer for 1-2 years. In a preliminary experiment a volunteer ingested NMF (0.1mmol/kg) and the analysis of his urine showed that 17% of the dose was excreted as AMCC (Mraz *et al*, 1989). This percentage is remarkably similar to the results obtained in mice, in which 15% of a dose of 3.4 mmol/kg was excreted in the urine.

In view of this it was proposed to investigate CYP2E1 activity in human liver microsomes. The 6-hydroxylation of chlorzoxazone has been shown to be catalysed by CYP2E1 in incubations with human liver microsomes (Peter *et al*, 1990), therefore rendering it a useful tool for *in vitro* comparison with other reactions potentially catalysed by CYP2E1. The chlorzoxazone assay is approximately 10-fold more sensitive than the colorimetric assays such as that employed in the determination of PNP metabolism.

If two reactions are catalysed by the same enzyme there should be a linear correlation between the rates of the reactions when microsomal preparations with varying enzyme activities are compared. Guengerich *et al* (1991) used this approach as part of a study into the role of CYP2E1 in the oxidation of low molecular weight cancer suspects. Correlation of catalytic activity of chlorzoxazone 6-hydroxylation in 12 different human liver samples, with

metabolism of a number of compounds including vinyl chloride, acrylonitrile and styrene were recorded.

In the study here the rates at which microsomes isolated from 6 human livers catalysed the metabolism of NMF and chlorzoxazone were measured. The relative amounts of CYP2E1 were also measured in these microsomes by immunoblot analysis using antibody against rat-CYP2E1. In other studies excellent correlations have been established between rates of chlorzoxazone 6-hydroxylation and both immunochemically quantified CYP2E1 and NDMA demethylation (Peter *et al*, 1990). Similar linear regression analysis was performed for NMF oxidation.

4.4.2 Results

5-Fluorobenzoxazolone was synthesised by the method of Peter *et al* (1990).

White crystals were obtained with mp 172.5-174.5°C (Lit 174-175°C).

^{1}H-NMR: 6.90 (1H, 8 lines, $^3J_{H\text{-}H}$ 8.8Hz, $^4J_{H\text{-}H}$ 2.7Hz, $^3J_{H\text{-}F}$ 4.3Hz, H6), 7.01 (1H, dd, $^4J_{H\text{-}H}$ 2.7Hz, $^3J_{H\text{-}F}$ 8.3Hz, H4), 7.30 (1H, dd, $^3J_{H\text{-}H}$ 8.8Hz, $^4J_{H\text{-}F}$ 4.3Hz, H7), 11.82 (1H, br s, N-H).

Electron Impact Mass Spectra, *m/z* (rel abundance): 153 ($M^{\cdot+}$, 100), 124 ($M^{\cdot+}$ —CHO, 5), 109 ($M^{\cdot+}$ —CO_2, 3), 97 ($M^{\cdot+}$—2CO, 88), 70 (*m/z* 97—HCN, 50).

The 5-fluorobenzoxazolone was then used as an internal standard for the quantification of chlorzoxazone metabolism as indicated in section 3.6.5.

The metabolism of chlorzoxazone was investigated in suspensions of microsomes isolated from 6 human livers. The rates of metabolism were calculated in terms of metabolite formed/ mg microsomal protein as opposed to nmol P-450. Due to the limited amounts of tissue available there was insufficient material in some samples to perform a cytochrome P-450 assay. The rates of formation of 6-hydroxychlorzoxazone were between 0.168 and 0.408 nmol/mg protein/min (mean±SD: 0.299 ± 0.09 nmol/mg protein/min). These results are summarised in table 4.4.1. There did not appear to be any correlation between either sex or age in the ability of the microsomal enzymes to metabolise chlorzoxazone.

The same table shows the results of an investigation into the metabolism of NMF in the same microsomes. Here values varied from between 0.071 to 0.263 nmol/mg protein/min (mean±SD: 0.183 = 0.07 nmol/mg protein/min). Again there was no correlation with either sex or age of the donors from whom the liver samples were obtained.

Table 4.4.1

Rate of generation of 6-hydroxychlorzoxazone and SMG in incubations of human liver microsomes with chlorzoxazone (400μM) and NMF (10mM), respectively.

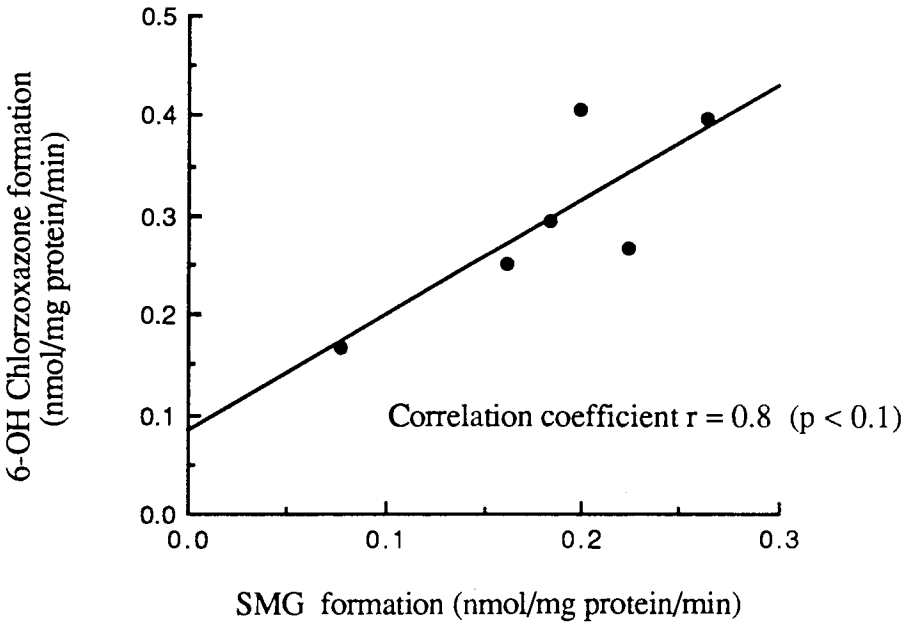
FEMALE			MALE		
	Metabolite Formation (nmol/mg protein/min)			Metabolite Formation (nmol/mg protein/min)	
Age	6-OH chlorzoxazone	SMG	Age	6-OH chlorzoxazone	SMG
10	0.296	0.184	12	0.400	0.263
28	0.168	0.071	45	0.408	0.198
34	0.268	0.223	55	0.252	0.161

Interestingly however, when the rate of chlorzoxazone metabolism was correlated with the rate of NMF metabolism linear regression analysis showed considerable linearity, with r = 0.8 (Figure 4.4.1). To substantiate the involvement of CYP2E1 further the relative amounts of CYP2E1 was determined by immunoblot analysis (Figure 4.4.2). The blot intensities were determined by laser densitometry and a correlation with NMF metabolism was observed (Figure 4.4.3).

NMF was also co-incubated with anti-rat CYP2E1 IgG in suspensions of human liver microsomes. This was performed in duplicate with one human liver sample since there was a lack of tissue available in the other five samples. The presence of anti-rat CYP2E1 inhibited metabolism maximally by 82% (Figure 4.4.4), whilst non-specific IgG was not inhibitory at this concentration.

Figure 4.4.1

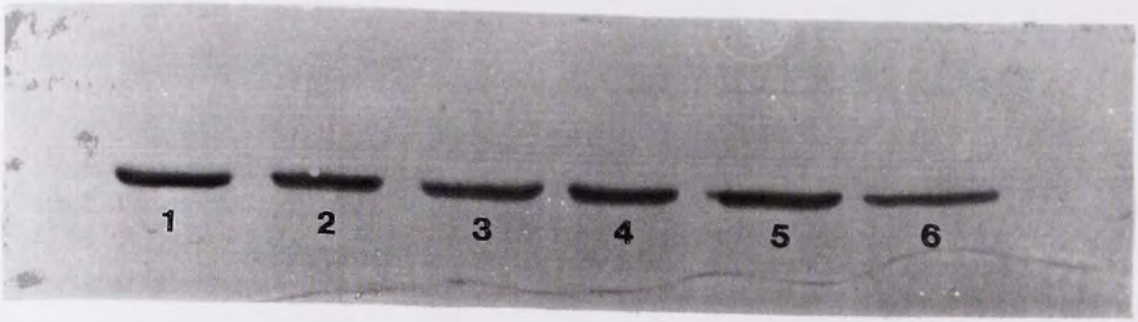
Correlation between the rates of metabolism of chlorzoxazone (400μM) and NMF (10mM) in suspensions of 6 human liver microsome samples.



Values are the mean of 2 separate experiments.

Figure 4.4.2

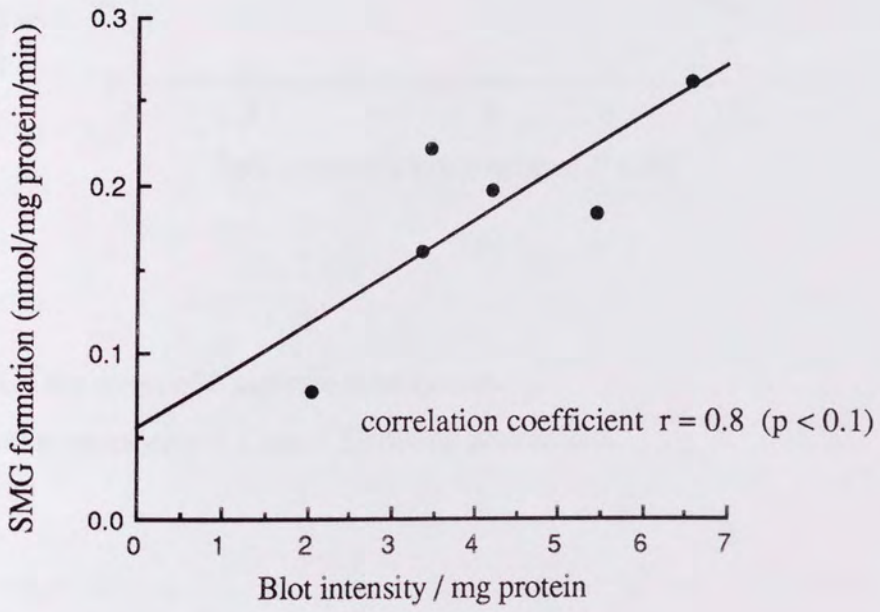
Western blot of 6 human liver microsome samples probed with anti-rat CYP2E1 IgG



Lane 1: Female, 10yrs
Lane 2: Male, 55yrs
Lane 3: Female, 36yrs
Lane 4: Male, 43yrs
Lane 5: Male 12yrs
Lane 6: Female, 28yrs

Figure 4.4.3

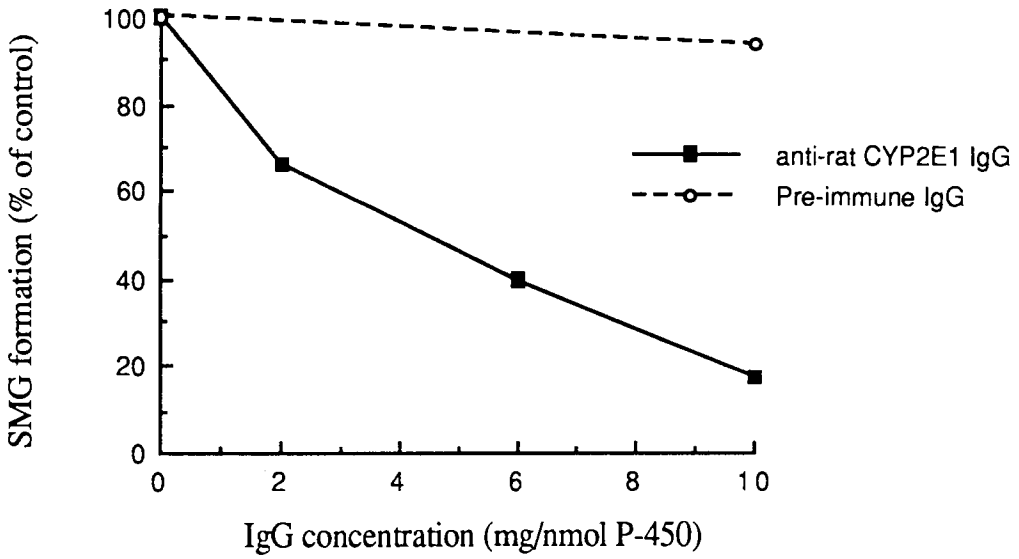
Correlation between rate of NMF (10mM) metabolism and relative amount of immunodetectable CYP2E1 in suspensions of 6 human liver microsome samples.



Values are the mean of 2 separate experiments.

Figure 4.4.4

Effect of coincubation with anti-rat CYP2E1 IgG or pre-immune IgG on the metabolism of NMF (10mM) in suspensions of human liver microsomes.



Values are the mean of 2 separate experiments.

100% value represents 0.18nmol SMG/mg protein/min.

4.4.3 Discussion

Human hepatic microsomes metabolised NMF to SMG in the presence of NADPH and GSH. Interestingly, there was some variability between the ability of microsomes isolated from human livers to metabolise NMF, values varied between 0.071 and 0.263 nmol/mg protein/min. All livers were obtained from healthy humans (3 male and 3 female donors) of varying ages. However, the differences in their metabolising ability could not be correlated with either sex or age. In animal studies there are a number of contradicting reports with respect to sex differences in CYP2E1 expression. Waxman *et al* (1989) reported that CYP2E1 was predominant in the female rat, the differences being attributed to suppression of CYP2E1 by testosterone. Conversely, Yamazoe *et al* (1989) reported male predominance of CYP2E1, whilst Thummel and Schenkman (1990) reported that there were no significant differences between CYP2E1 in male and female rats. However, there is a large sex related difference in expression of CYP2E1 in mouse kidney (Hu *et al*, 1990), where levels of CYP2E1 in males was much increased over females, which correlated well with NDMA demethylase activity. From the limited data available it appears that in human studies there are no sex related differences in CYP2E1 activity.

In animal studies hepatic cytochrome P-450 exhibits an age-related decrease in activity. Few studies directly evaluating age-related alterations in the activity of human hepatic cytochrome P-450s have been performed to date. Hunt *et al* (1990) looked at the effect of normal aging on NDMA demethylase activity in human liver microsomes isolated from 17 liver samples. They found no correlation between CYP2E1 activity and age and also failed to show any correlation with sex. This observation compares favourably with results in section 4.4.2.

Several *in vitro* studies have reported wide variations in the activity of various drug metabolising enzymes using human liver microsomes (Kapitulnik *et al*,1977, Von Bahr *et al*, 1980). There are also similar variations in the metabolism of drugs *in vivo* (Vessell, 1983). These differences may stem from both genetic, hormonal or environmental factors. In the case of the CYP2E1 isozyme factors such as ethanol consumption or exposure to solvents are likely to influence activity. It is therefore not surprising that there was such a wide variation in the apparent ability of hepatic microsomes from the 6 donors to metabolise

NMF. Diet has also been implicated in CYP2E1 control. Rat hepatic CYP2E1 levels have been shown to be proportional to the "fat-to-carbohydrate ratio" in the diet, and levels of ketone bodies in the animal (Yoo *et al*, 1991). Human CYP2E1 activity may similarly be expected to be influenced by the diet of the liver donor.

