# STUDIES OF THE METABOLISM AND THE TOXICITY OF N-METHYLFORMAMIDE AND RELATED AMIDES

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# Studies of the metabolism and the toxicity of N-methylformamide and related amides

by

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The hepatotoxicity of the industrial solvent and investigational antitumour agent N-methylformamide (NMF, $HOCNHCH_3$) and several structural analogues was assessed in mice. NMF and its ethyl analogue (NEF) were equipotent hepatotoxins causing extensive centrilobular necrosis and damage to the gall bladder. Pretreatment of mice with SKF525A did not influence the toxicity of these N-alkylformamides. Replacement of the formyl hydrogen of NMF with deuterium or methyl significantly reduced its hepatotoxicity.

An *in vitro* model for the study of the toxicity and metabolism of N-alkylformamides was developed using isolated mouse hepatocytes. The cytotoxicity of NMF *in vitro* was concentration-dependent with maximal toxicity being achieved at concentrations of 5mM or above. The cytotoxic potential of related amides correlated well with their *in vivo* hepatotoxic potential. Pretreatment of mice with buthionine sulphoximine (BSO), which depleted hepatocytic levels of glutathione to 15% of control values, exacerbated the cytotoxicity of NMF towards the hepatocytes.

NMF (1mM or above), incubated with isolated mouse hepatocytes, depleted intracellular glutathione levels to 26% of control values within 4h. Depletion of glutathione was quantitatively matched by the formation of a carbamoylating metabolite. Metabolism was dependent on the concentration of NMF and was drastically reduced in incubations of hepatocytes isolated from mice pretreated with BSO. The carbamoylating metabolite, S-(N-methylcarbamoyl)glutathione (SMG), was identified *in vitro* using FAB-MS. The generation of SMG was subject to a large primary kinetic H/D isotope effect when the formyl hydrogen was replaced with deuterium. Likewise, glutathione depletion and metabolite formation were reduced or abolished by the deuteration or methylation of the formyl moiety of NMF. NEF, like NMF, depleted hepatocytic glutathione levels and was metabolised to a carbamoylating metabolite.

Radioactivity derived from $^{14}C$-NMF and $^{14}C$-NEF, labelled in the alkyl moieties, was found to be irreversibly associated with microsomal protein on incubation *in vitro*. Binding was dependent on the presence of NADPH and was mostly abolished in the presence of reduced glutathione. SKF525A failed to influence the binding.

Keywords: N-alkylformamide hepatotoxicity, electrophilic metabolite, glutathione conjugation, covalent binding, isolated hepatocytes.

TO MY PARENTS

AND

SUSAN

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## Abbreviations

| | |
|---|---|
| ALT | alanine aminotransferase |
| APAP | N-acetyl-p-aminophenol, paracetamol, acetaminophen |
| AST | aspartate aminotransferase |
| BCF | benzylchloroformate |
| BCF-SMG | N-benzyloxycarbonyl derivative of SMG |
| BCF-SMG-E | N-benzyloxycarbonyl dimethylester derivative of SMG |
| BSA | bovine serum albumin |
| BSO | DL-buthionine-[S,R]-sulphoximine |
| BSTFA | bis(trimethylsilyl)-trifluoroacetamide |
| $^{14}$C-NMF | N-methylformamide labelled with $^{14}$C in either the formyl or methyl moiety |
| $^{13}C_2{}^{15}$N-NMF | N-methylformamide labelled with $^{13}$C and $^{15}$N ($HO^{13}C^{15}NH^{13}CH_3$) |
| cpm | counts per minute |
| DEM | diethylmaleate |
| DMF | N,N-dimethylformamide |
| dpm | disintergrations per minute |
| DTC | diethyldithiocarbamate |
| DTNB | 5,5'-dithiobis-(2-nitrobenzoic acid) |
| EDTA | ethylenediaminetetraacetic acid |
| EI-MS | electron impact mass spectrometry |
| ethyl-$^{14}$C-NEF | N-ethylformamide labelled with $^{14}$C in the ethyl moiety ($HOCNH^{14}CH_2CH_3$) |
| F | formamide |
| FAB-MS | fast atom bombardment mass spectrometry |

| | |
|---|---|
| formyl-$^{14}$C-NMF | NMF labelled with $^{14}$C in the formyl moiety |
| g | acceleration due to gravity |
| G6P | glucose-6-phosphate |
| G6PDH | glucose-6-phosphate dehydrogenase |
| GC-MS | gas chromatographic mass spectrometry |
| GLC | gas-liquid chromatography |
| GSH | reduced glutathione |
| GSSG | oxidised glutathione, glutathione disulphide |
| h | hours |
| HBSS | Hanks buffered salt solution |
| HEPES | N-2-hydroxyethylpiperazine-N'-2-ethane-sulphonic acid |
| HMA | N-(hydroxymethyl)acetamide |
| HMF | N-(hydroxymethyl)formamide |
| HMMF | N-(hydroxymethyl)-N-methylformamide |
| $^{2}$H-NMF | N-methyldeuteroformamide |
| $^{2}H_3$-NMF | N-(trideuteromethyl)formamide |
| $^{1}$H-NMR | proton nuclear magnetic resonance |
| HPLC | high pressure liquid chromatography |
| i.p. | intraperitoneal |
| i.v. | intravenous |
| LDH | lactate dehydrogenase |
| LSI-MS | liquid secondary ion mass spectrometry |
| methyl-$^{14}$C-NMF | N-methylformamide labelled with $^{14}$C in the methyl moiety |
| MIC | methyl isocyanate |
| min | minutes |

| | |
|---|---|
| MS | mass spectrometry |
| n | number of experiments |
| NAD/NADH | nicotinamide adenine dinucleotide (oxidised/reduced) |
| NADP/NADPH | nicotinamide adenine dinucleotide phosphate (oxidised/reduced) |
| NAPQI | N-acetyl-p-benzoquinoneimine |
| NEF | N-ethylformamide |
| NMA | N-methylacetamide |
| NMF | N-methylformamide |
| p | probability |
| rpm | revolutions per minute |
| s | seconds |
| S.D. | standard deviation |
| SDH | sorbitol dehydrogenase |
| SEC | S-(N-ethylcarbamoyl)cysteine |
| SEG | S-(N-ethylcarbamoyl)glutathione |
| SEM | S-(N-ethylcarbamoyl)mercapturate, N-acetyl-S-(N-ethylcarbamoyl)cysteine |
| SKF525A | proadifen, 2-diethylaminoethyl-2,2-diphenylvalerate |
| SMC | S-(N-methylcarbamoyl)cysteine |
| SMG | S-(N-methylcarbamoyl)glutathione |
| SMM | S-(N-methylcarbamoyl)mercapturate, N-acetyl-S-(N-methylcarbamoyl)cysteine |
| TLC | thin layer chromatography |
| TMS | trimethylsilyl |
| TNB | 5-thio-2-nitrobenzoate |
| tris | tris(hydroxymethyl)methylamine |

u.v. ultraviolet

All other abbreviations refer to SI units.

# SECTION 1

# INTRODUCTION

## 1.1 Metabolism of N-methylformamide (NMF) and structural analogues

The biological properties of NMF (fig 1) have been studied since the 1950's when its antitumour activity was first demonstrated. It now seems clear that the pharmacological and toxicological properties of NMF are linked to its metabolism (see sections 1.2 and 1.3). Therefore in the following, details of the metabolism of NMF and its congeners are reviewed. This short review constitutes the background for the hypotheses which have been tested in the work presented in this thesis.

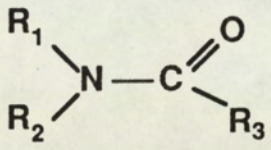
### 1.1.1 NMF

NMF possesses excellent solvent properties and is used in industrial processes (Gescher, 1986b). It is also an experimental antitumour agent. The fate of this N-alkyl-formamide in biological systems has been studied in recent years in order to clarify the relationship between the metabolism of this compound and its toxicological and antitumour activities.

The bioavailability of NMF in mice (Brindley et al, 1982) and man (Brindley et al, 1983b) is high. In mice and rats NMF is metabolised mainly to carbon dioxide, which is exhaled in the breath, and to methylamine ($CH_3NH_2$) which is excreted in the urine (Kestell et al, 1985a, b). NMF is metabolised more extensively in Balb/C mice than in Sprague Dawley rats (Tulip et al, 1985). Of the radiolabel injected

Figure 1

**Structures of NMF and related compounds.**




| Amide | Abbr. | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|---|
| Formamide | F | H | H | H |
| N-Methylformamide | NMF | $CH_3$ | H | H |
| N,N-Dimethylformamide | DMF | $CH_3$ | $CH_3$ | H |
| N-Methylacetamide | NMA | $CH_3$ | H | $CH_3$ |
| N-Ethylformamide | NEF | $C_2H_5$ | H | H |
| N-(Hydroxymethyl)formamide | HMF | $CH_2OH$ | H | H |
| N-(Hydroxymethyl)-N-methyl formamide | HMMF | $CH_2OH$ | $CH_3$ | H |
| N-(Hydroxymethyl)acetamide | HMA | $CH_2OH$ | H | $CH_3$ |
| N-Methyldeuteroformamide | $^2$H-NMF | $CH_3$ | H | D |
| N-(Trideuteromethyl)formamide | $^2H_3$-NMF | $CD_3$ | H | H |

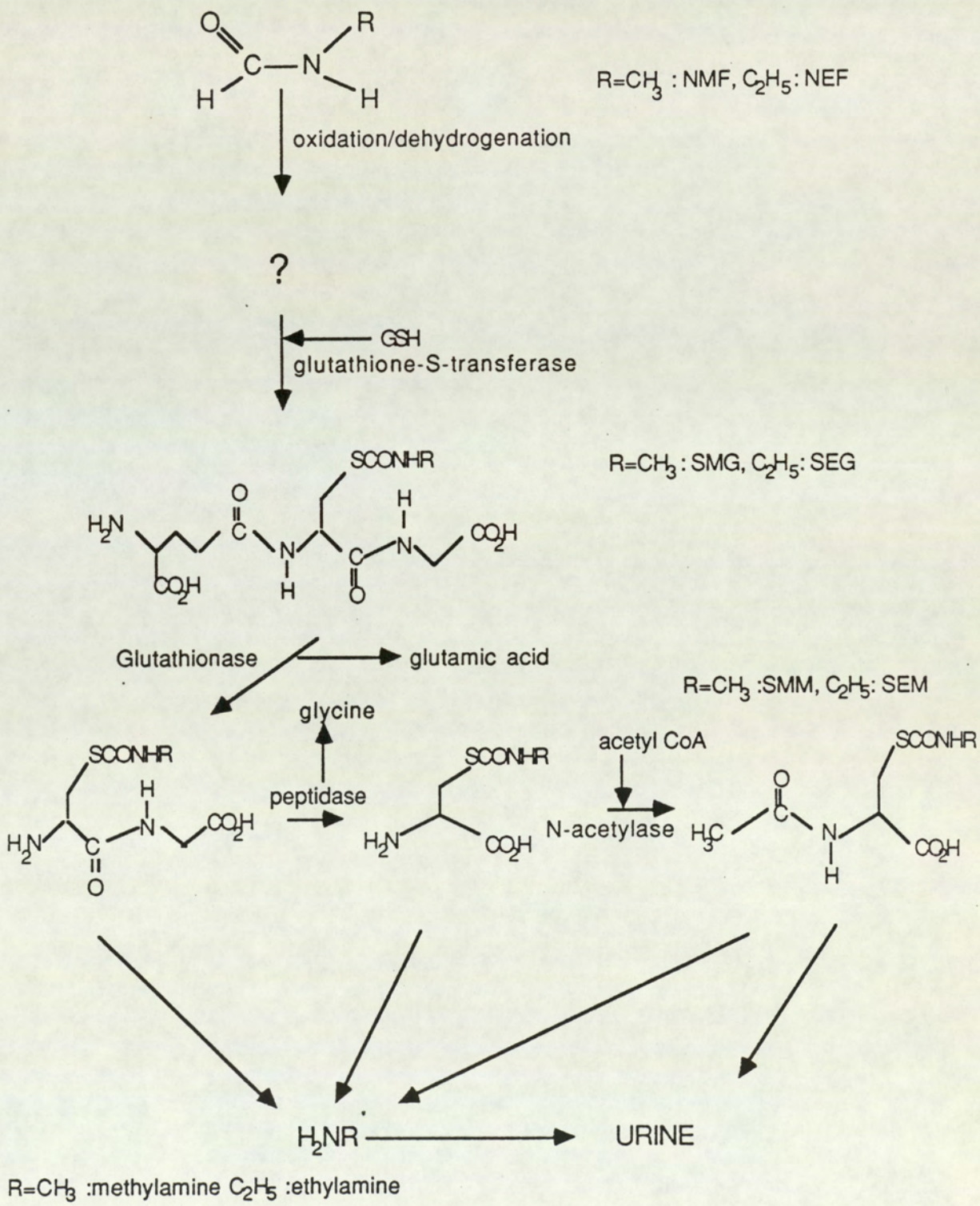
into CBA/CA mice as formyl-$^{14}$C-NMF ($HO^{14}CNHCH_3$) or methyl-$^{14}$C-NMF ($HOCNH^{14}CH_3$), 39% and 14% respectively was exhaled as carbon dioxide. The amount of the drug excreted unchanged with the urine in CBA/CA mice was 26% (Brindley et al, 1982) and 15% of the dose was excreted as methylamine (Kestell et al, 1985a). NMF was not hydrolysed to methylamine and formic acid by liver fractions in vitro (Brindley, 1983). It is therefore improbable that methylamine is generated by hydrolysis from the parent compound in vivo. Formate was not detected in the urine of mice which had received NMF (Kestell et al, 1985a) however it has been reported in the urine of rats after administration of NMF (Tulip et al, 1986).