Quantitative comparisons of rates of NMF metabolism *in vitro* between rodents and humans was only possible after conversion of rodent data to nmol SMG/mg protein /min. It had not been possible to calculate cytochrome P-450 content in the human liver samples, due to a lack of material in some samples. Under these conditions it was shown that mouse microsomes metabolised NMF at a rate (0.63 ± 0.26 nmol/mg protein/min) which was approximately 3-6 times greater than that observed in individual human samples (3.5 times that of the mean value). Interestingly, the rate of metabolism in rats (0.25 ± 0.07 nmol/mg protein/min) was much more comparable to the results obtained with the human liver microsome samples possessing the higher metabolising ability. The rate recorded here was only approximately 1.4 times greater than the mean values in humans. These result are not surprising in view of the generally lower drug metabolising enzyme activities in human liver compared to animal liver (Lorenz *et al*, 1984).

The variability of NMF metabolism compared favourably with metabolism of chlorzoxazone, a probe for CYP2E1, where a correlation of r=0.80 was observed. In similar studies performed by Peter *et al* (1990) a correlation between chlorzoxazone hydroxylation and NDMA demethylation in 14 human samples was recorded (r=0.94). There was also considerable variability in metabolic activity of these microsomes with respect to NDMA and chlorzoxazone metabolism. A five fold difference was recorded across the samples.

The relative amounts of CYP2E1 detected by Western blot analysis with anti-rat CYP2E1 IgG correlated with the rate of NMF metabolism. This correlation further confirmed that the observed variability in the rate of NMF metabolism was related to differences in CYP2E1 content.

A concentration dependent effect of anti-rat CYP2E1 was observed in incubations of human liver microsomes with NMF (10mM). Maximal inhibition was recorded as 82%. This is very similar to the effects seen in incubations with rat liver microsomes. This result reflects the similarity in specificity of the antibody to CYP2E1 across species. The cDNA for rat and human CYP2E1 were isolated and sequenced in 1986 (Song *et al*, 1986). The coding sequence of both rat and human CYP2E1 contained 1489 base pairs, and the deduced amino acid sequence for both species contained 493 amino acids. Human CYP2E1 shares 75% nucleotide and 78% amino acid similarity with the orthologous rat CYP2E1.

The results obtained in this section have established that NMF is also a substrate for the human CYP2E1. Hence factors modulating the activity of this human form would result in varying susceptibilities of individuals exposed to NMF. This effect is discussed in more detail in section 5.

4.5 Metabolism of DMF *in vitro*.

4.5.1 Introduction

The hepatotoxicity of DMF in workers occupationally exposed to DMF has already been discussed in section 1.4.2. As in the case of NMF the hepatotoxicity of DMF is believed to be associated with its metabolism. The major urinary metabolite of DMF in rodents and humans is HMMF (Scailteur *et al*, 1984, Brindley *et al*, 1983a, Kestell *et al*, 1986a). The mercapturate AMCC is another important metabolite (Mraz *et al*, 1989). As is believed to be the case with the oxidative metabolism of NMF, the formation of AMCC should be the corollary of conjugation of GSH with a reactive metabolite, probably MIC (Pearson *et al*, 1990). The overall metabolism of DMF to AMCC *in vivo* is quantitatively more prominent in humans than in rodents (Mraz *et al*, 1989).Therefore occupationally exposed workers may be at a higher risk of DMF induced toxicity than that which might be inferred from experimentation in rodents.

Little is known about the enzymes which catalyse the oxidation of DMF to HMMF, and about the steps which lead from DMF to AMCC. The metabolic generation of HMMF from DMF, and of SMG from NMF can be studied *in vitro* in suspensions of rodent or human liver microsomes. However, it has not been possible to observe the formation of SMG from DMF under these conditions (Cross *et al*, 1990).

In view of the results described in sections 4.1 to 4.4 which indicate CYP2E1 as the microsomal enzyme involved in the metabolism of NMF, the role for CYP2E1 in DMF oxidation was investigated. It is interesting to note that the DMF molecule is isoelectronic with the CYP2E1 substrate NDMA. In view of this structural similarity the possibility that DMF is metabolised by CYP2E1 was considered.

In order to characterise the isozyme of cytochrome P-450 responsible for the metabolism of DMF to HMMF induction and inhibition studies were performed using modulators of CYP2E1 activity. Further investigations involving co-incubation of anti-rat CYP2E1 IgG with rat and human liver microsomes and purified CYP2E1 were also carried out, similar to

those outlined above with NMF (section 4.3).

4.5.2 Results

To test the hypothesis that CYP2E1 catalyses the N-methyl oxidation of DMF its metabolism was measured in microsomes isolated from rat livers in which CYP2E1 activity had been induced by administration of acetone. Figure 4.5.1 shows the time course of metabolism of DMF to HMMF in both control and acetone- treated animals. The rate at which microsomes from acetone-treated rats oxidised DMF to HMMF was increased by approximately 200%, from 2.38 ± 0.61 to 8.0 ± 1.93 nmol HMMF/nmol P-450/min (n=4). The formation of SMG from DMF in incubations with microsomes in which CYP2E1 activity had been induced was also investigated. In all incubates tested SMG could not be detected.

The metabolism of DMF was also investigated in incubations with microsomes and inhibitors of CYP2E1. DEDTC was incubated with DMF at concentrations at which it is a relatively specific inhibitor of CYP2E1. Figure 4.5.2 shows that at the maximum concentration investigated (100μM DEDTC) the metabolic generation of HMMF from DMF was inhibited dramatically, by 87.9 ± 2.3% (n=3). The effect of another substrate, DMSO, was also investigated (Figure 4.5.3). Clearly the rate of oxidation of DMF to HMMF was inhibited in microsomal incubations with DMSO. At 1mM DMSO the metabolic formation of HMMF was inhibited to 71.34 ± 7.7% (n=3) and increasing the concentration of DMSO to 100mM reduced the rate of metabolism to 37.5 ± 18.2 % (n=3) compared to control values.

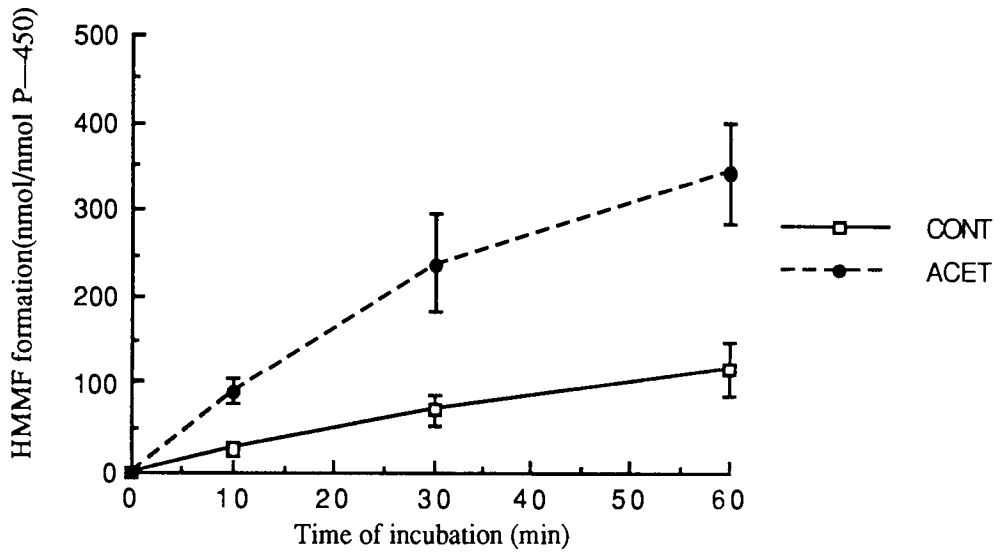
Since results indicated a role for CYP2E1 in DMF metabolism, the effect of coincubation of DMF and NMF (also a CYP2E1 substrate) was investigated with the assistance of Dr. J. Mraz (Aston University, Birmingham). This investigation was only possible since SMG cannot be detected in microsomal incubations with DMF. Therefore any SMG measured can be assumed to have originated from the metabolism of NMF. Figure 4.5.4 shows the dramatic effect that DMF has on the oxidation of NMF. At 0.2mM DMF the rate of NMF metabolism was inhibited by 51.5% (n=2) and increasing the DMF concentration to 10mM inhibited the formation of SMG to only 4.5% (n=2) of control values.

The monospecific antibody against rat CYP2E1 (described in section 4.3) was co-incubated with DMF in suspensions of human and rat liver microsomes. At antibody concentrations which did not inhibit aminopyrine N-demethylation the metabolism of DMF by human liver microsomes was inhibited substantially, as illustrated in Figure 4.5.5. Maximal inhibition was by 67.5% (n=2) as compared to control values. A non-specific antibody isolated from pre-immune animals had no influence on DMF metabolism at the same concentrations. The effect of this antibody on the oxidation of DMF in suspensions of rat liver microsomes was also investigated. Figure 4.5.6 shows the results of this experiment. Maximal inhibition of DMF metabolism was observed to be 68.4% (n=2). This result is remarkably similar to that observed in incubations with human liver microsomes.

HMMF was also detected in incubations of DMF (10mM) with purified rat CYP2E1 after reconstitution with cytochrome P-450 reductase and cytochrome b_5. In a 1 hr incubation period 555nmol HMMF/nmol P450 was generated. This value was 5 times greater than the amount of HMMF formed in identical incubations with microsomes isolated from streptozotocin-treated rats, which served as the source of the purified enzyme. In these incubations 110nmol HMMF/nmol P-450 were generated. This 5 fold difference in the generation of HMMF mirrored that observed in similar incubations with NMF where SMG generation was determined (section 4.3.2).

Figure 4.5.1

Effect of induction of CYP2E1 by acetone on the metabolism of DMF (10mM) in suspensions of rat liver microsomes.

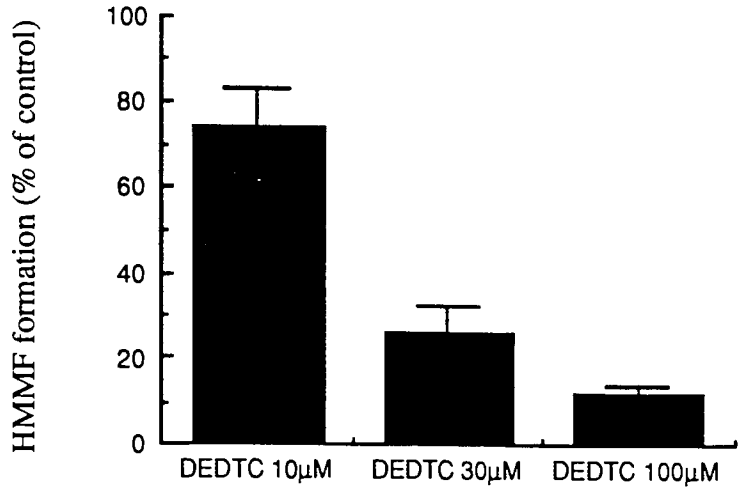


Pretreatments were as described in section 3.7.1.

Values are mean ± SD of 4 experiments.

Figure 4.5.2

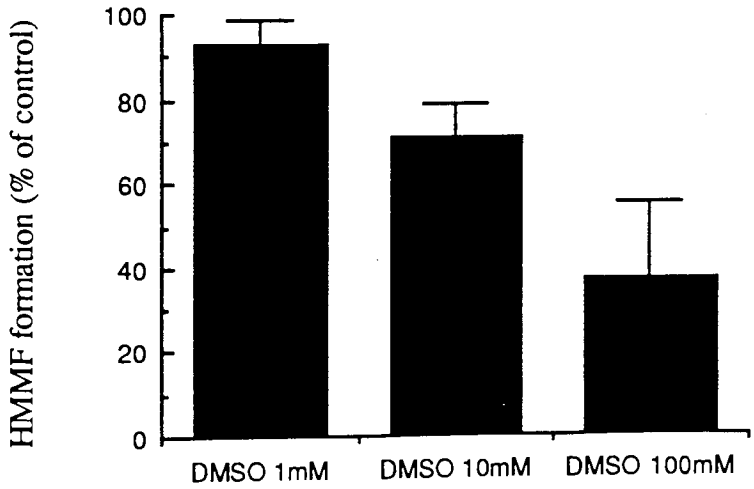
Effect of DEDTC on the metabolism of DMF (10mM) in suspensions of rat liver microsomes.



Values are the mean± SD of 3 experiments.

Figure 4.5.3

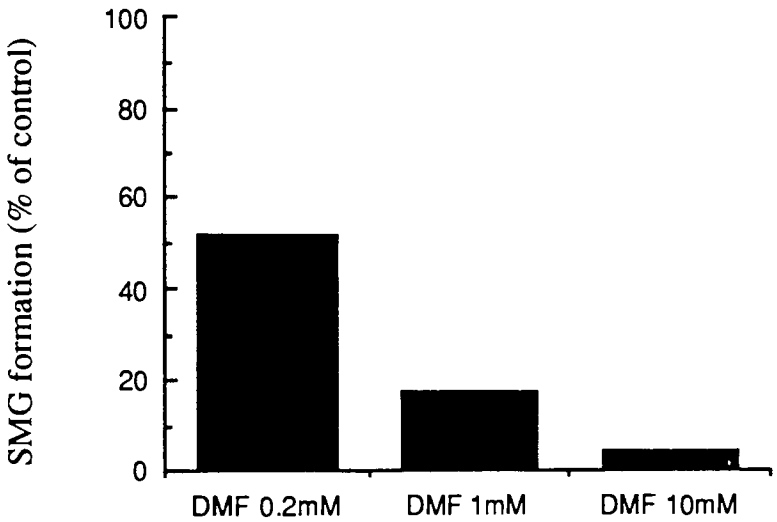
Effect of DMSO on the metabolism of DMF (10mM) in suspensions of rat liver microsomes.



Values are the mean ± SD of 3 experiments.

Figure 4.5.4

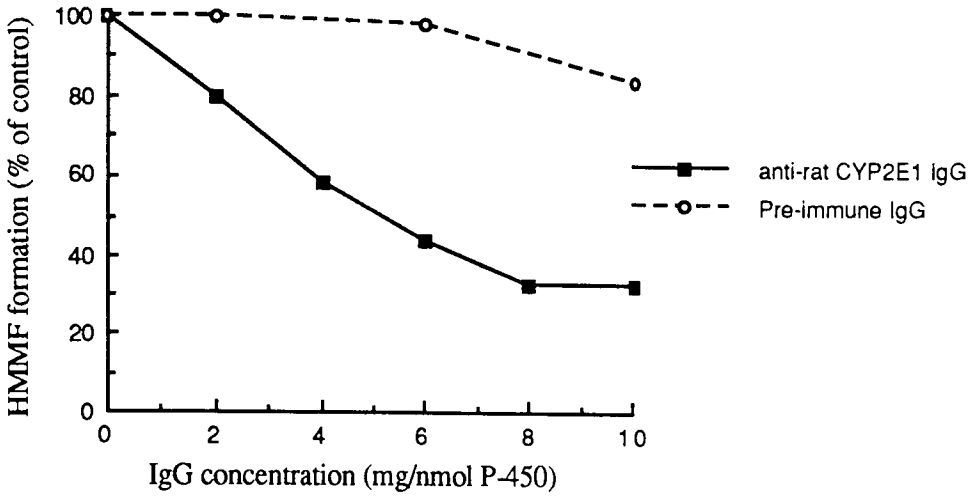
Effect of coincubation with DMF on the metabolism of NMF (1mM) in suspensions of rat liver microsomes.



Values are the mean of 2 separate experiments.

Figure 4.5.5

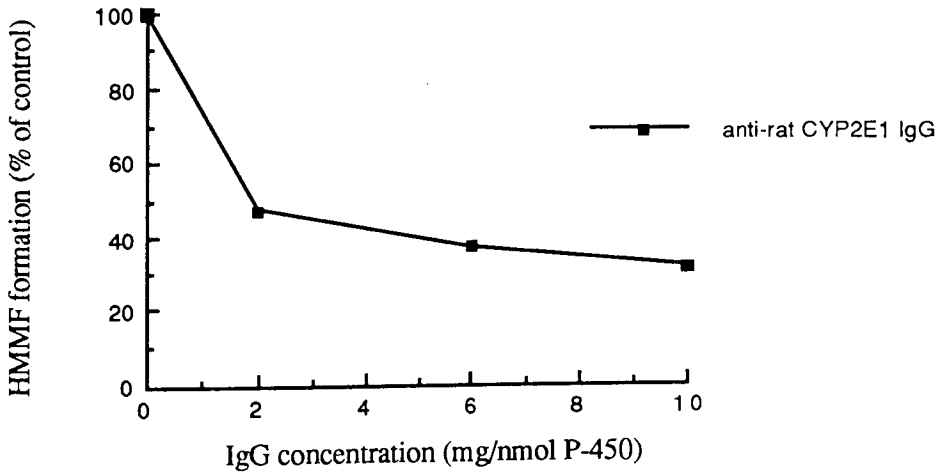
Effect of coincubation with anti-rat CYP2E1 IgG or pre-immune IgG on the metabolism of DMF (10mM) in suspensions of human liver microsomes.



Values are the mean of 2 separate experiments.

Figure 4.5.6

Effect of coincubation with anti-rat CYP2E1 on the metabolism of DMF (10mM) in suspensions of rat liver microsomes.



Values are the mean of 2 separate experiments.

4.5.3 Discussion

Previous studies into the the metabolism of DMF have been unable to detect the formation of SMG as a metabolite *in vitro*. AMCC however has been determined as a urinary metabolite of DMF (Mraz and Turecek, 1987, Mraz *et al*, 1989) and the precursor of this would be expected to be SMG. In light of the evidence that CYP2E1 metabolises NMF and NDMA, structural analogues of DMF, the ability of CYP2E1 to catalyse DMF oxidation has been investigated.

Induction of CYP2E1 resulted in an increase in the formation of HMMF. However, even using microsomes from induced animals the presence of SMG could not be detected. Microsomal incubations with HMMF alone have been shown to generate SMG as a metabolite (Cross *et al*, 1990). It was therefore thought that by increasing the amount of HMMF formed it may be possible to detect SMG from incubations with DMF. SMG however remained elusive.

HMMF formation was also inhibited by DEDTC and DMSO. With DEDTC maximal inhibition was comparable to that seen with the formation of SMG from NMF. However DMSO (100mM) inhibited NMF metabolism by 95% whilst formation of HMMF from DMF was only inhibited by 60%. Since the inhibition of NDMA demethylation by DMSO has been reported to be competitive (k_i = 0.39mM) it is probable that this is also the case with inhibition of NMF and DMF metabolism. The CYP2E1-catalysed metabolism of DMF proceeds with a k_m of 0.2 ± 0.06 mM whilst the rate of metabolism of NMF shows a k_m of 4.28 ± 1.35 (Mraz *et al*, 1992). Therefore DMF is a better substrate for CYP2E1 than NMF. Hence in the case of a competitive inhibitor such as DMSO, a higher concentration would be required to produce the same effect as observed with NMF. This would explain why DMSO is a less efficient inhibitor of DMF metabolism as compared to inhibition of NMF metabolism.

Coincubation of DMF with NMF led to inhibition in the formation of SMG. This result is very interesting as it suggests that DMF may in fact be an inhibitor of its own metabolism. DMF is believed to be metabolised to a GSH conjugate via a two step process. Firstly

oxidation at a methyl group to form either HMMF and/or NMF, and subsequently formyl C oxidation to a reactive intermediate which on conjugation with GSH yields SMG. If DMF itself inhibits the formation of SMG from NMF or HMMF then this fact could provide an explanation for the observed lack of generation of measurable SMG in incubations with DMF *in vitro* and the retarded excretion of AMCC as a urinary metabolite of DMF (Mraz *et al*, 1989).

The experiments described here show that at 0.2mM DMF the metabolism of NMF is inhibited to 50% and by increasing the concentration of DMF to 10mM NMF metabolism is inhibited down to 4.5% of controls. The metabolism of HMMF to SMG is also inhibited by DMF in a similar way (Mraz *et al,* 1992), and is expected to be a CYP2E1 substrate. The ability of DMF to inhibit the CYP2E1-mediated metabolism of its own metabolites is consistent with the observation that DMF inhibits the N-demethylation of NDMA (k_i = 66μM), another CYP2E1 substrate (Yang *et al*, 1991).

A monospecific antibody against rat-CYP2E1 decreased the metabolic formation of HMMF from DMF in incubations with human liver microsomes by 67.5% compared to 68.4% in rats. This finding again reflects the similarity between CYP2E1 in rats and humans. CYP2E1 enzymes characterised in rats, humans and rabbits have been shown to possess 80% similarity in their amino acid sequence.

The result shows clearly that oxidation of the methyl groups of DMF is catalysed via CYP2E1.

4.6 Metabolism of DMF *in vivo*

4.6.1 Introduction

Recently an analytical method was developed by Dr. T.A. Baillie and Ms M.R. Davis (Department of Medicinal Chemistry, University of Washington, Seattle) for the quantification of SMG in the bile collected from NMF treated rats. Due to the low concentrations of SMG present in bile, the chemical instability of SMG and the complexity of the biological matrix in which SMG is found, a rapid highly sensitive and specific analytical technique was required. The high sensitivity of ionspray mass spectrometry and the high selectivity of selective reaction monitoring were combined to make tandem ionspray mass spectrometry the ideal technique for the quantification of SMG in bile.

In experiments preceding my stay in the laboratory of Dr. T.A. Baillie the value of this method in monitoring the biliary excretion of SMG from NMF-treated animals was studied. Following the administration of an hepatotoxic dose of NMF to rats (400mg/kg ip) 0.066 ± 0.028% of the dose was excreted as SMG in bile collected over a 4 hour time period immediately post-dose (M.R.Davis, personal communication). This analytical technique provided a rapid and reproducible analysis of samples in the 30-300 picomole range. Due to the high sensitivity of the method it was proposed to look at the biliary metabolites of DMF in the hope of identifying the still elusive SMG molecule. The influence of CYP2E1 inducers and inhibitors on biliary excretion was also investigated.

Mechanistic details of formation of SMG from DMF were also considered to be amenable to study utilising stable isotopes of DMF. Substitution of hydrogen atoms with deuterium, which is thought not to perturb the electronic potential energy surfaces of molecules, has been used to address questions concerning the enzymatic mechanisms underlying the biotransformation of xenobiotics.

Such studies have been applied when investigating the mechanism of bioactivation of NMF *in vitro* (Baillie *et al*, 1988) and *in vivo* (Threadgill *et al*, 1987). The *in vivo* studies demonstrated that the formation of the GSH conjugate of NMF was subject to an apparent

intermolecular primary kinetic deuterium isotope effect. Metabolism was slowed by a factor of 7 ± 2 when the hydrogen in the formyl group was replaced by deuterium. Similar studies were proposed with DMF by replacing methyl group hydrogens and/or the formyl group hydrogen with deuterium, in order to obtain further mechanistic details of the bioactivation of DMF. Animals were dosed with the following isotopic mixtures (1:1) of DMF (500mg/kg i.p. total dose).

i) a) $[^2H]_6$ DMF: DMF b) $[^2H]_6$ DMF: $[^2H]_1$ DMF

ii) a) $[^2H]_7$ DMF: DMF b) $[^2H]_7$ DMF: $[^2H]_1$ DMF

The ratios of SMG:deuterated SMG generated were measured in order to calculate isotope effects.

4.6.2 Results

A dose of 500mg DMF/kg ip failed to produce a measurable amount of SMG in bile collected in the first 4 hr following administration. However pooled bile collected 24-28 hr post dose was observed to contain a measurable amount of SMG. Figure 4.6.1 shows the daughter ion spectrum obtained from collisional activation of the MH^+ ion of synthetic SMG. This spectrum is almost identical to that illustrated in Figure 4.6.2 which was obtained from a bile sample collected from a DMF treated rat. The major difference between these two spectra is the 189 peak in the bile sample. This is attributed to a natural constituent of bile also having a MH^+ of 365 and fragmenting to 189 on collisional activation. To overcome this interference all samples for the standard curve were subsequently prepared in bile. Figure 4.6.3 shows the raw data obtained for the calibration. The samples were analysed via flow injection and the data represent the ion currents recorded for the transition *m/z* 365--->236 (for SMG) and *m/z* 368--->239 (for $[^2H]_3$ SMG) this representing the loss of a glutamic acid moiety. These resulting ion currents were integrated over a range of 50 scans for quantification. Figure 4.6.4 is an example of a typical calibration curve which was obtained by this method.

Figure 4.6.1

Daughter ion spectrum obtained by collisional activation of the MH^+ ion of synthetic SMG.

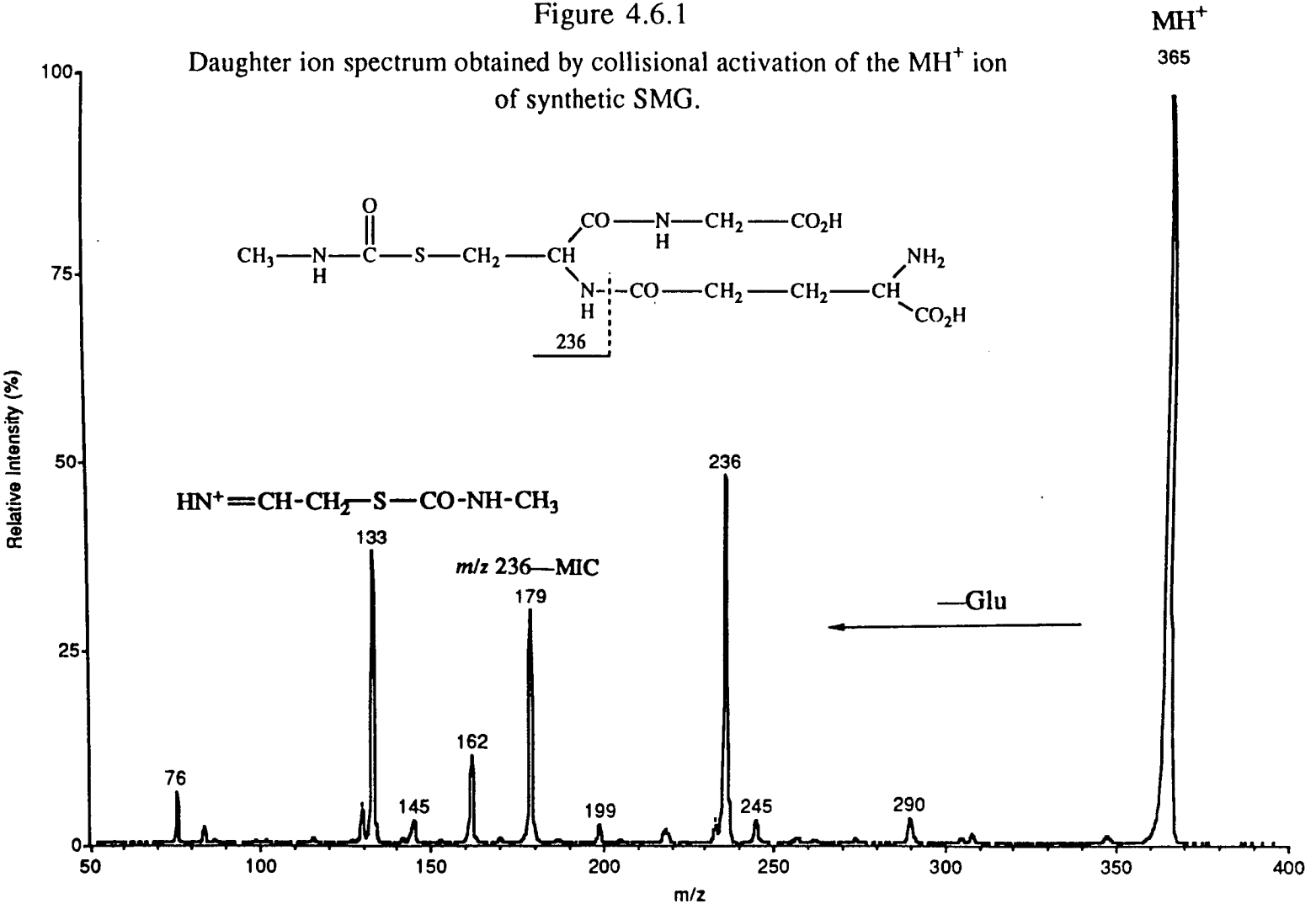


Figure 4.6.2

Daughter ion spectrum obtained by collisional activation of the MH^+ ion of SMG collected in a bile sample.

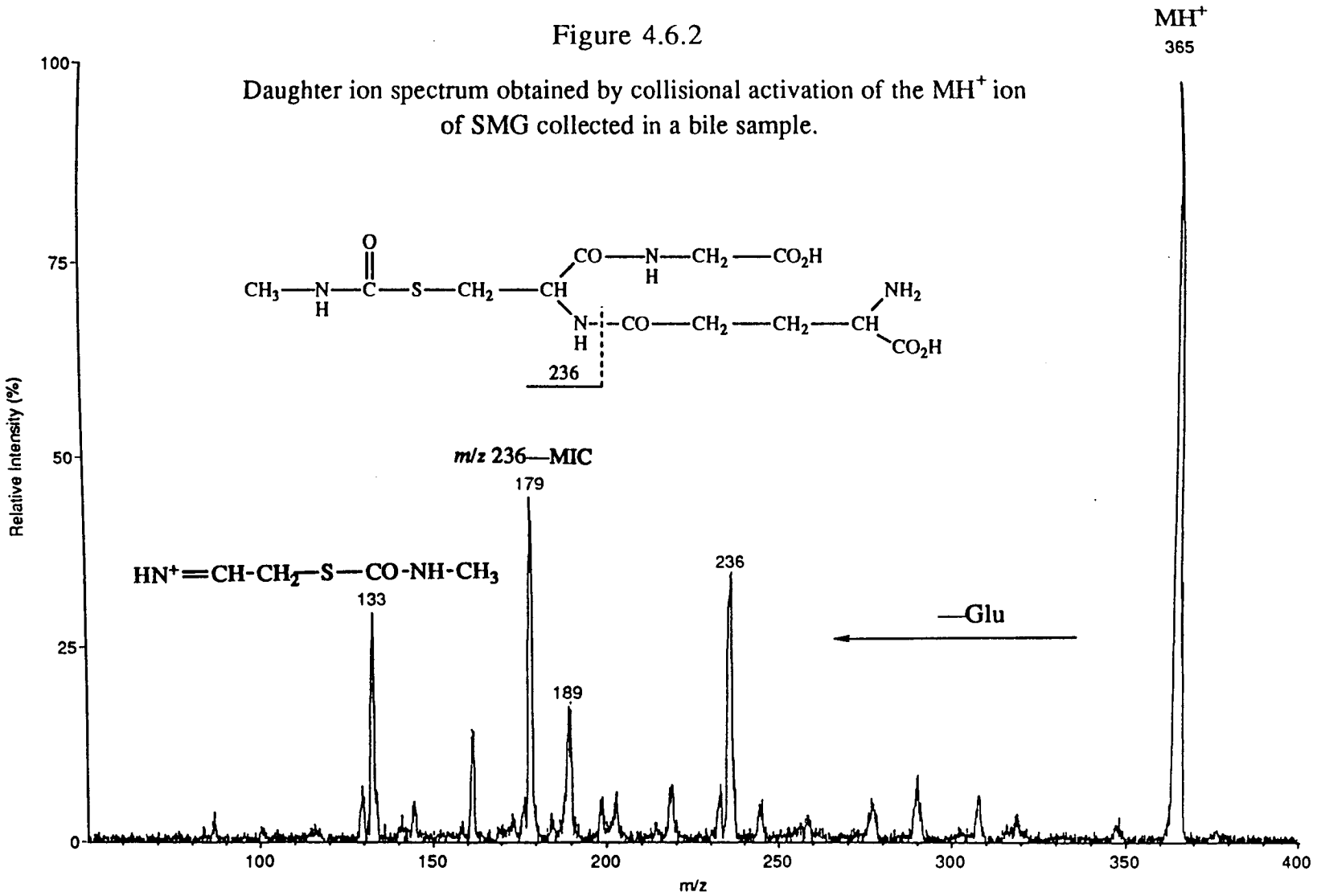


Figure 4.6.3

Ion currents obtained for transitions *m/z* 365--->236 and *m/z* 368--->239 from standard samples prepared for construction of a calibration curve.