On GLC analysis of urine samples obtained from mice which had received NMF, formamide (F, fig 1) was detected as a minor metabolite (Brindley et al, 1982). This metabolite was later tentatively identified as N-(hydroxymethyl)-formamide (HMF, fig 1), the immediate product of N-methyl-C-hydroxylation of the parent compound (Kestell et al, 1985a). HMF is thermally labile and gives F and formaldehyde on the GLC column. The extent of metabolism of NMF to HMF in vivo is small and constitutes less than 7% of the dose (Kestell et al, 1985a). All the NMF metabolites are rapidly eliminated into the urine (Brindley et al, 1982).

A further urinary metabolite of NMF, namely N-acetyl-S-(N-methylcarbamoyl)cysteine (SMM, fig 2) was identified by Kestell et al (1986b) in the urine of mice, rats and humans after exposure to NMF. The mercapturate was

Figure 2

**Proposed pathway for the generation of urinary alkylamines and mercapturates of N-alkylformamides.**




References: Threadgill et al (1987), Neal (1980).

characterised by high-field $^1$H-NMR and mass spectrometry by comparison with authentic reference material. The metabolite constituted 13-20% of the administered dose (400mg/kg) given i.p. to CBA/CA mice (Kestell et al, 1985a). More recently S-(N-methylcarbamoyl)glutathione (SMG, fig 2) has been identified using caesium ion liquid secondary ion mass spectrometry (LSIMS) as a biliary metabolite of NMF in Balb/C mice (Threadgill et al, 1987). NMF itself does not react chemically with reduced glutathione (GSH; Kestell et al, 1986b). Metabolism of NMF to urinary methylamine, urinary SMM and biliary SMG was found to be subject to a large primary kinetic deuterium isotope effect when the hydrogen atom was replaced by deuterium in the formyl moiety ($K_H/K_D$ = 5.5 $\pm$ 0.2, 4.5 $\pm$ 1.0 and 7 $\pm$ 2 respectively). This result indicates the existence of a common metabolic precursor of these metabolites. The glutathionyl or N-acetylcysteinyl moieties were found to be linked to the "NMF" molecule solely through the formyl carbon. Threadgill et al (1987) have proposed that glutathione ($\gamma$-glutamyl-cysteinylglycine) is conjugated with a reactive metabolite of NMF in the liver and that the conjugate is further metabolised, presumably in the kidney, to SMM (fig 2). Any of the thiocarbamates generated by this metabolic pathway and their reactive precursor could conceivably be hydrolysed to give the observed metabolic end products, methylamine and carbon dioxide.

Pearson et al (1987a) demonstrated that NMF (200mg/kg) caused a depletion of hepatic glutathione levels to 21% of control levels 2h after drug administration. Similarly

incubation of isolated mouse hepatocytes with NMF (7mM), which was the peak plasma concentration achieved after administration of NMF to mice (Brindley et al, 1982), led to a significant decrease in intracellular glutathione levels without an increase in the levels of glutathione disulphide (GSSG; Whitby et al, 1984b).

Contrary to an earlier report which claimed that NMF (1.7mM) was transformed to formaldehyde by rat liver homogenate (Barnes and Ranta, 1972), recent findings have strongly suggested that NMF does not undergo appreciable metabolism *in vitro*. Gescher et al (1982) failed to detect formaldehyde as a metabolite of NMF or to measure metabolism by the disappearance of the parent compound or by the production of F in either liver preparations or isolated hepatocytes. However, $^{14}$C-NMF was metabolised by Balb/C mouse liver microsomes in the presence of NADPH to a species which was bound covalently to microsomal proteins (Pearson et al, 1985). *In vitro* covalent binding was abolished by the presence of reduced glutathione (GSH, 10mM). Coincubation of microsomes with NMF and SKF525A (proadifen, 0.1mM), an inhibitor of cytochrome P450 monooxygenases, led to covalent binding which was almost four-fold the control level in the case of formyl-$^{14}$C-NMF but was similar to control binding in the case of methyl-$^{14}$C-NMF. *In vitro* binding of $^{14}$C-NMF to microsomes obtained from mice which had been pretreated with phenobarbital, an inducer of cytochrome P450 monooxygenases, was not different to binding observed in microsomes from control mice (Pearson et al, 1987b).

Radioactivity derived from $^{14}$C-NMF was incorporated into or bound to hepatic proteins, and to a much lesser extent to kidney proteins, when the radiolabel was administered i.p. to mice (400mg/kg, Pearson et al, 1984). Association of label was significantly reduced by pretreatment of mice with cycloheximide (2mg/kg), an inhibitor of protein synthesis. This result indicates that NMF may be incorporated to some extent into proteins via endogenous substrates. Depletion of hepatic glutathione by pretreatment of mice with buthionine sulphoximine (BSO, 1600mg/kg) or diethylmaleate (DEM, 0.3ml/kg) prior to administration of formyl-$^{14}$C-NMF enhanced the association of label with hepatic proteins measured 1h after drug injection.

Administration of NMF influences the metabolism of other drugs: Oral administration of 2 or 20mmol/kg NMF to rats 3 or 18h before oral administration of ethanol induced an elevation in blood acetaldehyde levels (Hanasono et al, 1977). In the case of the high dose of NMF administered 18h before alcohol the level of ethanol in the blood was also raised significantly over controls. A single case of NMF-induced alcohol flushing was reported in a cancer patient who drank a glass of wine after a course of NMF treatment (McVie et al, 1984). Administration of NMF on 4 consecutive days enhanced the sleeping time caused by pentobarbital in male rats to 565% of controls (Scailteur et al, 1981). Similar treatment of rats also reduced to a moderate extent the activities of hepatic cytochrome P450 and cytochrome c reductase (Scailteur et al, 1981).

## 1.1.2 N,N-Dimethylformamide (DMF)

The main application of DMF (fig 1) is as a solvent in industrial processes, especially for polar polymers such as polyvinylchloride, polyacrylonitrile and polyurethanes (Gescher, 1986a). The metabolism of DMF has been studied for over a decade primarily in the evaluation of the risk associated with the occupational exposure to this N-alkylformamide.

Urine samples of rodents (Barnes and Ranta, 1972; Kimmerle and Eben, 1975a; Kestell et al, 1987; Mraz et al, 1988), dogs (Kimmerle and Eben, 1975a) and man (Kimmerle and Eben, 1975b; Mraz et al, 1988) which had been exposed to DMF were examined by GLC analysis and NMF and F were detected as well as the parent compound. DMF was extensively metabolised *in vivo* with less than 5% of the administered dose excreted unchanged in the urine (Mraz et al, 1988). DMF, unlike NMF, was metabolised to alkylamines only to a minor extent by CBA/CA mice (Kestell et al, 1987).

The urinary metabolites of DMF identified as NMF and F on the basis of GLC analysis are most likely N-(hydroxymethyl)-N-methylformamide (HMMF, fig 1) and HMF, respectively. HMMF, the product of N-methyl-C-hydroxylation of DMF, and HMF are stable in aqueous solution but are thermally degraded on the GLC column. The evidence that HMMF is the major urinary metabolite of DMF is based on three studies: Brindley et al (1983a) found that a metabolite in the urine of mice which had received DMF liberated formaldehyde only after alkaline hydrolysis. In

aqueous solution, authentic HMMF also decomposed to formaldehyde only on alkaline hydrolysis. Scailteur et al (1984) isolated a urinary metabolite of DMF in rats by HPLC and subjected it to mass spectrometric analysis. The observed fragmentation pattern suggested the presence of HMMF, even though the mass fragments, including the one corresponding to the molecular ion, were also detected in control urine samples. Unequivocal evidence for the contention that HMMF and not NMF is the major metabolite of DMF was recently obtained by high-field $^{1}$H-NMR spectroscopy of urine samples of mice which had received DMF (Kestell et al, 1986a). HMMF exists in two rotameric forms and the methyl and formyl protons in the two rotamers are not equivalent. The resonance frequencies corresponding to the methyl and formyl protons of both rotamers were prominent signals in the NMR spectrum of the urine. However at the resonance frequency of the methyl protons of NMF only a minute signal was observed.

SMM was found as a metabolite of DMF in the urine of rodents, human volunteers and of workers in a factory after exposure to DMF (Mraz and Turecek, 1987; Mraz et al, 1988). The excretion of the metabolite in the urine was monitored by GLC analysis after conversion of SMM to the more stable and volatile N-methylurethane by reaction with ethanol under alkaline conditions. A quantitative difference in the metabolic pathway of DMF to SMM appears to exist between rodents and humans (Mraz et al, 1988). The portion of the dose (0.1, 0.7 or 7.0mmol/kg i.p.) which was metabolised to SMM in mice, rats and hamsters was only 1.1-5.2%. Ten human

volunteers who absorbed between 28 and 60µmol/kg DMF during 8h exposure to DMF vapours (60mg/m³) excreted 9.7-22.8% of the dose as SMM in the urine. SMM was detectable 15-150h after the start of a 6h exposure to airborne DMF. In contrast, SMM was detectable 2-4h after the commencement of exposure to NMF vapours (Mraz and Turecek, 1987). This indicates that the reactive metabolite of DMF which is conjugated with GSH may be generated via NMF as an intermediate.

N-Methyl-C-hydroxylation of DMF occurred at an exceedingly slow rate when DMF was incubated with liver fractions (Brindley et al, 1983a; Scailteur and Lauwerys, 1984a). It has been suggested that the metabolism of DMF to HMMF *in vitro* is mediated, at least in part, by hydroxyl radicals and/or hydrogen peroxide, as this metabolic route measured in rat liver microsomes was reduced in the presence of catalase, superoxide dismutase, and the radical scavengers DMSO, t-butanol, aminopyrine and hydroquinone (Scailteur and Lauwerys, 1984a). DMF itself inhibited the oxidation of DMSO, t-butanol and aminopyrine.

Symptoms comparable to those precipitated by alcoholic drinks in combination with disulfiram (antabuse), such as facial flushing, have been described after ingestion of alcohol in persons working with DMF (Reinl and Urban, 1965; Chivers, 1978; Lyle et al, 1979). Ethanol (2g/kg p.o.) given 18h after DMF (2mmol/kg) in rats led to raised blood levels of acetaldehyde while a higher dose of DMF increased blood levels of ethanol and not of acetaldehyde (Hanasono et al, 1977). The authors concluded that the lower dose of DMF

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impaired acetaldehyde metabolism, the larger dose ethanol metabolism. High concentrations of DMF inhibited mouse liver alcohol dehydrogenases *in vitro* in an uncompetitive fashion (Sharkawi, 1979). Ethanol given to rats and dogs prior to exposure to DMF vapours inhibited DMF metabolism as shown by both elevated DMF concentrations in the blood and a delay before the appearance of its major metabolite (Eben and Kimmerle, 1976).

DMF given i.p. caused a prolongation of the pentobarbital-induced sleeping time in mice (Weetman and Crossfield, 1982). This effect was probably not caused by the inhibition of mixed function oxidase enzymes as pretreatment of mice with the enzyme inhibitor SKF525A did not exacerbate the DMF effect. DMF also caused an increase in sleeping time induced by thiobarbital, a barbiturate the duration of action of which is not determined by oxidative metabolism. Therefore DMF appears to cause this effect by interference with the central nervous system.

### 1.1.3 Other formamides

F itself undergoes relatively little metabolism both *in vivo* and *in vitro* (Kennedy, 1986): Rats, dogs and cats which received F orally excreted a large proportion of the dose unchanged in the urine. The amide group was hydrolysed to a very slight extent by rabbit liver extracts.

Kestell et al (1987) studied the metabolism of F, N-ethylformamide (NEF, fig 1) and N-methylacetamide (NMA, fig 1), as well as NMF and DMF, in CBA/CA mice. In

freeze-dried urine samples of mice which had received NMF or NEF, thioesters were detected by TLC. Such species could not be found after administration of F, DMF or NMA. Evidence based on HPLC analysis and high-field $^{1}H$-NMR and mass spectrometry suggested that the thioester metabolite of NEF was N-acetyl-S-(N-ethylcarbamoyl)cysteine (SEM, fig 2). More recently S-(N-ethylcarbamoyl)glutathione (SEG, fig 2), the likely precursor of SEM, was identified as a bilary metabolite in mice which had received NEF (Pearson and Baillie, unpub.). NEF also underwent extensive metabolism to ethylamine ($CH_3CH_2NH_2$). In contrast, the urine of mice which had received NMA contained no detectable levels of methylamine. Instead, the major metabolite of NMA was N-(hydroxymethyl)acetamide (HMA, fig 1).

On incubation for 80min with isolated mouse hepatocytes, F and HMF, unlike NMF (7mM), failed to deplete intracellular glutathione (Whitby et al, 1984b).