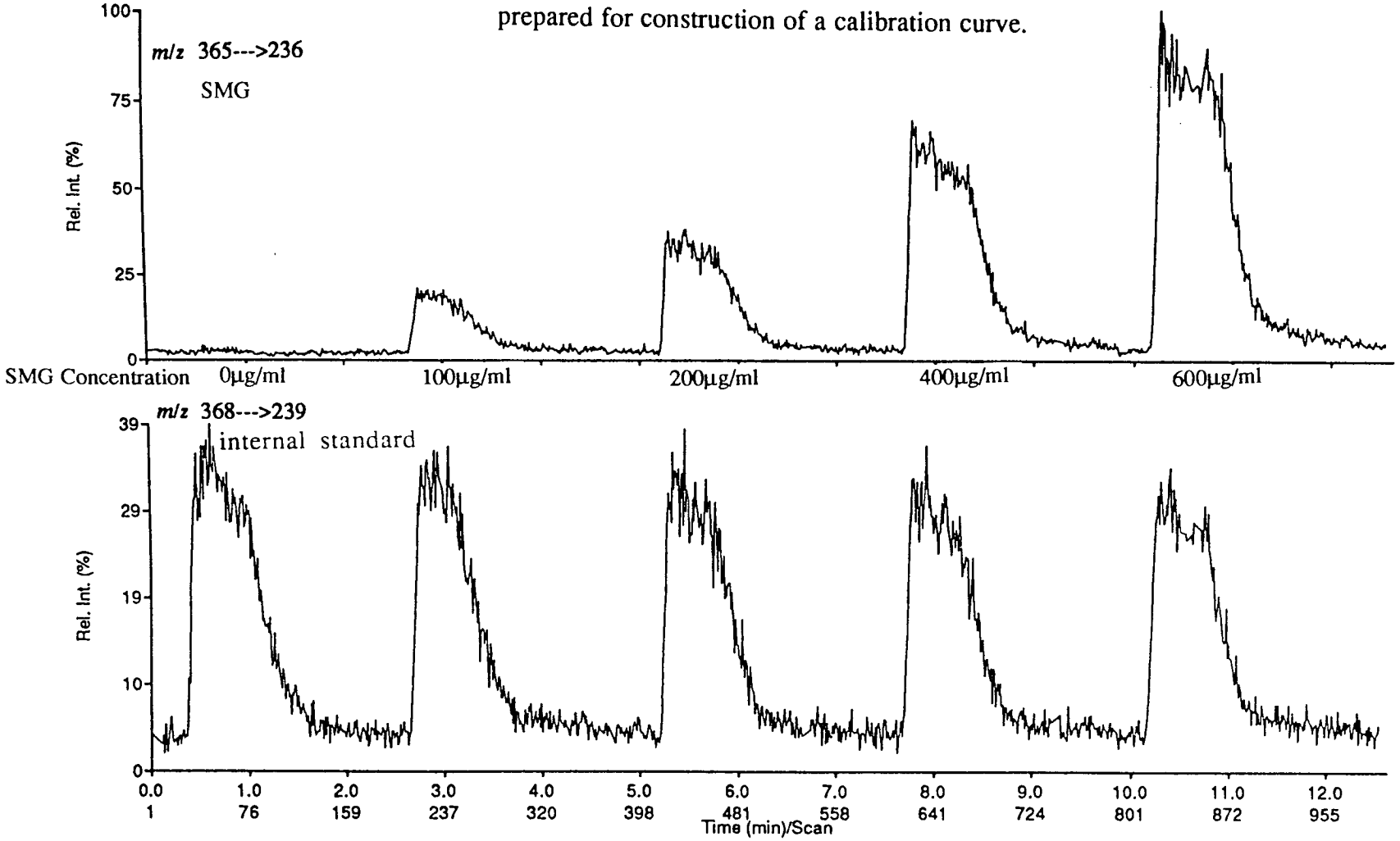
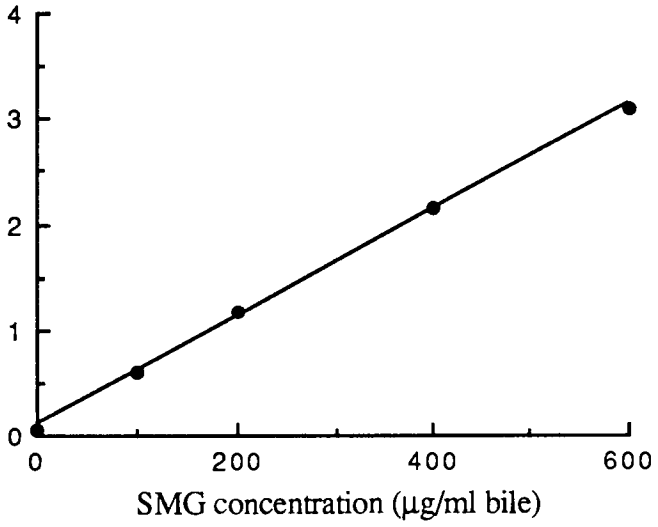


Figure 4.6.4

Standard curve obtained following integration of ion currents over a range of 50 scans.



Correlation coefficient, $r = 0.999$.

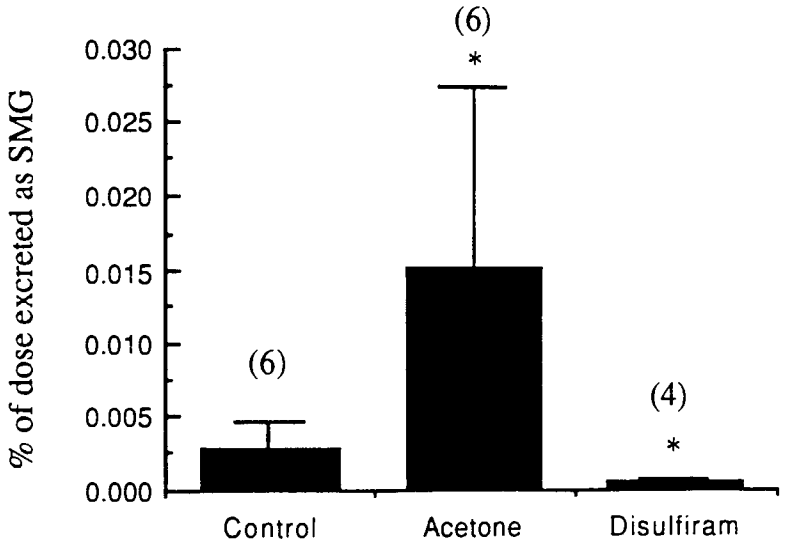
On quantification of bile samples SMG was found to be formed 24-28hr post dose at 0.0027 ± 0.0019 % of a dose of DMF (n=6) (Figure 4.6.5). Pretreatment of rats with acetone increased the generation of SMG to 0.015 ± 0.012 % dose of DMF (n=6), representing an increase of 500%, and disulfiram inhibited SMG production by 80%.

Since levels of SMG were so low the studies performed with DMF isotopes were performed with acetone-treated animals in order to maximise CYP2E1 activity, and an improved analytical technique was utilised. This involved the derivatisation of SMG in bile samples to the N-benzyloxycarbonyl methyl ester. Figure 4.6.6 is a daughter ion mass spectrum of this derivative. Loss of the glycine methyl ester (transitions *m/z* 527---> 438, and *m/z* 530---->441 for the deuterated compound) was monitored for quantification purposes. The ratio of responses in the resulting current profiles were used to calculate the apparent isotope effects in the conversion of DMF to SMG.

Studies which involved the administration of an equimolar mixture of $[^2H]_6$ DMF and unlabelled DMF (500mg/kg i.p. total dose) failed to yield detectable amounts of SMG in bile. Experiments involving the formyl deuterated analogues $[^2H]_1$ DMF and $[^2H]_7$ DMF revealed that there was a substantial isotope effect associated with deuterium substitution on the methyl groups of DMF. When a 1:1 mixture of $[^2H]_7$ DMF and unlabelled DMF was administered to rats at a total dose of 500mg/kg i.p., the SMG excreted in pooled bile was collected between 24-28 hr for determination of the relative amounts of SMG and $[^2H]_3$ SMG, as measured by conversion of the conjugate to its N-benzyloxycarbonyl derivative, prior to extraction. The two molecular species of derivatised SMG were detected by selected reaction monitoring (*m/z* 527--->438 for SMG and *m/z* 530--->441 for $[^2H]_3$ SMG), which indicated that the unlabelled conjugate was present in an approximate 10-fold excess over its deuterated counterpart. It was calculated that the metabolism of DMF to SMG *in vivo* exhibits a large apparent isotope effect of $K_H/K_D = 10.1 \pm 1.3$. When a similar experiment was carried out with a equimolar mixture of $[^2H]_1$ and $[^2H]_7$ DMF, the corresponding figure was 7.7 ± 0.7 (n=4).

Figure 4.6.5

Effect of induction or inhibition of CYP2E1 on the percentage of a dose of DMF (500mg/kg) excreted in the bile of rats as SMG.

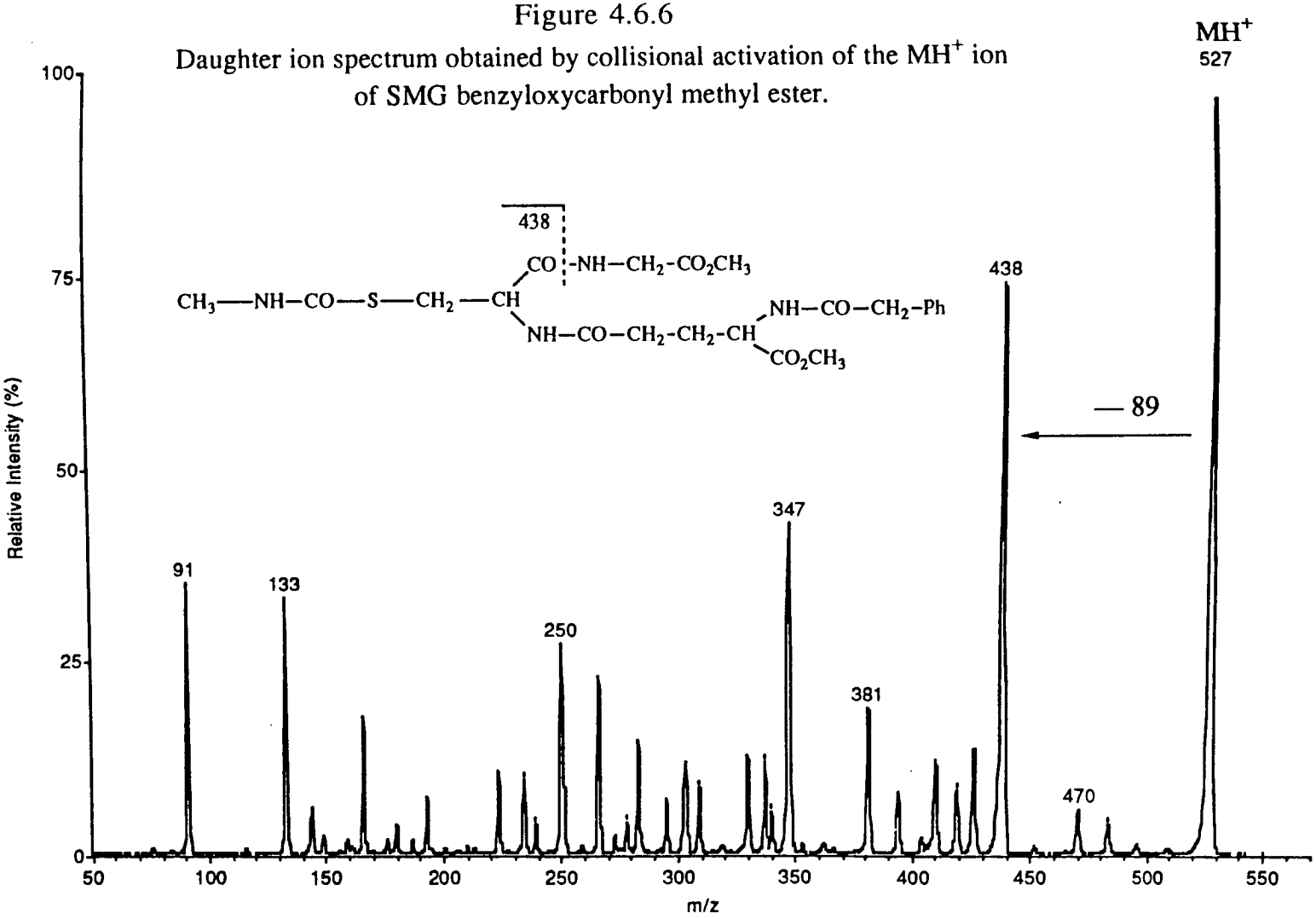


Pretreatments were as described in section 3.8.1. Values are the mean ± SD of 4-6 experiments, number of experiments in brackets.

Stars indicate significant difference from controls, *P<0.05.

Figure 4.6.6

Daughter ion spectrum obtained by collisional activation of the MH^+ ion of SMG benzyloxycarbonyl methyl ester.



4.6.3 Discussion

SMG has been identified as a biliary metabolite of DMF. This is the first time that SMG has been detected as a metabolite of DMF *in vivo*. The generation of SMG was much lower than that observed with NMF when results were expressed in terms of SMG excreted as % dose. This lower rate of metabolism to SMG is reflected in the observed reduced toxicity of DMF compared to that of NMF in a number of earlier studies (Kestell *et al*, 1987, Shaw *et al*, 1988).

DMF metabolism to SMG was inducible by acetone and inhibited by the mechanism based inhibitor disulfiram, the oxidised form of DEDTC, which was used in previous *in vitro* studies. These results confirmed the pivotal role for CYP2E1 in DMF metabolism *in vivo*. The delayed excretion of SMG correlated well with that seen for the urinary excretion of AMCC. Mraz *et al* (1989) observed that a dose of 7mmol/kg DMF given to rats resulted in appearance of AMCC in the urine only after a delay of 24 hours. The inhibitory effect of DMF on CYP2E1 mediated N-alkylformamide metabolism can help to explain why there is the delay in excretion of AMCC and SMG. This has previously been discussed in section 4.5.3 with respect to a lack of SMG generated in *in vitro* studies with DMF. The implication of this study together with the significant involvement of CYP2E1 in DMF metabolism *in vivo* is discussed in section 5.

The mechanism of DMF metabolism was also studied using stable isotopes of DMF. Replacement of the N-methyl hydrogens and formyl hydrogen with deuterium decreased substantially the rate at which SMG was formed from DMF *in vivo*. The apparent kinetic deuterium isotope effect was 10.1 ± 1.3, which most likely represents the product of 2 discrete isotope effects on N-demethylation and formyl oxidation reactions. The result obtained with an equimolar mixture of $[^2H]_7$:$[^2H]_1$ DMF gave an indication of the effects associated with the methyl groups (apparent kinetic deuterium isotope effect of 7.7 ± 0.7). To attempt to extract from these values deuterium isotope effects for each of the two discrete metabolic steps (N-demethylation and formyl oxidation) would be inappropriate. The relative concentrations of labelled and unlabelled substrates and intermediates in these experiments would be changing constantly, and as a result of this it would be expected that

the true isotope effects would be masked. Nevertheless an estimate of the limits of these isotope effects may be made. The value of 7.7 ± 0.7 represents a maximum for the N-demethylation reaction, and a value of 1.3 (i.e. 10.1/7.7) represents a minimum for the formyl oxidation.

It therefore appears that there is a substantial isotope effect associated with deuterium substitution on the methyl groups of DMF, whilst deuteration at the formyl position appears to have only a modest influence on conversion of DMF to SMG in rats. The findings in this *in vivo* study seem to be in good agreement with those of the *in vitro* work described by Mraz *et al* (1992). Here it was observed that replacement of N-methyl hydrogens with deuterium reduced the rate of DMF metabolism, and additional deuteration of the formyl group enhanced this effect.

This study has indicated that DMF oxidation is mediated via a mechanism involving abstraction of a hydrogen atom from a methyl group as opposed to abstraction of electrons from the nitrogen as the rate-limiting step in the generation of SMG.

Section 5

General Discussion

5 General Discussion

Guidelines as to the safe use of chemicals require, ideally, a large base of knowledge which allows for a rational safety assessment to be made. The results presented in this thesis might contribute to the scientific base which will help to judge the consequences of exposure of humans to formamides. A major objective of these studies was to clarify which cytochrome P-450 isozyme is involved with the metabolic toxification of NMF and DMF. Using *in vitro* methods the enzymatic details of the metabolism of NMF and DMF were investigated. *In vivo* studies were utilised in order to help elucidate the mechanism of DMF metabolism.

Like many chemicals that cause hepatotoxicity, formamides must be bioactivated to reactive species to initiate cell damage. The role of metabolism in the toxicity of N-alkylformamides has been previously studied in much detail (Threadgill *et al*, 1987, Kestell *et al*, 1986b). The mechanism by which NMF causes toxicity is thought to be specifically related to the metabolic pathway by which SMG is formed and which leads ultimately to the excretion of N-alkylcarbamic acid thioesters in the urine (Threadgill *et al*, 1987). Prior to this study there has been little work directed towards the elucidation of the enzymes catalysing the formation of SMG. *In vitro* studies utilising liver microsomal systems have demonstrated this approach to be a suitable one for studying formamide metabolism. From such studies cytochrome P-450 has been implicated in NMF metabolism, but not an isozyme which is inducible by either BNF or phenobarbitone (Cross *et al*, 1990).

The work presented in this thesis has established that NMF metabolism is catalysed by CYP2E1. To demonstrate this fact several approaches were used. Metabolic activity was induced by compounds known to induce CYP2E1. Not only was it established that NMF metabolism was indeed induced (section 4.1) but there was also a corresponding increase in the toxicity of NMF to isolated hepatocytes (section 4.2). However it is pertinent to point out that a correlation between an increase in the activity being measured and treatment with an inducer such as acetone is not definitive since acetone also induces CYP2B1. Although suitably induced microsomes can implicate CYP2E1 in catalysis *in vitro* the availability of specific inhibitors of the isozyme would also been extremely valuable. Mechanism-based

inhibitors offer the greatest potential for specificity since catalysis is required for inhibition. DEDTC and PEITC were used in this investigation and in addition the reversible inhibitors, DMSO and PNP, were also utilised. These inhibitors represent alternative substrates for CYP2E1 and their inhibition depends on their affinity for binding to CYP2E1. In section 4.1 and 4.2 it was shown that in most cases the inhibitors chosen decreased the rate of NMF metabolism, and the toxicity of NMF.

Another strategy used to characterise catalysis by CYP2E1 was the use of a specific antibody. The results clearly demonstrate that coincubation with this antibody inhibited the metabolism of NMF. The most direct approach was that of measuring catalytic activity with the purified isozyme in a reconstituted system. In such studies one should be aware of the possibility of contamination of a CYP2E1 preparation with a small amount of another form of cytochrome P-450 possessing high activity. This could lead to the false identification of an activity being associated with CYP2E1. It is possible to avoid this complication by expression of the enzyme in either bacterial or mammalian cells where CYP2E1 is the only isozyme expressed (Umeno *et al*, 1988a). However, from the multiple experimental approach in this investigation it would appear that NMF is metabolically activated by CYP2E1 in rodents.

In section 4.4 the results of a study into the metabolism of NMF by human liver microsomes is outlined. Here again a specific inhibitory antibody was utilised to demonstrate a reduction in the formation of SMG from NMF. This experiment also served to demonstrate the similarity of CYP2E1 across species. NMF metabolism was inhibited maximally by anti-rat CYP2E1 IgG to a similar extent in incubations with both rat and human liver microsomes.

An additional strategy was utilised to demonstrate the involvement of human CYP2E1. It involved the correlation of the activity under investigation with the metabolism of another substrate for which CYP2E1 has been shown to be the principle catalyst. In the study described in this thesis chlorzoxazone 6-hydroxylation was chosen. In such correlation studies the concentration of microsomal CYP2E1 should ideally vary over as wide a range as possible. This range is easily achieved in animal models where doses of inducers can be

manipulated, but this is not possible in humans. The absolute concentration of CYP2E1 need not be established, and as has been seen in section 4.4, the relative concentrations can be easily determined by immunoblot analysis of microsomal samples. NMF metabolism correlated with both the relative amount of immunoblottable CYP2E1 and chlorzoxazone 6-hydroxylation. Chlorzoxazone is an approved drug and not toxic at low doses (Peter *et al*, 1990). It therefore has a major advantage over other metabolic markers for CYP2E1 such as PNP or NDMA. This compound has the potential to be used as a non-invasive marker of CYP2E1 activity in animals and humans.

From all the results summarised above one can conclude that CYP2E1 participates in the metabolism of NMF. NMF may therefore be included in the increasing list of substrates for this P-450 isoenzyme. As more substrates for CYP2E1 are identified a greater understanding of the substrate structure requirements of this isozyme is achieved. A knowledge of the conditions under which CYP2E1 is induced or inhibited helps to explain, at least in part, many previously observed interactions between chemicals, for example, the potentiation of carbon tetrachloride toxicity by alcohol. Involvement of CYP2E1 in NMF metabolism may also help to explain the variation observed in the toxicity of the compound when it was given to patients in the clinical trials (McVie *et al*, 1984, Eisenhauer *et al*, 1986). One of the side effects reported was alcohol intolerance similar to the disulfiram response. This effect could be attributed to NMF competitively inhibiting acetaldehyde metabolism, since acetaldehyde has recently been identified as a substrate of CYP2E1 (Terelius *et al*, 1991). Patients also varied in their susceptibility towards hepatotoxicity as measured by liver function tests. Such toxicity, which may now be attributed to CYP2E1 activity, resulted in the termination of any further clinical trials.

Probably more important than the toxicity of NMF is that of DMF, which is used extensively as an industrial solvent. In 1990 a NIOSH Alert was published by the US department of Health and Human Services concerning the health risks to workers exposed to DMF (NIOSH, 1990). In the past 10 years there have been a number of reports of adverse health effects of DMF following occupational exposure (Potter, 1973, Redlich, 1988). Recently AMCC was confirmed to be present in the urine of humans and rodents after exposure to

DMF (Mraz and Turecek, 1987). This finding indicated that one metabolic route for DMF may involve bioactivation to form MIC, with subsequent GSH conjugation, as is believed to occur with NMF.

In section 4.5 the possible involvement of CYP2E1 in DMF metabolism was investigated. The results obtained allow the following conclusions to be made: CYP2E1 is responsible for catalysing the metabolism of DMF to HMMF; DMF is a potent inhibitor of formamide formyl oxidation, which is catalysed by CYP2E1. These conclusions are the result of findings according to which pretreatment of rats with acetone increased DMF metabolism, whilst coincubations with DEDTC decreased the metabolic formation of HMMF from DMF. DMF metabolism was catalysed by purified rat liver CYP2E1 and it was dramatically inhibited in rat and human liver microsomes by the anti-rat CYP2E1 antibody. In preliminary experiments it was observed that DMF competes with the CYP2E1 substrate NMF for the active site of the enzyme, therefore the metabolism of NMF to SMG was inhibited. The ability of DMF to inhibit the CYP2E1-mediated metabolism of its own metabolites is consistent with the observation that DMF blocks the N-demethylation of NDMA, another CYP2E1 substrate (Yang *et al*, 1991). The k_i reported in that paper, 0.07mM, is close to that measured in recent work in our laboratory for the inhibition by DMF on NMF formyl oxidation (k_i = 0.11mM) (Mraz *et al*, 1992).The metabolic generation of HMMF from DMF in rat liver microsomes is characterised by a k_m of 0.2mM, whilst formation of SMG from NMF has a k_m of 4.28mM (Mraz *et al*, 1992). Therefore DMF is a better substrate for the isozyme than NMF and hence is a stronger inhibitor.

It is not surprising that DMF has been confirmed to be a CYP2E1 substrate. The molecule is isoelectronic with NDMA which is often used as a specific metabolic marker of CYP2E1. Recently Lucas *et al* (1990) concluded that of 12 different activities the demethylation of NDMA was one of the best indicators of CYP2E1 induction. What is perhaps of more interest is the ability of DMF to inhibit the CYP2E1-mediated metabolism of its own metabolites (see section 4.5). This result provides an explanation for why *in vitro* studies on the metabolism of DMF have failed to yield measurable amounts of SMG. It also explains why the start of urinary excretion of AMCC after exposure to DMF *in vivo* is delayed. An

occupational corollary of this conclusion is the possibility that manifestations of detrimental effects on the health of workers after over-exposure to DMF may develop well beyond the end of the work shift. Inhibition of metabolism of NMF by DMF also rationalises the observation that DMF decreased the severity and postponed the onset of NMF-induced hepatotoxicity measured by elevated SDH levels in rats (Lundberg *et al*, 1981).

In vivo studies into the metabolism of DMF have demonstrated here for the first time the formation of SMG as a biliary metabolite. SMG was found in bile of rats 24-28 hr after a dose of 500mg/kg DMF. This finding is significant since SMG is believed to be formed as a result of the conjugation of GSH with the toxic intermediate MIC. The delayed excretion of SMG in bile correlates well with the delayed excretion of AMCC in urine of similarly treated rats, further suggesting, as has been found to be the case with NMF, that SMG is the biliary precursor of urinary AMCC. There have been few reports of the effect of DMF on hepatic GSH; Imazu *et al* (1992) have shown that after a single dose of DMF the GSH pool in the liver is depleted, suggesting that GSH conjugation is involved.

Originally DMF was thought to undergo metabolism to NMF, which was regarded to be the hepatotoxic species. However it is now known that the metabolite of DMF which previously was thought to be NMF is actually HMMF, which accounts for up to 50% of a dose of DMF in rats (Scailteur and Lauwerys, 1984). The measurement of urinary levels of this metabolite has been used as a means of assessing human exposure to DMF in a number of studies in volunteers and in the work place (Kimmerle and Eben, 1975b, Yonemoto and Suzuki, 1980). It has previously been shown that the hepatotoxic properties of N-alkylformamides are associated with metabolism to N-acetyl-S-(N-alkylcarbamoyl)cysteine. DMF is also metabolised by this route, a pathway which appears to be more important in humans than rodents (Mraz *et al*, 1989). In the light of the identification of SMG as a biliary metabolite of DMF it would therefore appear that the monitoring of HMMF is second best and a more accurate indicator of the hepatotoxic risk to humans exposed to DMF might be the biomonitoring of AMCC in the urine.

The findings outlined in section 4.6 that DMF metabolism to SMG is catalysed by CYP2E1 has several implications. Humans exposed to DMF may run an increased risk of experiencing liver damage under conditions which may cause induction of CYP2E1, such as fasting, diabetes and alcohol consumption. There have also been a number of reports of incompatibilities when ethanol and DMF are present together, where DMF causes symptoms comparable to those observed when disulfiram is taken with alcohol. However DMF unlike disulfiram, has not been shown to inhibit alcohol dehydrogenase. As has previously been discussed, the interaction responsible for the incompatibility between DMF and alcohol may occur at the level of hepatic CYP2E1. DMF itself might increase the levels of acetaldehyde via inhibition of CYP2E1. These speculations and their relevance to the mechanisms of the adverse effects of DMF need further verification.

A vexing question associated with the link between DMF toxicity and its metabolic conversion to N-methylcarbamic thioesters has been whether HMMF is a substrate for the formyl-oxidising microsomal enzyme, or if production of SMG from DMF requires the intermediacy of NMF. NMF is only a quantitatively minute urinary metabolite of DMF, whereas its relatively stable N-hydroxymethyl precursor, HMMF, is the major biotransformation product in the urine (Kestell *et al*, 1986b, Brindley, 1983b). However, it has recently been shown in the Dupont-Haskell Toxicology laboratories that following administration of DMF, both NMF and HMMF were found in comparable quantities in the plasma of rat, mouse and monkey. In the urine HMMF was still the major metabolite whilst NMF was present only in traces. It is possible that the NMF present in the plasma is generated either by a separate metabolic pathway to that which catalyses HMMF formation, or as a result of further metabolism of HMMF, either hepatically or extra-hepatically. Since NMF is present only at minute levels in the urine it would appear that it is rapidly metabolised to other metabolites. It remains to be clarified whether NMF or HMMF is the immediate precursor of SMG and AMCC formed from DMF *in vivo*.

The results outlined above help to establish more strongly the link between metabolism and toxicity of NMF and DMF. They together with further studies will eventually supply the basis for rational advice as to the occupational safety of this class of compounds. At present,

it may be prudent to minimise the occupational use of N-alkylformamides as they undergo fairly rapid CYP2E1-mediated hepatic metabolism to poisonous intermediates, probably alkylisocyanates. In contrast, N-methylacetamide is metabolised solely by N-methyl oxidation, almost certainly a detoxification pathway (Kestell *et al*, 1987). Therefore N-methylacetamide or N,N-dimethylacetamide might be occupationally safer than the related formamides.