## 1.2 Antitumour activity of N-alkylformamides

The antitumour activity of NMF in mice was first shown more than 30 years ago in the sarcoma 180 (Clarke et al, 1953) and the Ehrlich ascites tumour models (Furst et al, 1955). Of over 100 formamides and related amides, NMF was the most potent inhibitor of the growth of Ehrlich ascites tumour cells *in vivo*. The activities of DMF and F were marginal while NEF exhibited no antineoplastic activity. More recently NMF and a series of derivatives were tested for activity against the M5076 ovarian sarcoma and the TLX5 lymphoma, also in mice (Gate et al, 1986). NMF was again by far the most potent antitumour agent in both models. N-(Trideuteromethyl)formamide ($^2H_3$-NMF, fig 1) and NEF showed activity against the TLX5 lymphoma while DMF and HMF showed marginal activity against the M5076 tumour. The inactivity of most analogues tested by Furst et al (1955) and Gate et al (1986) indicates that there is a rigorous structural requirement for antitumour activity.

The mode of antitumour activity of NMF is still unclear. Gescher et al (1982) proposed that bioactivation of NMF may be required for antineoplastic activity. Coadministration of NMF and diethyldithiocarbamate (DTC), an inhibitor of cytochrome P450 monooxygenases, decreased the antitumour activity of NMF against sarcoma 180 and Ehrlich ascites tumours (Masuda et al, 1986). Though NMF and HMF both demonstrated inhibitory activity against the MX-1 mammary xenograft in mice (Cooksey et al, 1983), HMF lacked activity against the TLX5 lymphoma and sarcoma 180 *in vivo*

suggesting that N-methyl-C-hydroxylation of the parent compound is not required for the activation of NMF (Gate et al, 1986). The abolition of the antitumour activity of NMF by the replacement of either the hydrogen or oxygen atoms in the formyl moiety is consistent with the notion that bioactivation may occur in this portion of the drug (Gate et al, 1986). As described in section 1.1.1, NMF undergoes metabolism in the formyl moiety to generate a species which reacts with GSH to form SMG. This unknown reactive metabolite may be involved in the antitumour activity of NMF. Furthermore, thiocarbamates related to SMM such as S-carbamoylcysteine and S-ethylcarbamoylcysteine have antitumour activity (Skinner et al, 1958; Nemeth et al, 1978), which suggests that SMM and its precursors may possess antineoplastic activity. Results of experiments to test this hypothesis have so far been equivocal (Chubb and Gescher, unpub.).

NMF, though effective against a number of murine tumour models in vivo, including the human cancer cell lines HCT-15 and DLD-2 (Dexter et al, 1982), is surprisingly free from the commonly observed deleterious effects of antitumour drugs on the host bone marrow (Newman et al, 1981; Langdon et al, 1985b). This is an almost unique property for an antineoplastic agent. NMF is a drug which induces terminal differentiation of certain cell lines in vitro (Spremulli and Dexter, 1984). However it is not clear whether tumour growth is limited in vivo by the promotion of terminal differentiation of the tumour to viable, non-proliferating cells, or by cytotoxicity. Cytotoxicity observed in vitro

appears to be non-specific as NMF and NEF are equipotent cytotoxins against TLX5 cells in vitro whereas NMF is markedly more active as an antitumour agent in vivo (Gescher et al, 1982). The ability of NMF to induce differentiation in HL-60 human leukaemia cells in vitro is a property shared by polar solvents including DMF which are inactive as antitumour agents in vivo (Langdon and Hickman, 1987). Further evidence suggesting that NMF does not act as an antitumour agent in vivo by inducing tumour cells to differentiate is the disparity between the plasma concentrations of NMF achieved in vivo and those required to induce terminal differentiation in vitro: Peak plasma concentrations seen in mice after administration of an effective antitumour dose of NMF (400mg/kg) was 7mM (Brindley et al, 1982) while the induction of differentiation in vitro required concentrations of NMF in the range of 100mM (Spremulli and Dexter, 1984). This disparity also supports the suggestion that a metabolite of NMF may be responsible for antitumour activity.

Maturation of DLD-1 clone A cells in culture by NMF was accompanied by a fall in intracellular glutathione (Cordeiro and Savarese, 1984). The growth and glutathione depletory effects of NMF in this cell type were restored to control values on coincubation with 0.5mM L-cysteine. Furthermore, BSO depleted cellular glutathione and mimicked the ability of NMF to cause cytostasis together with the acquisition of a more mature phenotype (Cordeiro and Savarese, 1986). A similar fall in cellular glutathione was observed in TLX5 cells treated with NMF in vitro (Bill et al, 1988).

However, when a glutathione depletion of similar magnitude was elicited by BSO, there was no significant effect on cell replication or viability. Also the addition of cysteine to NMF-treated cultures was unable to restore the depleted cellular glutathione levels of TLX5 cells or restore cellular growth. These results suggest that the relationship between cell growth and glutathione metabolism is cell type dependent.

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; Elsenhauer et al, 1986) clinical trials. The results have been singularly disappointing. Dosing was often limited by severe nausea and gastrointestinal and liver toxicity. Several other reactions occurred including skin rashes, abdominal pain and gastritis and at least one patient died as a result, at least in part, to NMF toxicity. The clinical importance of the toxicity of NMF, especially the hepatotoxicity, and the lack of understanding of the underlying mechanisms and their relationship to antitumour activity have led to this investigation of NMF toxicity.

## 1.3 Toxicology of NMF and its structural analogues

### 1.3.1 NMF

The therapeutic limitations of NMF as an anticancer drug due to its hepatotoxicity were predicted in the first studies with NMF in mice bearing the sarcoma 180 (Clarke et al, 1953). This prediction was shown to be true in preliminary clinical studies with NMF in 5 patients with advanced cancer (Laird Myers et al, 1955). All 5 patients after oral or i.v. administration of NMF showed symptoms of toxicity, chiefly liver damage, anorexia, nausea and vomiting, while no inhibition of tumour growth was observed. In more recent phase I trials where NMF was administered by the oral or i.v. route, anorexia, malaise, nausea and vomiting were the most common side effects, often severe enough to necessitate the termination of therapy with NMF (McVie et al, 1984; Ettinger et al, 1985; Wiemann et al, 1985; Murphy et al, 1987). Reversible hepatotoxicity, evidenced by elevation of bilirubin and/or transaminases, was also commonly observed in the patients (table 1a). The maximum tolerated dose (MTD) of NMF in humans was 800-900mg/m$^2$/day (21-24mg/kg/day; McVie et al, 1984; Murphy et al, 1987). Patients treated at the MTD or higher showed no therapeutic benefit. As in the phase I trials, toxic effects during phase II evaluation of NMF were primarily nausea, vomiting and elevation of hepatic enzymes (table 1b) while inhibition of tumour growth was not apparent (Tauer et al, 1985; Elsenhauer et al, 1986; Planting et al, 1987).

Table 1

## The hepatotoxicity of NMF in clinical trials.

(a) Phase I trial (Murphy et al, 1987).

| Iv dosage ($mg/m^2$ day × 5) | No. of patients/ No. of courses | No. of toxic patients* | No. of courses associated with— | | | |
|---|---|---|---|---|---|---|
| | | | Nausea/ vomiting | Hyperbilirubinemia | Hepatic transminase elevation | Chest pain and/or abdominal pain |
| 800 | 4/7 | 1 | 4 | 0 | 2 | 3 |
| 900 | 3/5 | 1 | 2 | 0 | 0 | 1 |
| 1000 | 6/9 | 5 | 9 | 0 | 3 | 3 |
| 1100 | 2/2 | 1 | 1 | 1 | 1 | 0 |
| | 15/23 | 8 | 16 (70%) | 1 (4%) | 6 (26%) | 7 (30%) |

*Patients experiencing grade 3 or grade 4 or subjectively intolerable degrees of toxicity.

(b) Phase II trials (Elsenhauer et al, 1986).

| Dose ($mg/m^2$) | Total No. of courses | No. of courses with toxicity grade— | | | | | Total No. of courses with toxicity |
|---|---|---|---|---|---|---|---|
| | | 0 | 1 | 2 | 3 | 4 | |
| 600 | 11 | 6 | 4 | | 1 | | 5 |
| 800 | 29 | 18 | 6 | 2 | 2 | 1 | 11 |

| | Toxicity grading* | | | | |
|---|---|---|---|---|---|
| | Grade 0 | Grade 1 | Grade 2 | Grade 3 | Grade 4 |
| Bilirubin | < 1.25 × N | 1.25-2.5 × N | 2.6-5.0 × N | 5.1-10.0 × N | > 10.0 × N |
| SGOT | < 1.25 × N | 1.25-2.5 × N | 2.6-5.0 × N | 5.1-10.0 × N | > 10.0 × N |
| Alkaline phosphatase | < 1.25 × N | 1.25-2.5 × N | 2.6-5.0 × N | 5.1-10.0 × N | > 10.0 × N |

*N = normal.

The hepatotoxicity of NMF has been investigated in mice (Newman et al, 1981; Whitby et al, 1984b; Langdon et al, 1985a; Pearson et al, 1987a,b), rats (Lundberg et al, 1981; Tulip et al, 1985) and dogs (Newman et al, 1981). A correlation was established in rodents which had been administered NMF between the elevation of liver-associated enzymes in the plasma, namely sorbitol dehydrogenase (SDH), alanine aminotransferase (ALT) and aspartate aminotransferase (AST), and histopathological evidence of liver damage (Scailteur and Lauwerys, 1984; Whitby et al, 1984b; Pearson et al, 1987a). Plasma activities of these enzymes reached a peak 24h after administration of a hepatotoxic dose of NMF. Histopathological evidence of liver damage, primarily centrilobular necrosis, was apparent 12h and later after dosing with NMF. NMF is considerably more toxic to mice than to rats: Single doses of 2g/kg NMF given i.p. to Sprague Dawley rats caused little hepatic damage whereas doses of 400mg/kg NMF given i.p. to Balb/C mice caused extensive centrilobular necrosis (Tulip et al, 1985). Male rats were more sensitive to NMF-induced hepatotoxicity than female rats (Scailteur et al, 1981). Mice display a marked strain difference in sensitivity towards NMF-induced hepatotoxicity: The threshold i.p. doses of NMF to elicit liver damage in Balb/C, CBA/CA and $BDF_1$ mice were 100-200, 200-400 and 800mg/kg, respectively as measured by elevation of SDH, ALT and AST in the plasma 24h after administration on NMF (Pearson, 1985).

Pearson et al (1987a,b) have postulated that, in analogy to the mechanism by which hepatotoxins such as

paracetamol and bromobenzene cause liver damage (see appendix 1), NMF may be metabolised to an electrophilic intermediate, the proximate or ultimate hepatotoxic species, and conjugation of this metabolite with glutathione constitutes a detoxification pathway (fig 2). NMF-induced hepatotoxicity and glutathione status appear to be mechanistically linked (Pearson et al, 1987a): In Balb/C mice, the strain most susceptible to NMF toxicity, a hepatotoxic dose of NMF (200mg/kg) caused a depletion of hepatic glutathione to 21% of control levels 2h after drug administration. In CBA/CA and $BDF_1$ mice the same dose of NMF was not hepatotoxic and depleted hepatic glutathione to only 53% of control levels. In Balb/C mice, depletion of hepatic glutathione by pretreatment with the γ-glutamyl-cysteine synthetase inhibitor BSO decreased the hepatotoxic dose threshold of NMF from 150mg/kg to 100mg/kg. Conversely, pretreatment of mice with cysteine and N-acetylcysteine protected against both hepatic glutathione depletion and NMF-induced hepatotoxicity.

Reactive metabolites of hepatotoxins such as paracetamol and bromobenzene can bind covalently to proteins, nucleic acids and polysaccharides, and are believed to alter the biological properties of one or more of these cellular macromolecules in a detrimental fashion (Bridges et al, 1983). However, the contention that covalent binding plays a pivotal role in initiating a cascade of events leading to cell injury and ultimately cell death is still controversial and is often based on the correlation between the extent of binding and the severity

of the accompanying lesion *in vivo* (Gillette, 1974). NMF-induced hepatotoxicity has been linked by three pieces of evidence to the generation of a reactive metabolite of $^{14}$C-NMF capable of binding covalently to hepatic protein (Pearson et al, 1987b): (i) The binding of $^{14}$C-NMF metabolites to hepatic proteins *in vivo* was appreciably higher in the target organ of toxicity, namely the liver, than in other tissues, (ii) the rank order of association of metabolites of $^{14}$C-NMF with liver macromolecules *in vivo* in three strains of mouse, Balb/C, CBA/CA and $BDF_1$, parallelled differences between the sensitivity of these strains towards NMF-induced hepatotoxicity, and (iii) depletion of hepatic glutathione with BSO or diethylmaleate (DEM) prior to administration of NMF not only exacerbated toxicity but also increased binding of metabolites of formyl-$^{14}$C-NMF *in vivo*. Conversely, pretreatment of mice with thiol compounds protected their livers against glutathione depletion and toxicity and likewise the presence of GSH in microsomal incubations abolished binding *in vitro*.