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APPENDIX

Publications

Abstracts

A1 Hyland, R. and Gescher, A. (1990)
Characterization of the cytochrome P-450 isozyme which catalyses the oxidation of formamides. 16th Annual Open Meeting, Drug Metabolism Discussion Group, York. 20th-23rd Sept.

A2 Hyland, R., Jheeta, P., Mynett, K. and Gescher, A. (1991)
The role of CYP2E1 in the toxicity of N-methylformamide. The British Toxicology Meeting, Edinburgh. 18th-20th Sept.

A3 Hyland, R., Thummel, K., Smith, A. and Gescher, A. (1992)
Formamides- A new class of substrate of the cytochrome P-450 isoenzyme CYP2E1. Society Of Toxicology, 31st Annual Meeting, Seattle, Wa. 23rd-27th Feb.

A4 Hyland, R., Mraz, J., Davis, M.R., Baillie, T.A., Jheeta, P. and Gescher, A. (1992) Characterization of the metabolic toxification of N,N-dimethylformamide. Gordon Research Conference on Drug Metabolism, Plymouth, NH. 12th-17th July.

Papers

P1 Cross, H., Dayal, R., Hyland, R. and Gescher, A. (1990) N-alkylformamides are metabolized to N-alkylcarbamoylating species by hepatic microsomes from rodents and humans. Chem. Res. Toxicol. 3, 357-362.

P2 Hyland, R., Gescher, A., Thummel, K., Schiller, C., Jheeta, P., Mynett, K., Smith, A.W. and Mraz, J. (1992) Metabolic oxidation and toxification of N-methylformamide catalyzed by the cytochrome P450 isoenzyme CYP2E1. Mol. Pharmacol. 41, 259-266.

P3 Mraz, J., Hyland, R., Jheeta, P., Gescher, A. and Thummel, K. (1992)
Investigation of the mechanistic basis of N,N-dimethylformamide toxicity. I: characterization of N,N-dimethylformamide metabolism and its autoinhibition in rat and human liver microsomes. Chem. Res. Toxicol. In Press.

CHARACTERISATION OF THE CYTOCHROME P-450 ISOZYME WHICH CATALYSES THE OXIDATION OF FORMAMIDES

Pages 192 - 231

Therapeutics, 1987; 241: 265-270

Liver Unit, Queen Elizabeth Hospital, and Dr. J.K. Chipman, School of Biochemistry (both University of Birmingham, U.K.), for access to human liver samples, Dr. R.P. Hanzlik (University of Kansas) for provision of N-trideuteromethyl-N-methylamine hydrochloride and for guidance with the interpretation of some results, Drs. T.A. Baillie and W. Trager (School of Pharmacy, University of Washington) for stimulating discussions, and Mr. G.H. Smith for help with the figures.

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Table I. Kinetic Parameters for the Metabolic Oxidation of Formamides in Rat and Human Liver Microsomes

substrate	product	app.K_m (mM)		app.V_{max} [nmol·(mg microsomal protein)$^{-1}$·min^{-1}	
		rat	human	rat	human
DMF[a]	HMMF	0.20±0.06[b,c]	0.12±0.06[d]	0.54±0.20[e]	0.57±0.49[e]
NMF[f]	SMG	4.28±1.35[g]	3.92±2.11[h]	0.34±0.08[g]	0.24±0.17[i]
HMMF[f]	SMG	2.52±0.34[k]	1.25	0.016±0.005[k]	0.033

[a]Substrate concentration range: 0.02-5mM. [b]Values are the mean±SD. Details of metabolite analysis and calculation of kinetic constants are described under "Experimental Procedures". [c]Number of separate experiments: 12. [d]Number of separate experiments: 4. Individual values: 0.19, 0.14, 0.06, 0.09mM. [e]Number of separate experiments: 4. Individual values: 0.23, 1.25, 0.61, 0.19nmol HMMF·(mg microsomal protein)$^{-1}$·min^{-1}. [f]Substrate concentration range: 0.4-10mM. [g]Number of separate experiments: 10. [h]Number of separate experiments: 4. Individual values: 6.73, 3.78, 3.55, 1.63mM. [i]Number of separate experiments: 4. Individual values: 0.10, 0.46, 0.28, 0.09nmol SMG·(mg microsomal protein)$^{-1}$·min^{-1}. [k]Number of individual experiments: 4.

Table II. Kinetic Parameters of the Oxidation of DMF and Its Isotopomers in Rat Liver Microsomes

substrate	concentration range (mM)	app.K_m (mM)	$V_{max isotopomer}/V_{max DMF}$ [a]	n [b]
DMF	0.02-5	0.21±0.04 [c,d]	-	12
[$^2H_1$]DMF	0.02-5	0.14±0.06	1.04±0.39	6
[$^2H_6$]DMF	0.02-5	0.21±0.04	0.46±0.08 [e]	6
[$^2H_7$]DMF	0.02-0.2	0.15±0.06	0.20±0.08 [e]	4
	0.2-5	0.76±0.30	0.61±0.11	6

[a]The variability for the V_{max} values between experiments was considerable, therefore the ratio $V_{max isotopomer}/V_{max DMF}$ was calculated in each individual experiment. The values shown are the mean±SD of these ratios. The apparent V_{max} for DMF was 0.54±0.20 nmol HMMF·(mg protein)$^{-1}$·min^{-1}. [b]Number of experiments. [c]Values are the mean±SD. Details of metabolite analysis and calculation of kinetic constants are described under "Experimental Procedures". [d]The difference between the K_m values for the high affinity oxidations is not significant ($P>0.05$). [e]Significantly different from the ratio for [$^2H_1$]DMF ($P<0.05$).

Table III. Intermolecular Kinetic Isotope Effect on DMF N-Methyl Oxidation

substrate conc. (mM)		KDIE[a]
DMF	$[^2H_6]$DMF	
2.5	2.5	5.8±0.6
0.5	0.5	4.6±0.2
1	4	5.2±0.1
0.2	0.8	4.3±0.2

[a]Ratio of formation of HMMF to $[^2H_5]$HMMF. Details of metabolite analysis are described under "Experimental Procedures". Each value is the mean±SD of 4 separate experiments. Mean KDIE±SD of the mean values shown is 5.0±0.7.

Table IV. Intramolecular Kinetic Isotope Effect on DMF N-Methyl Oxidation

[$^2H_3$]DMF conc. (mM)	KDIE[a]
5	5.7±0.4
1	5.0±0.5
0.2	4.9±0.2

[a]Ratio of formation of [$^2H_3$]HMMF to [$^2H_2$]HMMF. Details of metabolite analysis are described under "Experimental Procedures". Each value is the mean±SD of 4 separate experiments. Mean KDIE±SD of the mean values shown is 5.2±0.4.

Table V. Inhibition of Metabolic Generation of SMG from NMF in Rat Liver Microsomes by Low-Molecular Weight Amides

inhibitor $R_1R_2NCOR_3$			app. K_i (mM)[a]	n[b]
R_1	R_2	R_3		
H	H	H	44±7	4
C_2H_5	H	H	0.34±0.09	3
CH_3	CH_3	H	0.11±0.02	9
C_2H_5	C_2H_5	H	0.054±0.027	4
C_4H_9	C_4H_9	H	0.56±0.14	4
H	H	CH_3	158±11	3
CH_3	H	CH_3	6.52±1.16	3
CH_3	CH_3	CH_3	0.25±0.04	3
C_2H_5	C_2H_5	CH_3	2.51±0.33	3

[a]Details of metabolite analysis and calculation of apparent Ki values are described under "Experimental Procedures". Values are the mean±SD. [b]Number of separate experiments, each of which consisted of 6 to 8 individual incubations.

Figure Legends

Figure 1. Metabolism of DMF and NMF. The methyl isocyanate ion in brackets is a postulated intermediate. "GS-" denotes glutathionyl.

Figure 2. Metabolic oxidation of DMF isotopomers [$^2H_6$]DMF and [$^2H_7$]DMF (**A**), [$^2H_3$]DMF (**B**) and [$^2H_1$]DMF (**C**).

Figure 3. HPLC separation of HMMF (retention time 3.9min) from NMF (retention time 3.4min) (top trace) and formaldehyde (bottom trace). Formaldehyde content in the fractions of the HPLC elueate was determined using the colorimetric method of Nash (*24*). Values on the y-axis are arbitrary.

Figure 4. GLC separation of NMF and [$^2H_3$]NMF in extracts of incubates of DMF and [$^2H_6$]DMF with rat liver microsomes. (i), (ii) and (iii) denote peaks with the retention times of DMF/[$^2H_6$]DMF, [$^2H_3$]NMF and NMF, respectively. Note that NMF and [$^2H_3$]NMF are the products of of hydrolysis of HMMF and [$^2H_5$]HMMF, respectively, generated during sample preparation. For details of incubation and metabolite analysis see "Experimental Procedures".

Figure 5. Effect of pretreatment of rats with acetone (**A**), and of coincubation of microsomes with DEDTC (**B**) on the metabolism of DMF (10mM) to HMMF in supensions of rat liver microsomes. The closed and crossed bars in **A** represent metabolism in microsomes from control and acetone-pretreated rats, respectively. Numbers in the bars in **B** indicate DEDTC concentrations. For details of incubation and metabolite analysis see "Experimental Procedures". Values are the mean±SD of 4 experiments.

Figure 6. Correlation of apparent V_{max} of metabolism of NMF with that of DMF in microsomes from 4 human livers. The V_{max} values used are the ones which describe the enzyme with high affinity for NMF and DMF. For details of incubation and analysis of metabolites see "Experimental Procedures". Standard linear regression analysis gave a correlation coefficient of r=0.99.

Figure 7. Effect of anti-rat P450 2E1 IgG (closed circles) or preimmune IgG (open squares) on the metabolism of DMF (10mM) in human liver microsomes. Values, which are expressed as percentage of the rate of metabolic production of HMMF from DMF in incubations without IgG, are the mean of two individual microsomal preparations, each conducted in duplicate. Details of incubation conditions and metabolite analysis are described under "Experimental Procedures".

Figure 8. Eadie-Hofstee plot of generation of HMMF from DMF in incubates of rat liver microsomes. The individual values are the mean of 2 incubations. They were obtained with substrate concentrations ranging from 0.02 to 100mM. The plot shown is representative of 4 experiments. Symbols "v" and "s" represent reaction rate and substrate concentration, respectively. For details of incubation and metabolite analysis see "Experimental Procedures", and for the mean apparent K_m and V_{max} values see Table I.

Figure 9. Eadie-Hofstee plot of metabolic generation of SMG from NMF in incubations of rat liver microsomes. The individual values are the mean of 2 incubations. They were obtained with substrate concentrations ranging from 0.2 to 200mM. The plot shown is representative of 9 experiments. Symbols "v" and "s" represent reaction rate and substrate concentration, respectively. For details of incubation and metabolite analysis see "Experimental Procedures". For the mean apparent K_m and V_{max} values see Table I.

Figure 10. Eadie-Hofstee plots of generation of HMMF, [$^2H_1$]HMMF, [$^2H_5$]HMMF [$^2H_6$]HMMF from DMF, [$^2H_1$]DMF, [$^2H_6$]DMF and [$^2H_7$]DMF, respectively, in incubations of rat liver microsomes. The individual values were obtained with substrate concentrations ranging from 0.02 to 5mM, and they are the mean of 2 incubates. The plots shown are representative of 4 experiments each. Symbols "v" and "s" represent reaction rate and substrate concentration, respectively. For details of incubation and metabolite analysis see "Experimental Procedures", and for the mean apparent K_m and V_{max} values computed from the

4 different plots see Table II.

Figure 11. Eadie-Hofstee plot of metabolic generation of SMG from NMF in the absence (closed circles) and presence of DMF (open squares) in incubations of rat liver microsomes. The individual values are the mean of 2 incubations. They were obtained with substrate (NMF) concentration ranging from 0.4 to 10mM and inhibitor (DMF) concentrations of 0.1mM. The plot shown is representative of 3 experiments. Symbols "v" and "s" represent reaction rate and substrate concentration, respectively. For details of incubation and metabolite analysis see "Experimental Procedures". The apparent K_i value for the inhibition of NMF oxidation by DMF computed from this experiment is 0.08mM.

Fig. 1

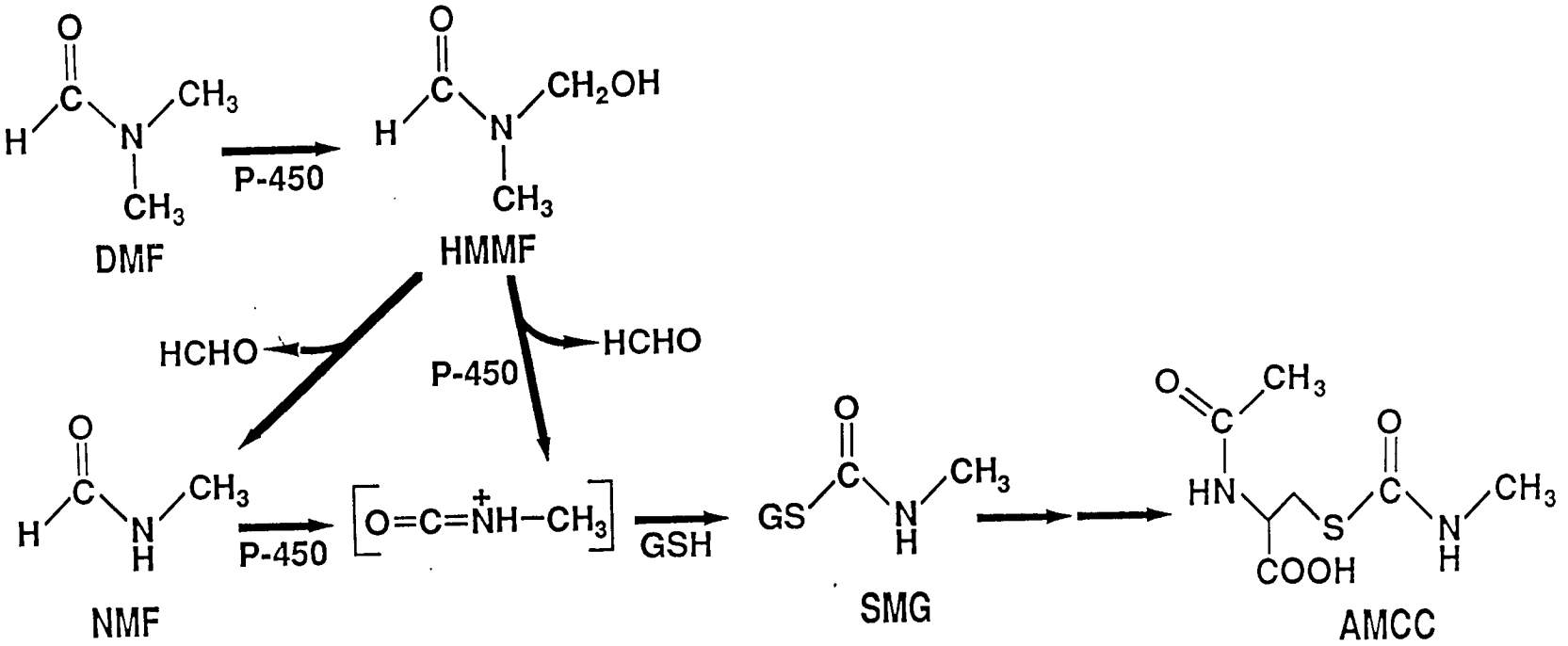
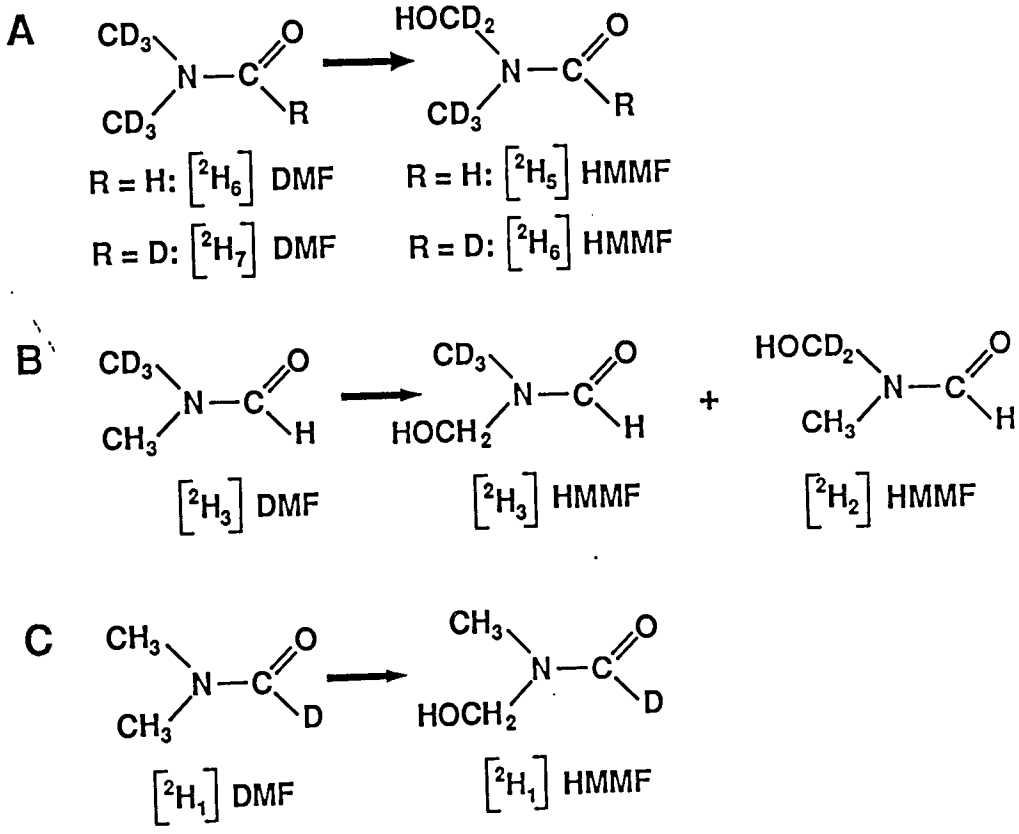


Fig. 2


A
CD3
N
C
O
R
CD3
HOCD2
N
C
O
R
CD3
R = H: [2H6] DMF
R = D: [2H7] DMF
R = H: [2H5] HMMF
R = D: [2H6] HMMF
B
CD3
N
C
O
H
CH3
[2H3] DMF
CD3
N
C
O
H
HOCH2
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+
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C
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H
CH3
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C
CH3
N
C
O
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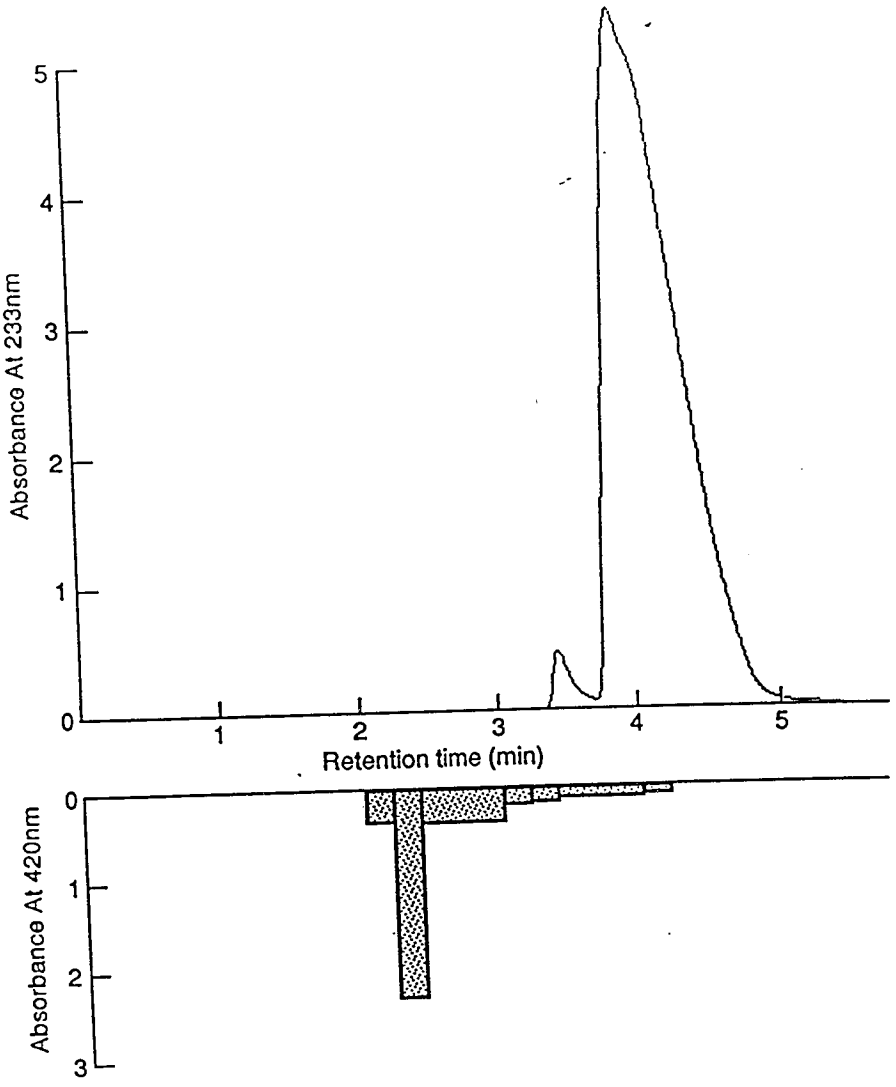
N
C
O
D
HOCH2
[2H1] HMMF

D58A02

Fig. 3


Absorbance At 233nm
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4
3
2
1
0
1
2
3
4
5
Retention time (min)
Absorbance At 420nm
0
1
2
3

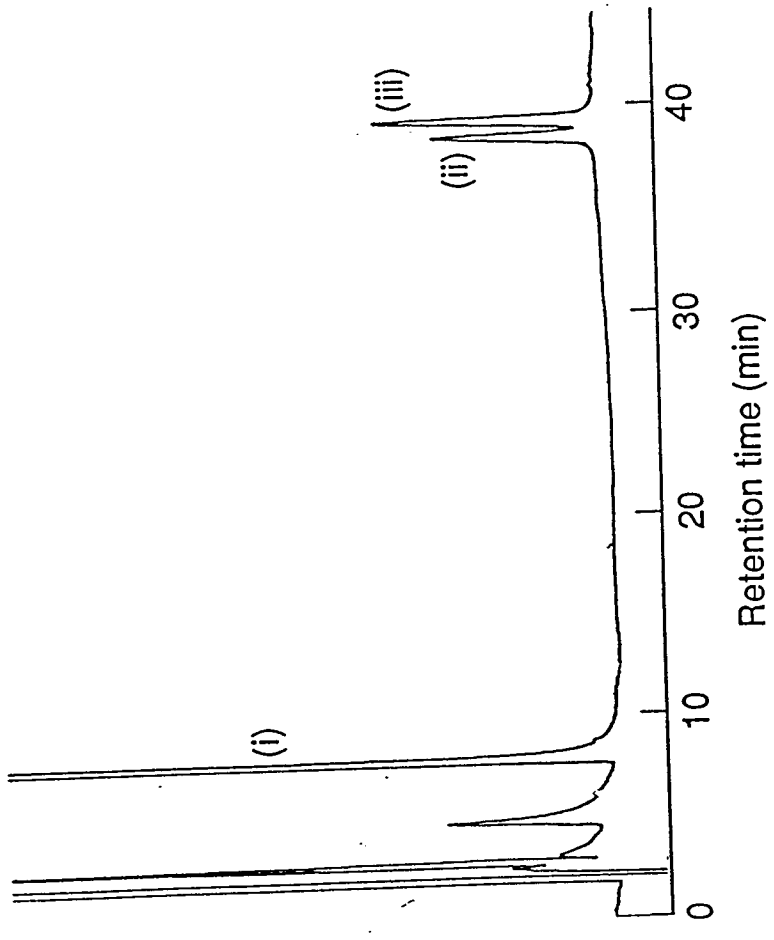


Fig. 4.

D55AG1

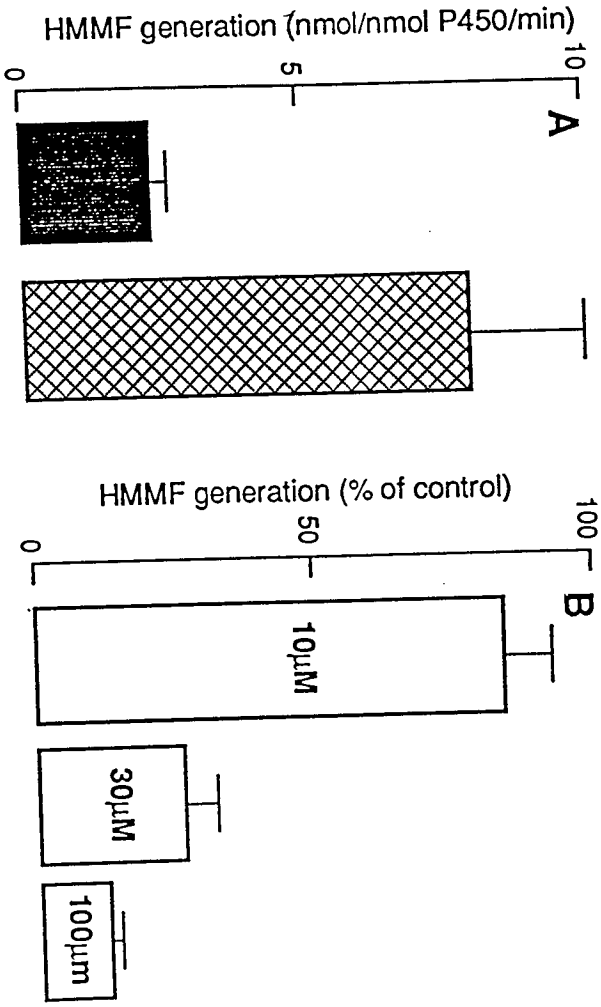
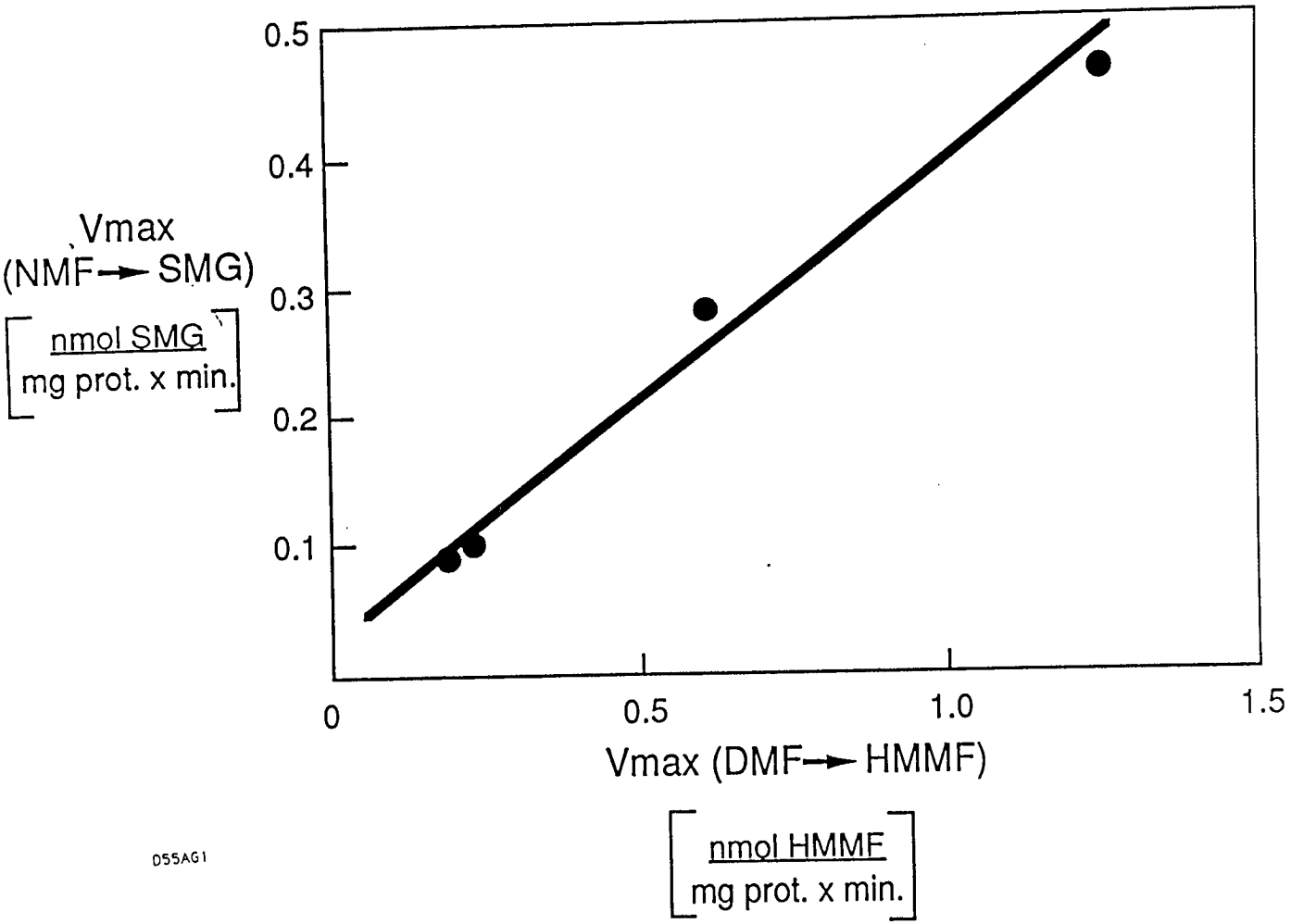

HMMF generation (nmol/nmol P450/min)
0
5
10
A
HMMF generation (% of control)
0
50
100
B
10μM
30μM
100μM

Fig. 5

Fig. 6


0.5
0.4
0.3
0.2
0.1
0
0.5
1.0
1.5
Vmax
(NMF → SMG)
nmol SMG
mg prot. x min.
Vmax (DMF → HMMF)
nmol HMMF
mg prot. x min.