Identification of SMG and SMM as metabolites of NMF (section 1.1.1) has prompted speculation as to the identity of their electrophilic precursor. One candidate is methyl isocyanate ($CH_3$-N=C=O) which reacts with GSH *in vitro* to form SMG (Baillie et al, 1988). The reactivity of isocyanates has been implicated in the cytotoxicity of the antitumour nitrosoureas (Gibson and Hickman, 1982) and could explain the fulminant hepatic necrosis seen after administration of NMF. The enzyme system responsible for the activation of NMF remains however a mystery. Oral

administration of DTC, an inhibitor of cytochrome P450 monooxygenases, to mice prior to dosing with NMF prevented glutathione depletion and hepatic necrosis and delayed the metabolism of NMF (Masuda et al, 1986). However NMF-induced toxicity was not enhanced by pretreatment of mice with the cytochrome P450 inducers phenobarbitone, 3-methylcholanthrene or β-naphthoflavone. Furthermore, inhibition of the cytochrome P450 mixed function oxidase system by pretreatment of mice with SKF525A prior to administration of a hepatotoxic dose of NMF failed to alleviate the hepatotoxicity of NMF or abolish the covalent binding of NMF metabolites to microsomal protein (Pearson et al, 1987b). Similarly, pretreatment with phenobarbitone prior to administration of NMF did not exacerbate either the toxicity of NMF or the binding of NMF metabolites to microsomal protein. These findings, while not an exhaustive characterisation of the role of cytochrome P450, are suggestive that this enzyme system is not involved in the bioactivation of NMF.

Mitochondria may be an intracellular target of NMF toxicity: $Ca^{2+}$ sequestration by mouse liver mitochondria was potently inhibited 1h after administration of a hepatotoxic dose of NMF (Whitby et al, 1984a). The effect was dose related and was not observed after either non-hepatotoxic doses of NMF or after equimolar doses of DMF, HMF or F. The inhibitory effect of NMF on the mitochondrial $Ca^{2+}$ pump appeared to be specific as mitochondrial respiration and microsomal $Ca^{2+}$ sequestration were unaffected.

As well as hepatotoxic properties, NMF is also

teratogenic and embryotoxic in the rat (Thiersch, 1962; Stula and Krauss, 1977) and the rabbit (Stula and Krauss, 1977). However, the mechanisms of toxicity involved are at present unknown.

### 1.3.2 DMF

Workers in frequent contact with DMF have been reported to exhibit various symptoms of toxicity including headache, nausea and vomiting as well as gastric and hepatic abnormalities (Massman, 1956; Tolot et al, 1958; Reinl and Urban, 1965). Gastritis was diagnosed in one textile worker who inhaled a large amount of DMF (Tolot et al, 1969) while other occupationally exposed persons have experienced severe abdominal pain after dermal contact with DMF (Reinl and Urban, 1965; Potter, 1973). Hepatic damage, as indicated by increased levels of transaminases and bilirubin in the plasma, and in some cases hepatitis have been observed in workers after respiratory or dermal exposure to DMF (Reinl and Urban, 1965; Tolot et al, 1969; Von Klavis, 1970; Finzel, 1972; Potter, 1973; Chivers, 1978). Recently, DMF was implicated in the etiology of some occupational testicular cancers (Ducatmen et al, 1986; Levin et al, 1987).

Histopathological examination of the livers of mice and rats after chronic dosing with DMF by the i.p. route revealed centrilobular necrosis with some areas of regeneration (Massman, 1956; Dexter et al, 1972; Matthew et al, 1980; Lundberg et al, 1981). DMF appears to be a less

potent hepatotoxin than NMF (Scailteur et al, 1981). Likewise, the $LD_{50}$ of DMF was four times greater than that of NMF in $BDF_1$ mice (Cooksey et al, 1983). Coadministration to rats of DMF and NMF delayed the onset of NMF-induced hepatotoxicity as determined by the elevation of SDH in the plasma (Lundberg et al, 1981).

The mechanism by which DMF causes toxicity is unclear. NMF-induced hepatotoxicity is thought to be caused by a reactive metabolite that can conjugate with GSH (section 1.3.1). The detection of SMM in the urine of rodents and humans exposed to either DMF or NMF (section 1.1) raises the possibility that both N-alkylformamides may cause liver damage via the same reactive intermediate. Furthermore, DMF may be metabolised to the toxic species via NMF. The intermediate formation of NMF may explain the delay in urinary excretion of SMM witnessed after exposure of a human to DMF compared to the rapid excretion of SMM after exposure to NMF (section 1.1.2). It may also explain the failure of DMF to deplete hepatic glutathione in vitro.

The teratogenic and mutagenic potential of DMF appears to be low (Gescher, 1986a).

### 1.3.3 Other formamides

The $LD_{50}$ value of F was only marginally higher than for NMF in female $BDF_1$ mice dosed by the i.p. route (Cooksey et al, 1983). However, the hepatotoxic potential of F was low in comparison to its N-methyl and N,N-dimethyl derivatives (Scailteur et al, 1981). Furthermore, F (7mM) failed to

deplete intracellular glutathione in isolated mouse hepatocytes exposed to the amide for 80min (Whitby et al, 1984b).

HMF was considerably less toxic than NMF in tumour-bearing mice though it was more cytotoxic than NMF to TLX5 lymphoma cells *in vitro* (Cooksey et al, 1983). Unlike NMF, HMF did not reduce mouse hepatic glutathione levels *in vivo* (Cooksey et al, 1983) or *in vitro* (Whitby et al, 1984b). In view of these results it appears unlikely that HMF is the hepatotoxic metabolite of NMF or a precursor of it.

NEF was slightly less toxic than NMF in female $BDF_1$ mice though there was no indication as to which was the target organ for toxicity (Cooksey et al, 1983). NMA appears to possess no liver toxicity as mice surviving one month following near-lethal doses of NMA showed no histopathological evidence of liver damage (Kennedy, 1986).

## 1.4 Aims of this investigation

As reviewed in sections 1.1-1.3 the biological activities of NMF are thought to be a consequence of metabolic activation for which there appears to be a very strict structural requirement. The primary aim of the experiments described in this thesis was to examine the importance of the chemical structure of NMF in relation to its hepatotoxicity and its ability to generate reactive, potentially toxic, metabolites. The hypothesis underlying this investigation was that a better understanding of the inter-relationships between chemical structure, toxicity and metabolism of N-alkylformamides might permit the elucidation of the mechanism of hepatotoxicity of this class of compound. Furthermore if, as seems likely, the hepatotoxic and antitumour activities of NMF result from separate metabolic pathways, a clearer knowledge of the mechanism of toxicity of NMF may aid the design of a less toxic antitumour agent.

The metabolism of NMF and a small number of analogues possessing only one functional alteration in either the $R_1$, $R_2$ or $R_3$ moiety of NMF (fig 1) has been studied in mice (section 1.1). NMF and its ethyl analogue NEF were extensively metabolised to among others alkylamine, S-(N-alkylcarbamoyl)glutathione and N-acetyl-S-(N-alkyl-carbamoyl)cysteine. However, the replacement of either hydrogen in the $R_2$ or $R_3$ positions with methyl dramatically reduced the production of these metabolites. Also a primary kinetic deuterium isotope effect was evident on replacement

of the formyl ($R_3$) hydrogen with deuterium in that the deuterated isotopomer was metabolised to methylamine, SMG and SMM *in vivo* at a significantly slower rate than the proteo form. NMF is thought to be metabolically activated to a reactive intermediate capable of covalently binding to hepatic macromolecules and initiating cell damage (section 1.3.1). The identification of SMG and SMM in mice which had received NMF strongly indicates that the reactive species binds to GSH. Thus the formation of SMG and SMM are presumably intimately linked with the hepatotoxic potential of NMF and structural analogues. To confirm this hypothesis, the hepatotoxic potential of NMF, F, NEF, DMF, NMA and of two isotopomers, $^{2}$H-NMF and $^{2}H_3$-NMF, are determined here in mice.

Although NMF is metabolised extensively *in vivo*, metabolism *in vitro* of this monoalkylformamide has so far only been observed indirectly by low level association of $^{14}$C-NMF derived radioactivity to microsomal protein (section 1.1.1). Previous attempts to measure NMF metabolism in isolated hepatocytes have been unsuccessful though NMF did appear to deplete intracellular glutathione in this model suggesting that a reactive metabolite may have been generated. *In vitro* studies using isolated hepatocytes have over recent years become an important alternative to whole-animal experimentation, both from a practical and ethical viewpoint. Isolated hepatocytes provide a unique experimental approach for the pharmacotoxicologist where the metabolic functions of the liver are available in an environment in which experimental conditions can be exactly

defined. Also, isolated liver cells from humans allow a direct evaluation of the hepatotoxicity and metabolism of xenobiotics in humans. Isolated hepatocytes are examined here as a potential *in vitro* model of the *in vivo* toxicity of NMF and related amides. As the model was found to be suitable it was used in a study of the inter-relationships between the chemical structure of NMF and its cytotoxicity, metabolism *in vitro* and effect on hepatocytic glutathione homeostasis.

The enzyme system(s) responsible for bioactivating NMF, and the product of this metabolic pathway are presently unknown. However an insight into the nature of the reactive intermediate(s) has been provided by the unambiguous identification of SMG as a bilary metabolite of NMF. One can propose probable species capable of forming such a conjugate with GSH however the very nature of such short-lived intermediates makes their detection and identification extremely difficult. The elucidation of this toxification pathway is attempted here in isolated hepatocytes using compounds labelled with stable-isotopes. These compounds are used in two separate approaches by determining either the removal of the isotope from, or incorporation of the isotope into, NMF during the bioactivation process. The possible role of cytochrome P450 in this toxification pathway, or in the detoxification of NMF, is investigated in a preliminary fashion by studying the effects of SKF525A on the hepatotoxicity of NMF *in vivo* and on the covalent binding of methyl-$^{14}$C-NMF to microsomal protein *in vitro*.

The results presented in section 4 enable conclusions

to be drawn as to the mechanism of toxicity of N-alkylformamides and provide a suitable *in vitro* system for the further study of this toxification pathway.

# SECTION 2

# MATERIALS

## 2.1 Animals and anaesthetics

Three strains of mice were used. Balb/C, CBA/CA and $BDF_1$ mice were supplied by Bantin and Kingman Ltd., Hull, U.K. Animals were male, between 10 and 20 weeks old and weighed between 18 and 25g. They were maintained in polypropylene cages under a 12h light/dark cycle and were provided with food (Heygate 41B breeding diet) and tap water *ad libitum*.

Fluothane was purchased from ICI, Macclesfield, U.K. and Sagatal (60mg/ml sodium pentobarbitone) was purchased from May and Baker Ltd., Dagenham, U.K.

## 2.2 Formamides

NMF and NMA were purchased from Aldrich Chemical Company Ltd., Gillingham, U.K. F and DMF were purchased from BDH Chemicals Ltd., Atherstone, U.K. NEF was purchased from Fluka Chemicals Ltd., Glossop, U.K. HMMF was synthesised by Dr A. Gledhill at the University of Aston, Birmingham, U.K. using a previously published method (Grady and Stott, 1967). All formamides were purified by distillation and were > 99% pure.

## 2.3 Stable isotope labelled compounds

Dr M.D. Threadgill synthesised N-methyldeuteroformamide ($^2$H-NMF, fig 1) and N-(trideuteromethyl)formamide ($^2H_3$-NMF) at the University of Aston, using previously

published methods (Threadgill et al, 1987; Threadgill and Gate, 1983). Deuterated starting materials were purchased from Aldrich Chemical Company Ltd., U.K. Electron impact mass spectrometry (EI-MS) showed both materials to be > 96% pure (see section 3.7.4).

$^{13}C_2{}^{15}N$-NMF ($HO^{13}C^{15}NH^{13}CH_3$) was purchased from MSD Isotopes, St. Louis, U.S.A. with an isotopic purity of 90 atom% excess $^{13}C_2$ and 95 atom% excess $^{15}N$.

$^{18}O_2$ was purchased from KOR Isotopes, U.S.A. with an isotopic purity of 98 mol% excess $^{18}O$.

## 2.4 Radiolabelled compounds

Methyl-$^{14}$C-NMF ($HOCNH^{14}CH_3$) and ethyl-$^{14}$C-NEF ($HOCNH^{14}CH_2CH_3$) were prepared by Dr M.D. Threadgill by treating $^{14}$C-methylamine hydrochloride and $^{14}$C-ethylamine hydrochloride respectively with ethylformate and sodium carbonate (Threadgill and Gate, 1983). The radiolabelled starting materials were purchased from Amersham International plc, U.K. The $^{14}$C-labelled products were purified by preparative thin layer chromatography until they were > 99% pure.

$^{14}$C-Hexadecane was purchased from Amersham International plc., U.K.

## 2.5 N-alkylcarbamoylating metabolites and their derivatives

S-(N-alkylcarbamoyl)glutathiones were synthesised by Dr P.G. Pearson and Dr D.-H. Han at the University of

Washington, Seattle, U.S.A. Methyl or ethyl isocyanate was reacted with GSH in aqueous buffer in the presence of acetonitrile ($CH_3CN$) to produce SMG or SEG respectively in good yield. Full details of this synthesis await publication. The benzyloxycarbonyl- and benzyloxycarbonyl dimethylester derivatives of SMG were synthesised and purified as detailed in section 3.7.2. Benzylchloroformate (BCF) and methylene chloride were purchased from Aldrich Chemical Co., Milwauwee, U.S.A. Methanolic hydrogen chloride (pH < 2) was produced by passing gaseous HCl, produced by dropping concentrated $H_2SO_4$ on NaCl, through methanol for 1h.

SMM was synthesised by Dr M.D. Threadgill using a previously published method (Kestell et al, 1986b).

## 2.6 Enzyme inducers and inhibitors

Sodium phenobarbitone was purchased from BDH Chemicals Ltd., U.K. SKF525A (proadifen, 2-diethylaminoethyl-2,2-diphenylvalerate) was kindly donated by Smith, Kline and French Ltd., Welwyn, U.K. DL-buthionine-[S,R]-sulphoximine (BSO) and disulfiram (antabuse) were purchased from Sigma Chemical Company Ltd., U.K.

## 2.7 Solutions for the preparation and incubation of liver fractions

All chemicals were purchased from Sigma Chemical Company Ltd, U.K.

Phosphate buffer (50mM, pH 7.4)

A solution of 340mg of $KH_2PO_4$ in 50ml of distilled water was added to a solution of 1.77g of $Na_2HPO_4$ in 250ml of distilled water until pH 7.4 was attained.

NADP (20mM)

NADP (34mg) was dissolved in 2ml of phosphate buffer (50mM, pH 7.4).

G6P (100mM)/G6PDH (40 units/ml)

G6P (62mg) was dissolved in 2ml of phosphate buffer (50mM, pH 7.4). To this solution was added 94µl of the commercial enzyme.

Aminopyrine (100mM)

Aminopyrine (115.65mg) was dissolved in 5ml of phosphate buffer (50mM, pH 7.4).

Magnesium chloride (100mM)

$MgCl_2.6H_2O$ (101.5mg) was dissolved in 5ml of phosphate buffer (50mM, pH 7.4).

NAD (20mM)

NAD (30.8mg) was dissolved in 2ml of phosphate buffer (50mM, pH 7.4).

## 2.8 Solutions for the preparation of isolated mouse hepatocytes

All chemicals were purchased from Sigma Chemical Company Ltd, U.K. unless otherwise stated.

### Calcium-free HBSS

Concentrated $Ca^{2+}$, $Mg^{2+}$-free Hanks buffered salt solution (HBSS) was purchased from Gibco Ltd., Paisley, U.K. 25ml was added to 200ml of distilled water. To this solution was added 525mg of $NaHCO_3$, 500mg of BSA (fraction V) and 2.5ml of 1M HEPES. The pH was then adjusted to pH 7.3 and the volume made up to 250ml. All solutions were sterile and preparation was performed in a Gelaire BSB3 lamniflow cabinet.

### Krebs-Henseleit buffer (pH 7.3)

The following were dissolved in 800ml of distilled water: NaCl (6.87g), KCl (0.4g), $MgSO_4.7H_2O$ (0.14g), $CaCl_2$ (0.28g), $NaH_2PO_4$ (0.14g) and β-D(+)-glucose (2g). To this solution was added 100ml of 2.1% $NaHCO_3$ and 10ml of 1M HEPES (pH 7.3). The pH was adjusted with dilute NaOH to pH 7.3 and the volume was made up to 1000ml. This solution was made up fresh every 2 weeks and was kept at 4°C.

### Collagenase solution

Collagenase (an extract of Clostridium histolyticum) was purchased from Boehringer Mannheim Ltd., Lewes, U.K. The dispersion efficiency of the enzyme preparation varied

between batches. Typically 80-100mg of collagenase was dissolved in 60ml of Krebs-Henseleit buffer (pH 7.3) within 1h of use. When low cell yields were obtained either the concentration of collagenase was increased or the medium was supplemented with 40mg of $CaCl_2.2H_2O$.

Medium for the incubation of hepatocytes

Krebs-Henseleit buffer was supplemented with 10% horse serum (mycoplasma screened, Gibco Ltd., U.K.) and 0.2% BSA (fraction V). The medium was made up either on the day of use or the previous day and kept frozen overnight. The preparation was performed in a Gelaire BSB3 lamniflow cabinet

Trypan blue solution (3% w/v)

Trypan blue (0.3g) was dissolved in 0.9% NaCl solution (10ml). The solution was vigorously shaken and then filtered before use.

## 2.9 Solutions for the culture of mouse hepatocytes

All ingredients were purchased sterile and preparation of solutions was performed in a Gelaire BSB3 lamniflow cabinet.

Calcium-free HBSS

The solution was prepared as previously described (section 2.8) but was supplemented with gentamycin (10µg/ml, Gibco Ltd., U.K.) and stored under refrigeration for less

than one week before use.

William's E medium

William's E medium was purchased from Gibco Ltd., U.K. and supplemented with gentamycin (10µg/ml) and HEPES (10mM, Sigma Chemical Co.) and the pH adjusted to pH 7.3.

Collagenase solution

Collagenase (50mg, Boehringer Mannheim Ltd.) was dissolved in 50ml of supplemented William's E medium within 1h prior to liver perfusion.

Culture medium

Commercial William's E medium was supplemented with foetal calf serum (1% v/v, heat inactivated, Gibco Ltd., U.K.), L-glutamine (266mg/l, Gibco Ltd., U.K.), insulin (200units/l, Sigma Chemical Co., U.K.), gentamycin (50mg/l) and HEPES (10mM). The medium was prepared within 24h prior to use and stored at 4°C.

## 2.10 Solutions for spectrophotometric enzyme assays

All chemicals were purchased from Sigma Chemical Company Ltd., U.K. unless otherwise stated.

### 2.10.1 Alanine aminotransferase (ALT) assay

L-Alanine (525mM)

L-Alanine (1.169g) was dissolved in 200ml of 125mM tris

base. pH was adjusted to pH 7.8 and the volume made up to 250ml.

Ketoglutarate (225mM)

∝-Ketoglutaric acid (1.65g) was dissolved in 30ml of 167mM Tris base. pH was adjusted to pH 7.8 and the volume made up to 50ml.

Lactate dehydrogenase (72000 units/l)

The commercial solution (20µl) was diluted with 2.50ml of 50% v/v glycerol in distilled water and was kept on ice throughout the assay.

NADH (6.5mM)

NADH disodium salt (25mg) was dissolved in 5ml of Tris buffer (100mM, pH 7.8).

Pyridoxal-5'-phosphate (4.5mM)

P5P (11.1mg) was dissolved in 10ml of Tris buffer (100mM, pH 7.8).

Tris base stock (1M)

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

Tris buffer (100mM, pH 7.8)

Tris base stock (1M, 50ml) was diluted with 300ml of distilled water and 5.5 ml of 6N HCl. pH was adjusted to pH 7.8 and the final volume made up to 500ml.

## 2.10.2 Aspartate aminotransferase (AST) assay

### L-Aspartate (228mM)

L-Aspartic acid (7.59g) was dissolved in 200ml of 125mM Tris base. pH was adjusted to pH 7.8 and the final volume made up to 250ml.

### Malate dehydrogenase (36000 i.u./l)

The commercial solution (20µl) was diluted with 1.40ml 50% v/v glycerol in distilled water and was kept on ice throughout the assay.

## 2.10.3 Formaldehyde (Nash) assay

### Formaldehyde (0.5M)

Paraformaldehyde (0.15g) was dissolved in 100ml of 0.1N NaOH. 1ml of this solution was then diluted with 100ml of distilled water.

### Nash reagent

This was prepared by adding 45g of ammonium acetate (6M), 0.6ml of acetylacetone (60mM) and 0.9ml of acetic acid (0.15M) to 100ml of distilled water. The pH was adjusted to pH 6.7. All three ingredients were purchased from BDH Chemicals Ltd., U.K.

## 2.10.4 Glutathione assay

### 5'5-Dithiobis-(2-nitrobenzoic acid) (DTNB, 6mM)

DTNB (23.8mg) was dissolved in 10ml of phosphate-EDTA buffer (pH 7.5). The solution was prepared immediately prior to use and was protected from the light and kept on ice throughout the assay.

### Glutathione, reduced (GSH, 1mM)

GSH (7.7mg) was dissolved in 25ml of 10% w/v metaphosphoric acid.

### Glutathione reductase (70000 i.u./l)

The commercial solution (180µl, type III, from yeast) was diluted with 500µl of phosphate-EDTA buffer (pH 7.5) immediately prior to use. The solution was kept on ice throughout the assay.

### NADPH (300µM)

NADPH (12.4mg) was dissolved in 50ml of phosphate-EDTA buffer (pH 7.5) immediately before use and the solution was kept on ice throughout the assay.

### Phosphate (125mM)- EDTA (6.3mM) buffer (pH 7.5)

$Na_2HPO_4$ (8.873g) and $Na_2EDTA$ (1.172g) were dissolved in 450ml of distilled water. The pH was adjusted to pH 7.5 and the volume made up to 500ml.

Trisodium orthophosphate (1.3M)

$Na_3PO_4.12H_2O$ (12.5g) was dissolved in 10ml of distilled water by stirring on a hot plate. Once dissolved the solution was made up to 25ml and replaced on the stirring hot plate to keep it in solution.

2.10.5 Lactate dehydrogenase (LDH) assay

NADH (3.5mM)

NADH disodium salt (24.8mg) was dissolved in 10ml of phosphate buffer (100mM, pH 7.4).

Phosphate buffer (100mM, pH 7.4)

$KH_2PO_4$ (100mM) was mixed with 100mM $Na_2HPO_4$ until pH 7.4 was attained.

Sodium pyruvate (32mM)

Sodium pyruvate (35.2mg) was dissolved in 10ml of phosphate buffer (100mM, pH 7.4).

Triton-X100 (16% v/v)

Triton-X100 (16ml) was dissolved in 84ml of distilled water.

2.10.6 Protein (Lowry) assay

Bovine serum albumin (BSA, 1mg/ml)

BSA (50mg, fraction V) was dissolved in 50ml of 0.5N NaOH.

Folin and Ciocalteu's phenol reagent

The working reagent was prepared by diluting 10ml of the commercial reagent (Fison's plc, Loughborough, U.K.) with 10ml of distilled water immediately before use.

Lowry reagent

This was prepared by mixing 2ml of 1% w/v copper sulphate ($CuSO_4.5H_2O$) solution and 2ml of 2% w/v potassium sodium tartrate ($KNaC_4H_4O_6.4H_2O$) solution with 200ml of 2% w/v sodium carbonate ($Na_2CO_3$) solution. This reagent was prepared immediately before use.

2.10.7 Sorbitol dehydrogenase (SDH) assay

β-D-Fructose (2.5M)

β-D-Fructose (4.5g) was dissolved in 10ml of Tris-HCl buffer (pH 6.6).

NADH (355μM)

NADH disodium salt (12.6mg) was dissolved in 50ml of Tris-HCl buffer (pH 6.6).

Tris-HCl buffer (100mM, pH 6.6)

Tris(hydroxymethyl)methylamine (6.05g) was dissolved in 400ml of distilled water. The pH was adjusted to pH 6.6 and the final volume was made up to 500ml.

## 2.11 Scintillation cocktails

Dimulume 30 was purchased from United Technologies, U.K. and Optiphase MP was purchased from LKB scintillation products, U.K.

## 2.12 Chromatographic solvents

All solvents used for gas-liquid and high pressure liquid chromatography were purchased from BDH Chemicals Ltd, Poole, U.K. and were of the highest grade.

# SECTION 3

# METHODS

## 3.1 Studies of the hepatotoxicity of NMF and structural analogues in mice.

### 3.1.1 Administration of compounds

All compounds were dissolved in sterile saline and an aliquot of 200µl was administered by the i.p. route between 9 and 10am. Some mice received sterile saline only. Mice were weighed before and 24h after dosing. NMF was given to CBA/CA mice at doses of 100, 200, 400 and 600mg/kg and to Balb/C mice at doses of 100 and 200mg/kg. CBA/CA mice also received NEF (200, 400, 600, 800 and 1000mg/kg), F (0.8, 1.6, 2.0 and 3.0mg/kg), NMA (0.8, 1.6 and 3.0mg/kg) and DMF (0.8, 1.6 and 3.0mg/kg). Balb/C mice received $^{2}H$-NMF (100, 200 and 300mg/kg) and $^{2}H_{3}$-NMF (100 and 200mg/kg).

The role of cytochrome P450 monooxygenases in the hepatotoxicity of NMF was investigated by pretreating Balb/C mice with SKF525A (60mg/kg i.p.) 1h before the administration of NMF.