D55AG1

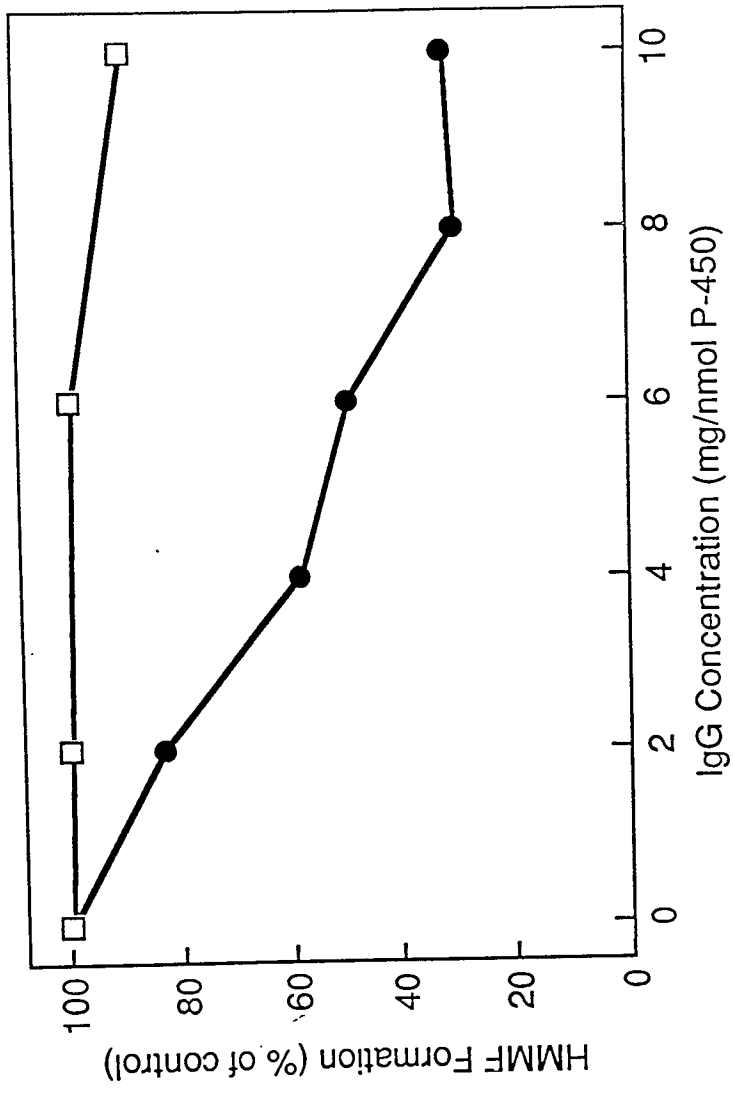

HMMF Formation (% of control)
100
80
60
40
20
0
0
2
4
6
8
10
IgG Concentration (mg/nmol P-450)

Fig. 7

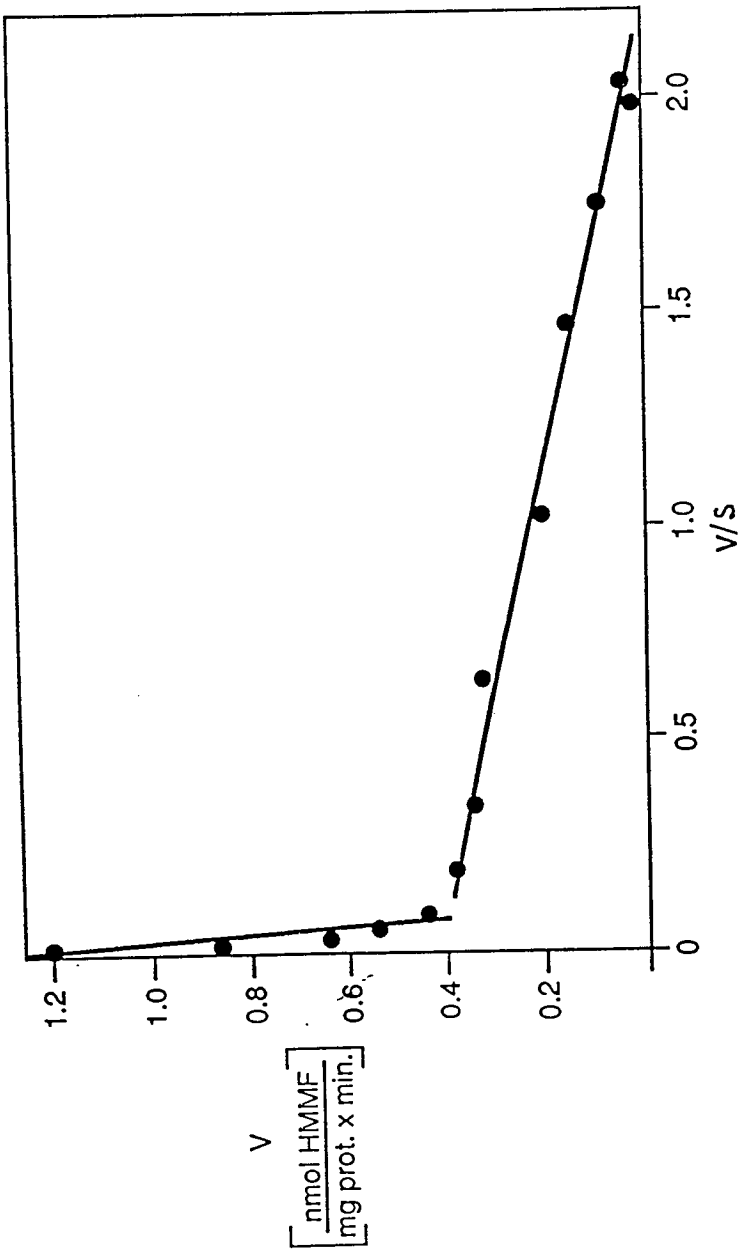
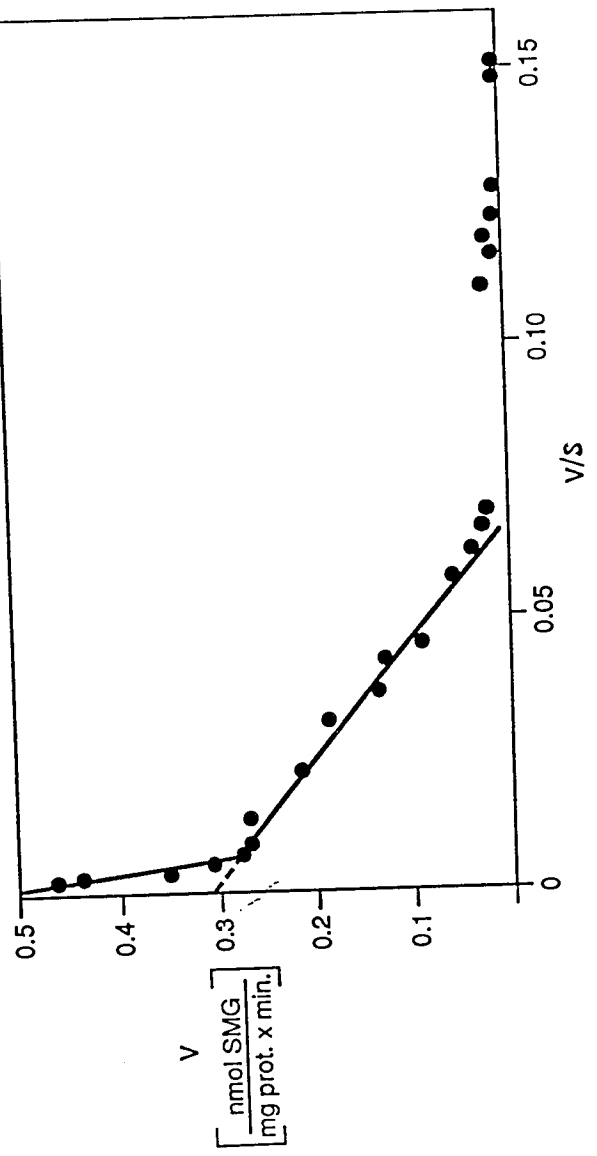


Fig. 8

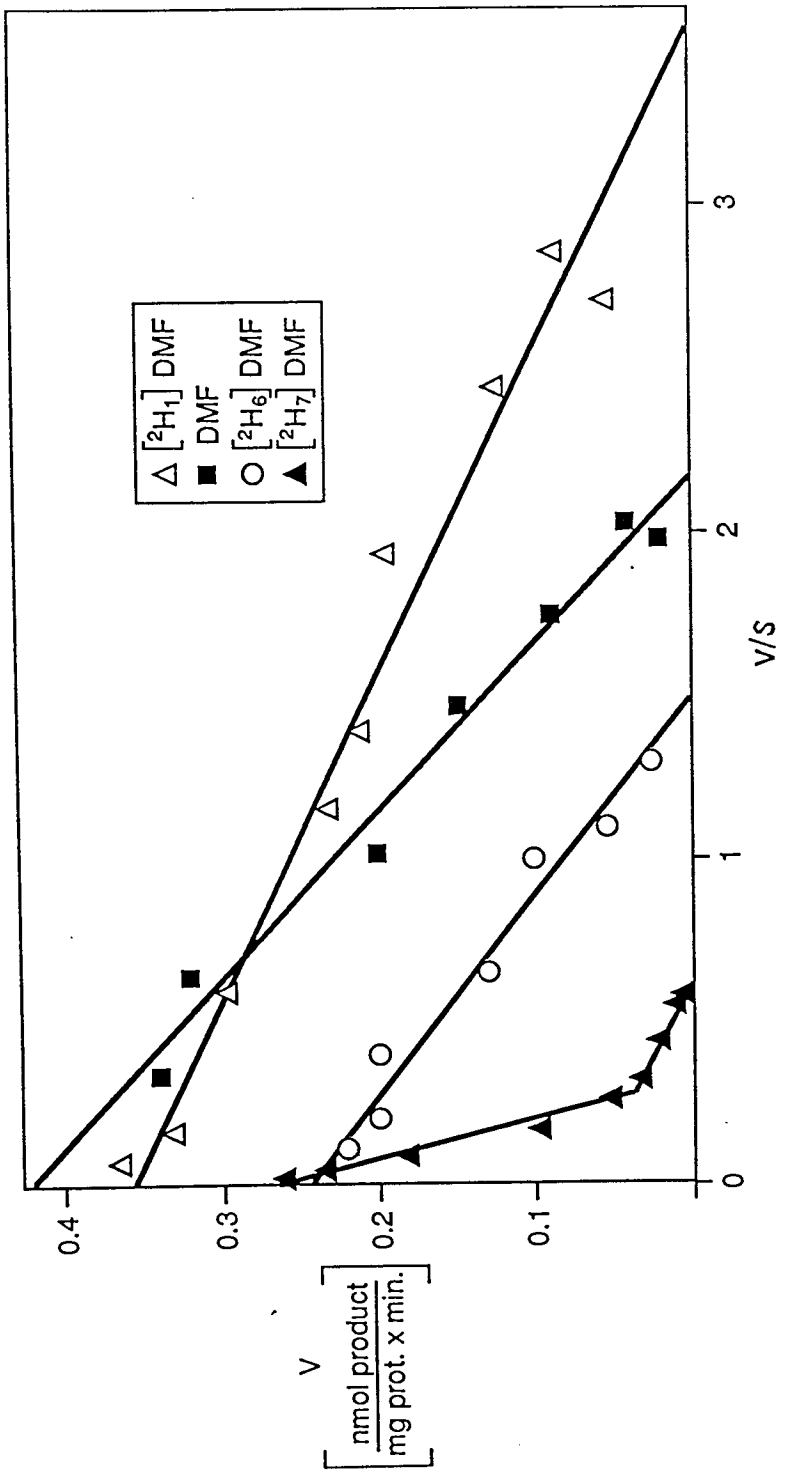
D55AG1

Fig. 9



D55AG1

Fig. 10



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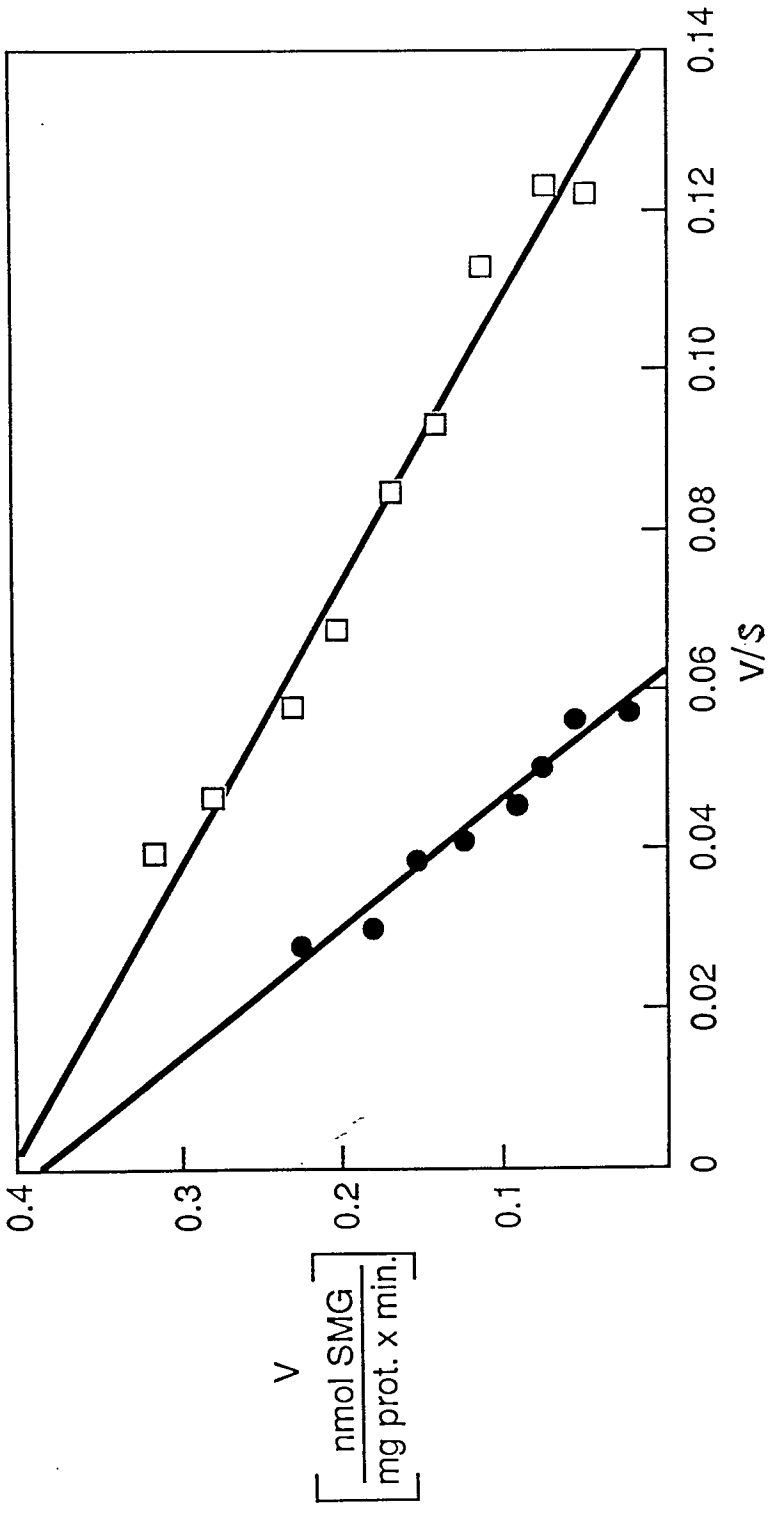

Fig. 11