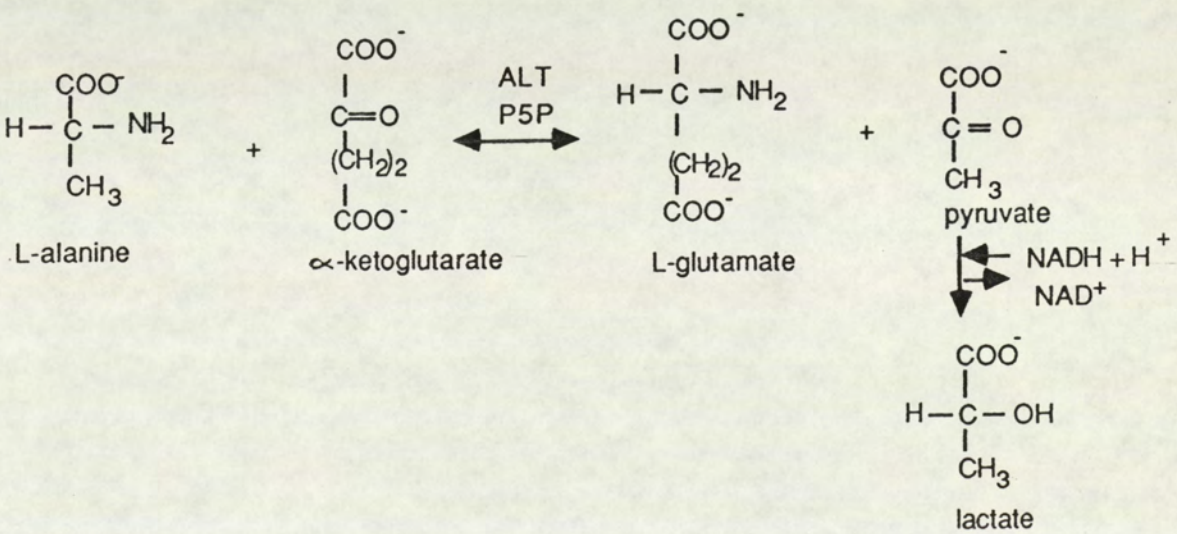
### 3.1.2 Collection of plasma samples

Mice were anaesthetised by inhalation of fluothane. Blood samples were collected by cardiac puncture and drawn into 1ml syringes containing 50µl of heparin solution (2500 u/l). Samples were centrifuged for 1min at 6500rpm in a MSE Microcentaur microfuge to spin down the red blood cells. Plasma samples were stored at 4°C and assayed within 6h of collection.

### 3.1.3 Alanine aminotransferase (ALT) assay

The activity of ALT in plasma was assayed according to a method described by Kachmar and Moss (1976). A plasma sample (67µl) was added to a 1cm pathlength cuvette designed to hold 1ml of liquid containing the following reagents: L-alanine (767µl, 525mM), NADH (33µl, 6.5mM), pyridoxal-5'-phosphate (P5P, 33µl, 4.5mM) and LDH (33µl, 72000u/l). The cuvette with its contents was preincubated in a thermostated cuvette holder of either a Beckman DU7 or Cecil CE594 spectrophotometer for 5min at 37°C to permit any endogenous side reactions to proceed to completion. The reaction catalysed by ALT (fig 3) was initiated by adding a solution of ketoglutaric acid (67µl, 225mM) to the cuvette and the absorbance change was measured at 340nm for 5min.

Figure 3: The reaction catalysed by ALT



P5P acts as a coenzyme in the amino transfer and is bound to the apoenzyme as a true prosthetic group (Kachmar and Moss, 1976). The transaminase reaction is monitored indirectly by the measurement of the rate of NADH oxidation of a specific dehydrogenase reaction coupled to the transaminase reaction.

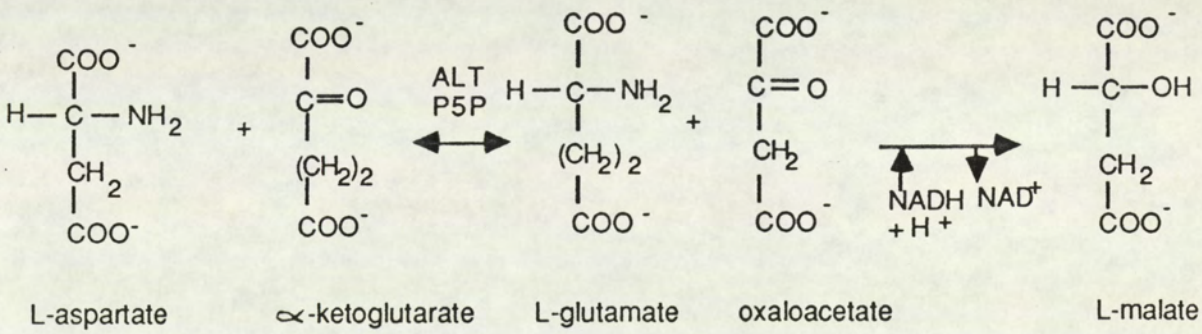
One unit of ALT activity is equal to the oxidation of 1µmol of NADH/l/min. The activity of ALT in this system is given by:

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

## 3.1.4 Aspartate aminotransferase (AST) assay

This assay is identical to the one for ALT described in section 3.1.3 except that L-aspartate replaced L-alanine as the amino group donor and malate dehydrogenase (MDH) replaced LDH as the indicator enzyme (fig 4).

Figure 4: The reaction catalysed by AST




L-aspartate ∝-ketoglutarate L-glutamate oxaloacetate L-malate

Thus the reaction mixture was the same as for the ALT assay except that L-aspartate (767µl, 228mM) and MDH (16µl, 36000u/l) were added in place of L-alanine, and LDH was present at a lower concentration (16µl, 72000u/l). The presence of LDH accelerates the completion of the endogenous side reactions.

One unit of AST activity is equal to the oxidation of 1µmol of NADH/l/min. The activity of AST in this system is given by:

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

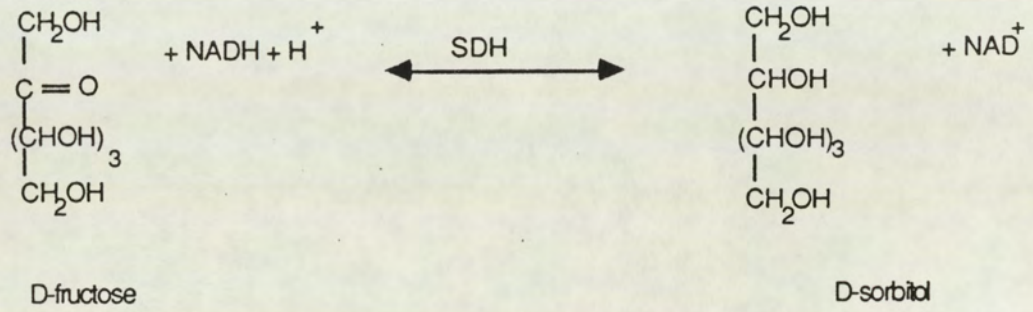
### 3.1.5 Sorbitol dehydrogenase (SDH) assay

The activity of SDH in plasma was assayed according to a method described by Rose and Henderson (1975). A plasma sample (100µl) was added to a reduced volume 1cm pathlength cuvette containing a solution of NADH (700µl, 355µM). The cuvette with its contents was preincubated in a thermostated cuvette holder at 37°C for 5min to permit any endogenous side reactions to proceed to completion. The reaction catalysed by SDH (fig 5) was initiated by addition of β-D-fructose (200µl, 2.5M) to the cuvette and monitored directly by the change in absorbance at 340nm for 5min.

One unit of SDH activity is equal to the oxidation of 1µmol of NADH/l/min. The activity of SDH in this system is given by:

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

Figure 5: The reaction catalysed by SDH




### 3.1.6 Preparation of liver sections

Immediately after extraction of blood samples from CBA/CA mice, the livers were removed and fixed in 10% formol saline for 7 days. Ms Rebecca Holt kindly prepared the liver sections as follows: Slices (4mm) were taken from the three major lobes of each liver and left in 10% formol saline for a further day. The liver slices were dehydrated in alcohol and cleared in xylene by a tissue processor (Shandon Southern Products Ltd.). The liver slices were embedded into wax blocks using Paramat wax in a tissue embedding system (Shandon Southern Products Ltd.). Sections (4µm) were prepared using an Anglia scientific rotary microtome and were mounted on glass slides. Sections were stained with Harris haematoxylin stain and counterstained with Navy eosin (Carelton, 1980). All slides were randomised prior to a "blind" pathological evaluation by light microscopy performed by Dr Iona Pratt of University College, Dublin.

## 3.2 Covalent binding of metabolites derived from methyl-$^{14}$C-NMF and ethyl-$^{14}$C-NEF to liver microsomes in vitro.

### 3.2.1 Preparation and incubation of microsomes

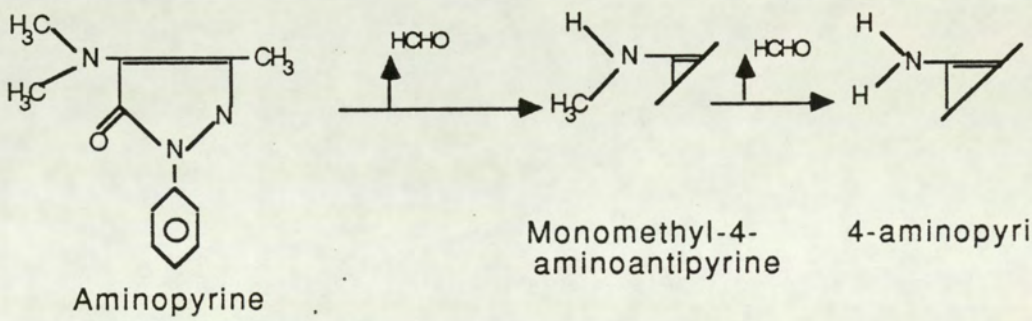
Balb/C or $BDF_1$ mice were killed by cervical dislocation between 9 and 10am. The livers were excised, washed with ice-cold phosphate buffer (50mM, pH 7.4), blotted and weighed. A 20% w/v homogenate was prepared from the livers after addition to an appropriate volume of phosphate buffer in an ice-cold homogenising tube. Homogenisation was achieved using a Camlab 563C homogeniser (5 return strokes, speed 6) fitted with a teflon pestle. The homogenate was transferred to pre-cooled centrifuge tubes and the post-mitochondrial supernatant prepared by centrifugation at 10 000g for 20min in a MSE Pegasus ultracentrifuge at 4°C. The supernatant was transferred to centrifuge tubes after removal of the floating lipid layer and the microsomal fraction was prepared by further centrifugation at 105 000g for 60min at 4°C. The cytosolic fraction was removed and the microsomal pellet resuspended by gentle homogenisation in fresh ice-cold phosphate buffer (7ml/g original liver weight).

Aliquots of the microsomal suspension (750µl) were added immediately to 20ml flasks containing phosphate buffer and the following cofactors: glucose-6-phosphate (G6P, 5mM), glucose-6-phosphate dehydrogenase (G6PDH, 2 i.u./ml), NADP (1mM) and $MgCl_2$ (5mM). The conversion of G6P to

6-phosphogluconic acid by G6PDH at the expense of $NADP^+$ ensures the continuous presence of NADPH in the incubation medium. The flasks were shaken for 5min in an incubator at 37°C before addition of either methyl-$^{14}$C-NMF or ethyl-$^{14}$C-NEF (50µCi/mmol) to give a concentration of 7mM in a final volume of 2ml. The incubation mixtures were shaken gently at 37°C for 2h. Incubations were terminated by addition of 2ml of acetone to precipitate the protein.

The functional viability of the microsomal preparations was assessed by the determination of formaldehyde (HCHO) generated by the metabolism of aminopyrine (4-dimethyl-aminoantipyrine). This compound is N-demethylated to 4-aminoantipyrine by the cytochrome P450-dependent mixed function oxidase system (fig 6; Hodgson and Dauterman, 1980).

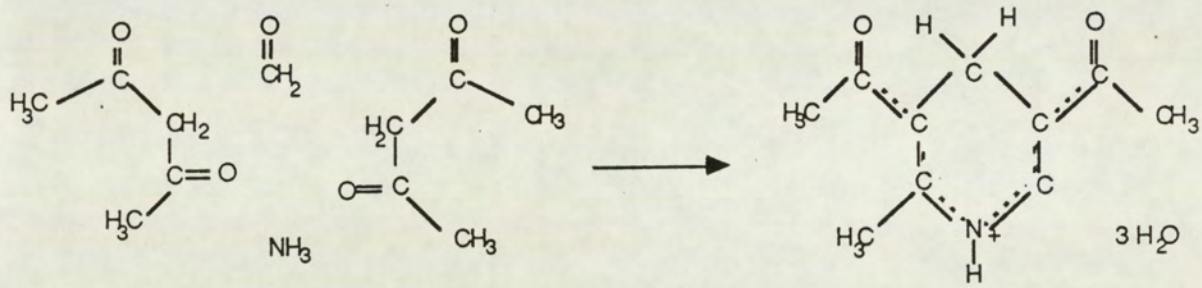
**Figure 6:** The N-demethylation of aminopyrine




Incubations containing aminopyrine (5mM) in place of the N-alkylformamides were terminated after 30min by the addition of 3ml of 12.5% trichloroacetic acid. The concentration of HCHO was determined by the method of Nash (1953) as described by Werringloer (1978). This

determination is based upon the Hantzsch reaction for the synthesis of pyrimidines (fig 7).

Figure 7: The Hantzsch reaction



2 acetylacetone
1 ammonia
1 formaldehyde

3,5-diacetyl-1,4-dihydrolutidine
3 water

## 3.2.2 Determination of covalent binding

After termination of microsomal incubations containing $^{14}C$-labelled N-alkylformamides, the precipitated protein was centrifuged at 500g in a Heraeus labofuge 6000 for 2min. The protein pellet formed was resuspended in 3ml of distilled water. The protein pellet was repeatedly washed (on average 10 times) in water and 50% methanol until no radioactivity could be removed. This was assessed by addition of an aliquot (1ml) of each washing fluid to 10ml of Optiphase MP and counting in a Packard Tricarb 2000CA scintillation counter (see section 3.3). The tissue pellet was solubilised in 2.5ml of 1N NaOH and two 1ml aliquots were each added to scintillation vials containing 10ml of Dimulume 30. These were used for the determination of the radioactivity bound to the microsomal protein (see section

3.3). Two 20µl aliquots of the alkaline protein solution were taken for protein determination (Lowry et al, 1951). The results were expressed as ng of N-alkylformamide equivalents bound/mg of microsomal protein.

### 3.2.3 Modification of covalent binding

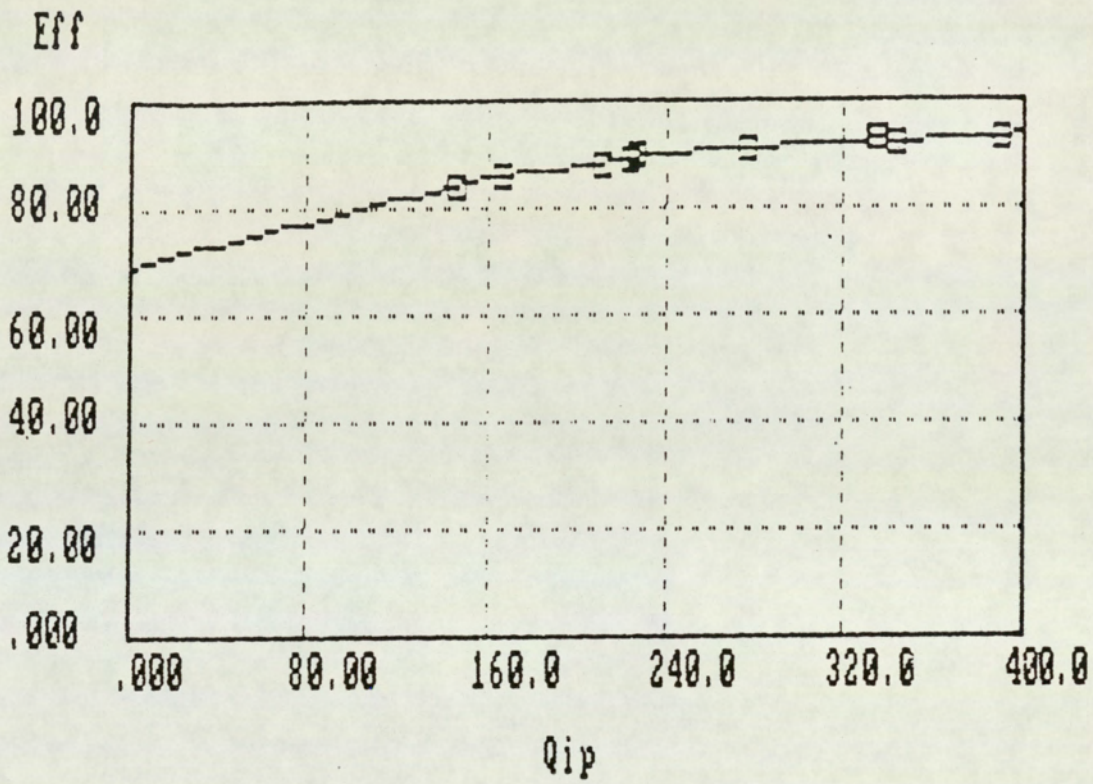
Incubations were performed in the presence or absence of NADP or with heat denatured microsomes (kept at 100°C for 90s) to assess the role of metabolism in covalent binding. The role of cytochrome P450 monooxygenases was investigated by incubating microsomes isolated from mice pretreated with phenobarbitone (50mg/kg/day i.p. for 4 days) or SKF525A (60mg/kg i.p.). Microsomes were also incubated in the presence of SKF525A (0.1 and 1mM). GSH (10mM) was added to incubations containing methyl-$^{14}$C-NMF or ethyl-$^{14}$C-NEF to evaluate its influence on the association of radioactivity to microsomal protein.

## 3.3 Liquid scintillation counting

$^{14}C$-labelled N-alkylformamides have been used in this investigation as markers in chromatographic procedures (see sections 3.7 and 3.8) and to determine covalent binding of NMF and NEF metabolites to liver macromolecules (section 3.2). In these experiments radioactivity was quantified in a Packard Tricarb 2000CA scintillation counter. The counter was operated in the external standardisation mode and the counting efficiency of the samples was determined by constructing a quench curve using the following general method: $^{14}C$-Hexadecane standard (5µl, 0.868 x $10^6$ dpm/ml) was added to 10 scintillation vials containing sequentially increasing volumes of the quenching agent, chloroform (0 - 800µl). The counting efficiency of the prepared samples, defined as the ratio of the observed cpm to the known dpm, was determined from the count rate using the transformed spectral index of the external standard (Qip:tSIE) from the calibration curve (fig 8). The counting efficiency of all samples was > 80% and always within the tSIE range of the quenched standards.

Figure 8

Calibration quench curve of counting efficiency (Eff).



## 3.4 Studies of the cytotoxicity of NMF and structural analogues in isolated and cultured hepatocytes.

### 3.4.1 Preparation of isolated mouse hepatocytes

The method used for the preparation of isolated hepatocytes was based on the collagenase perfusion methods of Berry and Friend (1969) and Seglen (1973) and adapted by the method of Klaunig et al (1981) for the particular problems posed by mouse livers.

The perfusate was pumped with a Gilson minipuls 2 peristaltic pump through tubing connected to a heating coil to maintain the perfusate at 37°C and to a bubble trap to prevent air from entering the vena cava during the retroperfusion. The flow rate was maintained at 8ml/min throughout the perfusion and the perfusate was not oxygenated or recirculated. The pH of the perfusate was kept at pH 7.3 by the addition of HEPES (10mM).

Balb/C mice were anaesthetised with an overdose of sagatal (sodium pentobarbitone, 200mg/kg) given via the i.p. route. A ventral mid-line incision was made and the abdominal skin pinned back. The intestines were deflected to the animals left and the liver and hepatic portal vein were exposed. The chest cavity was opened to expose the heart and a loose tie of cotton was placed around the inferior vena cava. The hepatic portal vein was cut before cannulating the inferior vena cava using a 20-gauge plastic cannula via an incision in the right atrium of the heart. This ensured that the perfusate flowed freely through the

liver and out of the portal vein thus preventing a build up of pressure in the organ which could damage the hepatocytes. The cannula was secured in place by tightening the loop of cotton around the vena cava and the initial perfusate, $Ca^{2+}$-free HBSS, was allowed to flow through the liver for 5min. This process clears the organ of extracellular $Ca^{2+}$ which is important for strengthening intercellular bonds (Guguen-Guillouzo and Guillouzo, 1986). Following perfusion with the $Ca^{2+}$-free medium, a solution of collagenase in Krebs-Henseleit buffer (80-100mg/60ml) containing $Ca^{2+}$ (5mM) was perfused to digest the liver. A honeycomb pattern appeared on the surface of the liver which was held together by the outer membrane. The liver was carefully cut from the animal and placed in a petri dish containing ice-cold incubation medium. The incubation medium was Krebs-Henseleit buffer supplemented with glucose (0.2%) horse serum (10%) and bovine serum albumin (0.2%) which have been reported to prolong the viability of isolated hepatocytes (Benford and Hubbard, 1987). The gall bladder was removed and the liver was gently dispersed. The suspension obtained was filtered through nylon mesh (125µm) to separate the cells from the white stringy connective tissue. The cells were allowed to sediment on ice and were washed three times in incubation medium to remove non-viable cells and debris. The cell yield was determined by counting using a Neubauer haemocytometer. Yields were between 34-41 x $10^6$ cells/g liver and on average 37 $\pm$ 2 x $10^6$ cells/g liver (n = 8).

### 3.4.2 Hepatocyte viability

Hepatocyte viability was determined by assessing the ability of cells to exclude trypan blue immediately after washing. Trypan blue (3%, 100µl), incubation medium (800µl) and cell suspension (100µl) were mixed together. After 2min the cells with or without a blue stained nucleus were counted using the haemocytometer and viability was calculated as the percentage of trypan blue-negative cells. Healthy hepatocytes were normally round and always had a well-defined refractive shape (fig 9). The latter phenomenon seems to be a good indicator of cell viability (Guguen-Guillouzo and Guillouzo, 1986). Cells with viabilities of > 80% were routinely obtained.

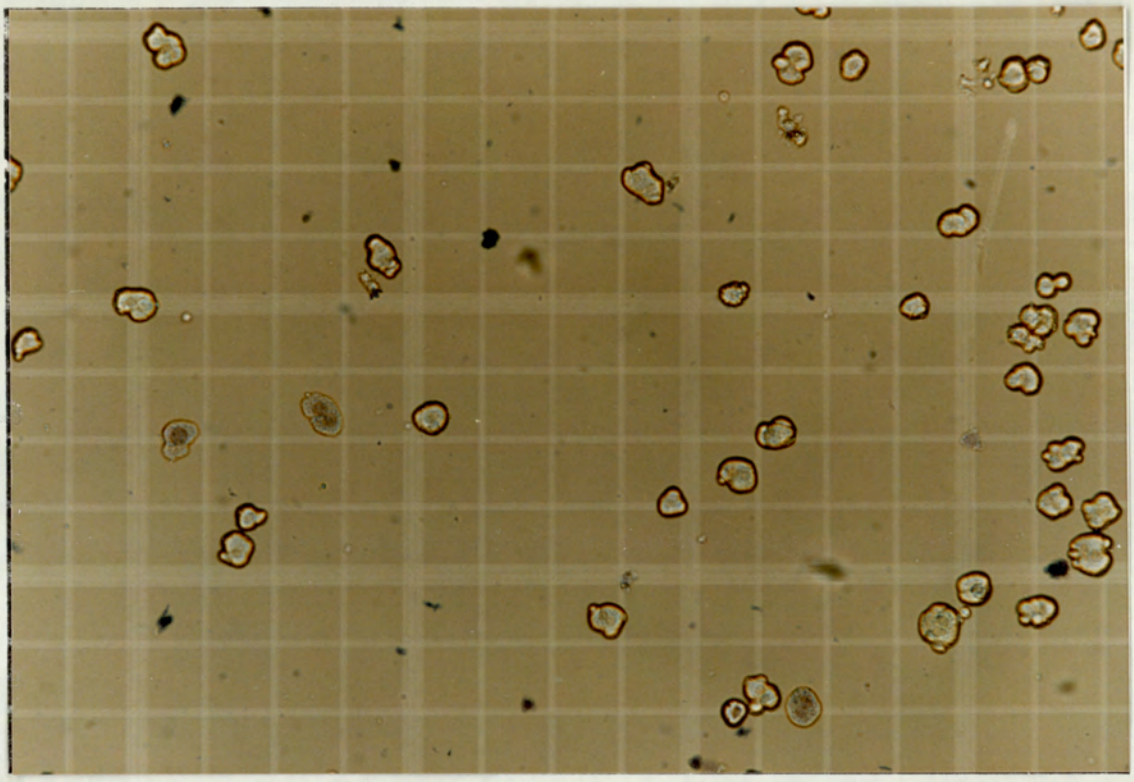
### 3.4.3 Preparation of isolated human hepatocytes

Hepatocytes were isolated by Dr. C. K. Chipman at the Department of Biochemistry, University of Birmingham from the liver of a 4 year old male accident victim (Guguen-Guillouzo et al, 1982). The cells were suspended in ice-cold $Ca^{2+}$-HBSS (pH 7.4) prior to incubation. Cell yield and viability were determined as described for mouse hepatocytes.

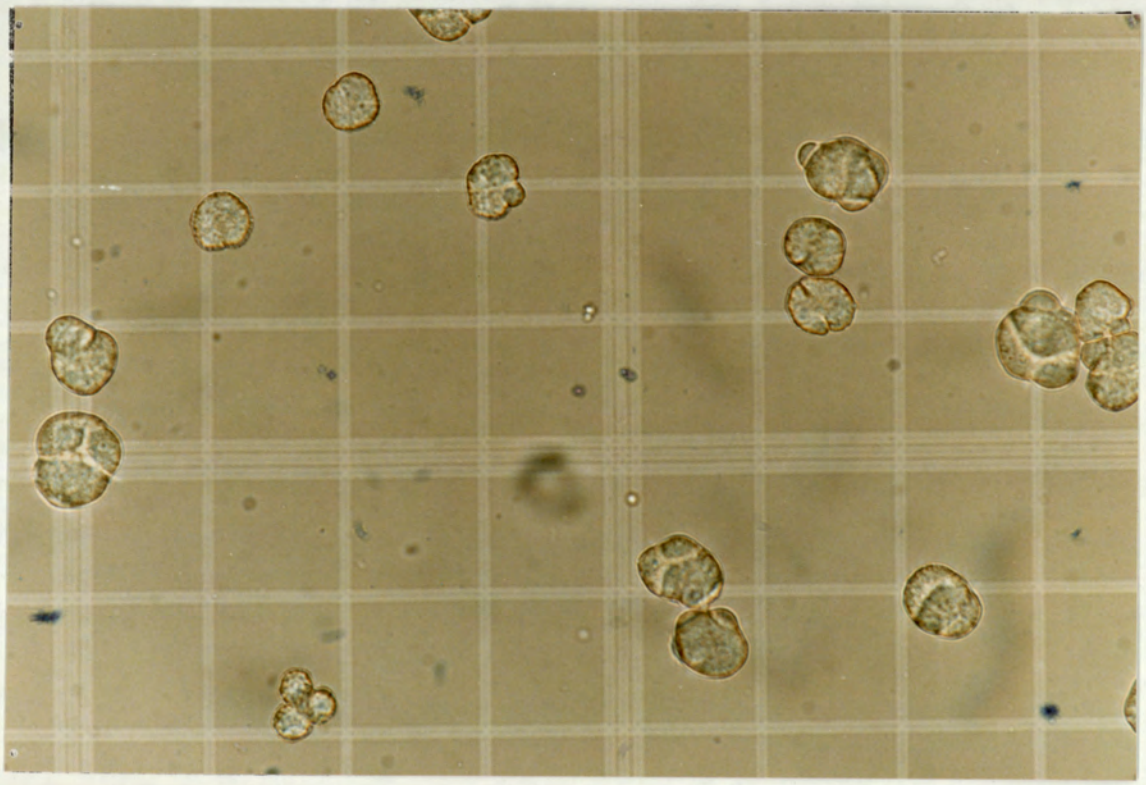
Figure 9

**Isolated mouse hepatocytes after exposure to trypan blue (0.3%w/v).**

(a) 60x Magnification



(b)120x Magnification



### 3.4.4 Suspension of hepatocytes

Suspended hepatocytes were incubated for up to 7h in 25ml silanised conical flasks gassed with 95% $O_2$: 5% $CO_2$. The incubation flasks were maintained at 37°C in a shaking incubator (Mickle Laboratory Engineering Co., Gomshall, U.K.). Incubations comprised of cell suspension, incubation medium and 100µl of diluted substrate to give a final volume of 3.5ml and an optimum cell density of 1.4 $\pm$ 0.1 x $10^6$ cells/ml (see section 4.3.2). Isolated hepatocytes were exposed for 6h to the following concentrations of formamides: NMF (0.1, 1, 5, 10 and 50mM), NEF (5mM), F (10, 50 and 100mM), DMF (10, 50 and 100mM), NMA (10, 50 and 100mM) and $^2$H-NMF (5mM). Aliquots of cell suspension (100µl) were removed for determination of extracellular LDH activity 2, 4 and 6h after the addition of the amides to the incubations.

The role of hepatic glutathione as protectant against formamide-induced cytotoxicity was assessed by incubation with NMF (10mM) of hepatocytes isolated from mice 4h after treatment with BSO (1600mg/kg).

### 3.4.5 Culture of mouse hepatocytes

Hepatocytes were cultured by the procedure outlined by Hayes and Pickering (1985) and adapted for mouse cells by the method of Maslansky and Williams (1982).

Mouse hepatocytes were isolated as previously described (section 3.4.1) with the following alterations: All

solutions were prepared in a Gelaire BSB3 lamniflow cabinet using sterile ingredients and perfusates and incubation media were supplemented with gentamycin. Collagenase was dissolved in William's E medium (pH 7.4) containing HEPES (10mM). The perfusion tubing was washed with 70% alcohol followed by sterile water prior to the perfusion. Following perfusion the digested liver was placed in a sterile beaker containing culture medium (section 2.9). All subsequent manipulations were conducted in a sterile cabinet. After sedimentation and washing three times the cells were plated out at a density of $5 \times 10^5$ cells/ml in 2.5ml of culture medium onto Linbro tissue culture wells (Gibco Ltd., Paisley, U.K.). The cells were incubated at 37°C in a 5% $CO_2$: 95% air (v/v) humidified incubator (Flow laboratories) for 3h to allow cell attachment to the plastic surface. After this time period medium with unattached cells was removed and the attached cells were refed with fresh culture medium. After incubation for a further hour NMF dissolved in culture medium (100µl) was added to the wells to give a final NMF concentration of 10mM. The cells were then incubated for up to 24h. An aliquot of the culture medium (250µl) was removed from the wells for analysis of LDH activity throughout the incubations.

### 3.4.6 Determination of cytotoxicity

Cytotoxicity of the amides was assessed by the determination of the release from cells of lactate dehydrogenase (LDH) according to Leathwood and Plummer

(1969). LDH catalyses the reduction of pyruvate to lactate at the expense of NADH. Aliquots of the incubation medium were centrifuged at 6500rpm for 2min in a MSE Micro Centaur centrifuge. The supernatant was removed and kept on ice. Supernatant (40-200µl) was added to a cuvette kept at 37°C containing phosphate buffer (pH 7.4) and NADH solution (100µl, 3.5mM) in a final volume of 3ml. After 2min the LDH reaction was initiated by addition of sodium pyruvate (100µl, 32mM) to the cuvette. The rate of reaction was monitored in a Cecil CE594 spectrophotometer by the change in absorbance at 340nm for 5min. Cytotoxicity was expressed as a percentage of maximal release of LDH. Maximal LDH release was determined after the addition of Triton-X100 (13µl, 16% v/v) to an aliquot of the cellular incubate (200µl) and the measurement of the change in absorbance at 340nm for 5min.

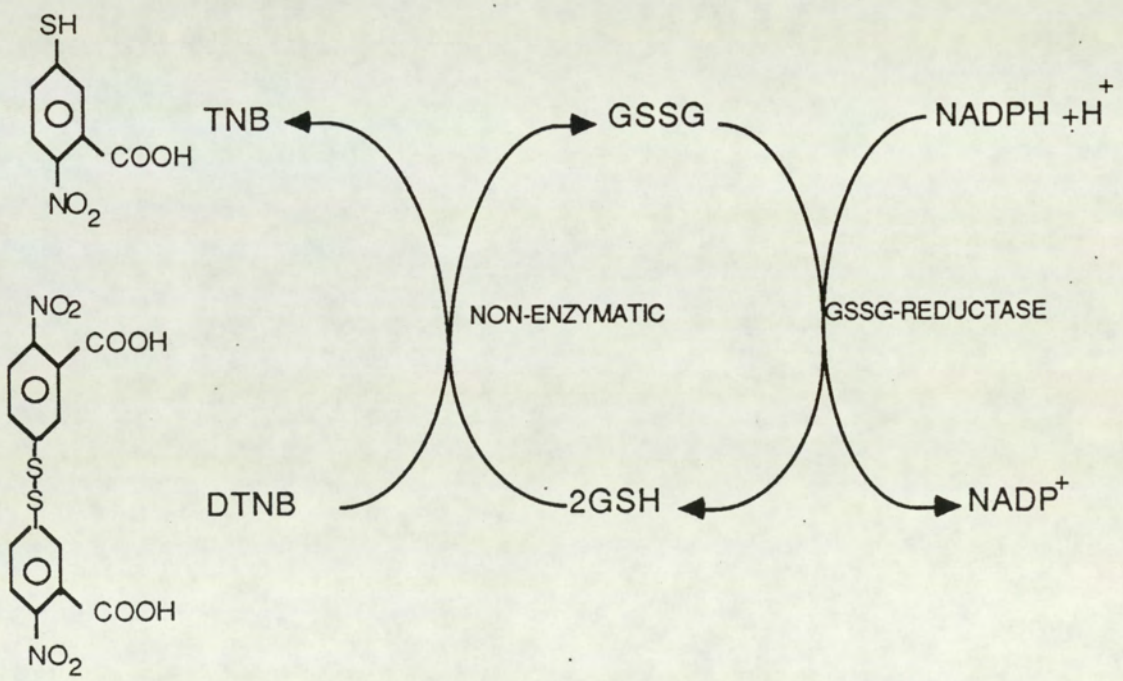
LDH activity was inhibited by high concentrations of amides (> 50mM) and thus made the determination of cytotoxicity in such cases inaccurate. In these instances cytotoxicity was determined by assessing trypan blue exclusion.

## 3.5 The effect of formamides on glutathione status in isolated hepatocytes

### 3.5.1 Determination of intracellular glutathione

Glutathione was measured by the recycling method originally developed by Tietze (1969) as described by Akerboom and Sies (1981). In this kinetic assay GSH brings about the continuous reduction of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) at the expense of NADPH:

Figure 10: The reaction for the determination of glutathione



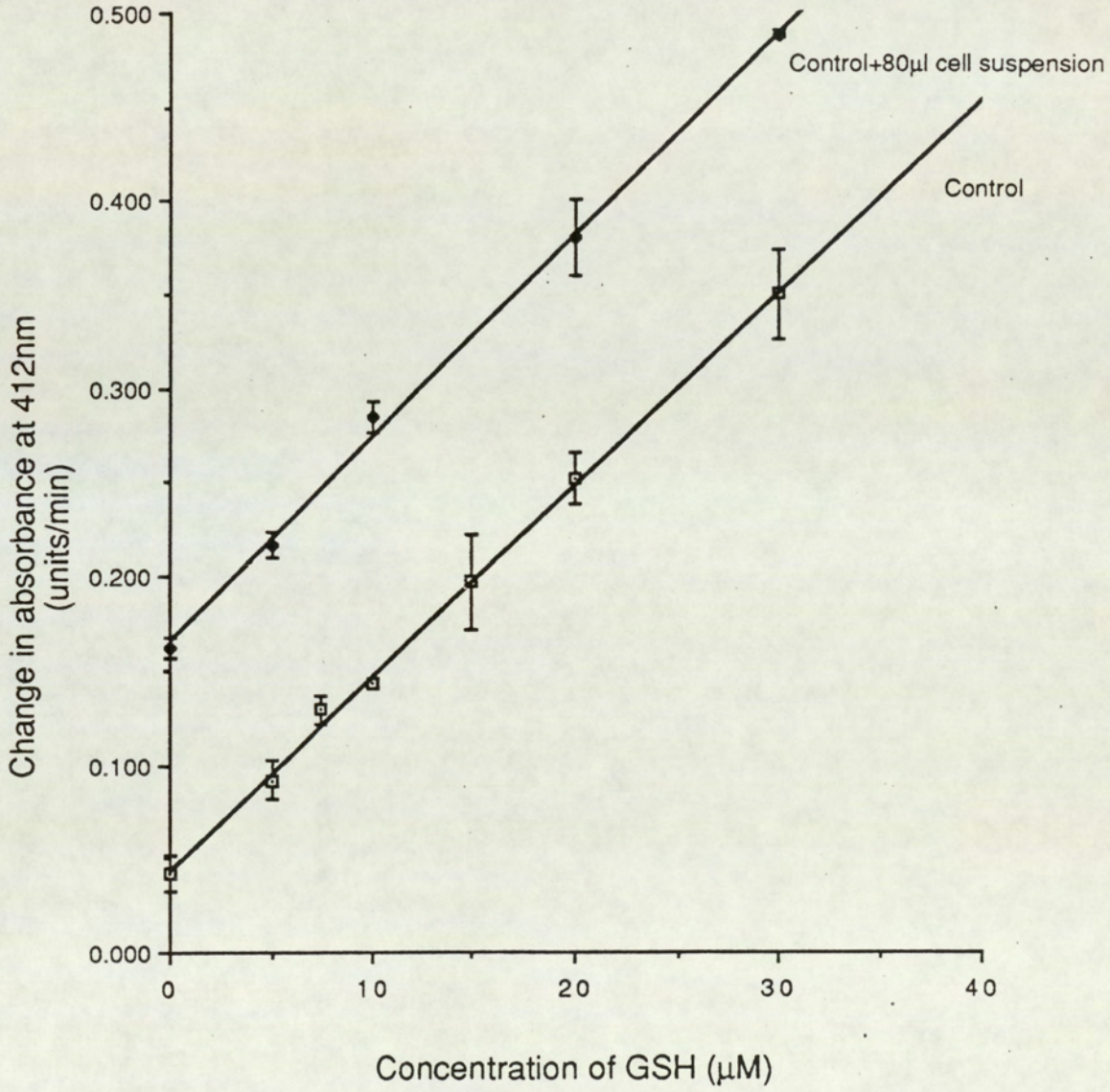

GSSG is reduced to GSH enzymatically by glutathione reductase. The reaction rate is proportional to the concentration of total glutathione (GSSG + GSH) and the formation of 5-thio-2-nitrobenzoate (TNB) is followed spectrophotometrically at 412nm.

To perform the determination, aliquots of the cell suspension (160µl) containing approximately 2 x $10^5$ cells were centrifuged at 6500rpm for 2min in a MSE Microcentaur centrifuge. The supernatant was discarded and the pellet was resuspended in 320µl of 10% w/v metaphosphoric acid. After vigorous shaking the suspension was centrifuged at 6500rpm for 2min. The acidic supernatant (250µl) was adjusted to pH 7.2-7.3 by the addition of a hot solution of trisodium orthophosphate (200µl, 1.3M). An aliquot (140µl) of this neutralised mixture was transferred to a reduced volume 1cm pathlength quartz cuvette in a thermostated holder (30°C) of a Cecil CE594 spectrophotometer. Solutions of NADPH (700µl, 300µM) and DTNB (100µl, 6mM) were added to the cuvette and the reaction was initiated by the addition of a solution of glutathione reductase (10µl, 70000 i.u./l). The change in absorbance at 412nm was monitored for 5min.

A series of glutathione standards (5-50µM) were prepared in 10% w/v metaphosphoric acid and subjected to the same analytical procedure to produce a calibration line. The rate of enzyme reaction was proportional to GSH concentration within the range of standards prepared. Addition of cell suspension (80µl) to glutathione standards produced a calibration line parallel to that produced by standards alone indicating that the cells did not interfere with the assay (fig 11).

Figure 11

**Calibration of glutathione concentration vs initial rate of reaction.**




Values are mean ± S.D. of 3-4 separate determinations.

### 3.5.2 Chemical depletion of GSH

The ability of NMF and analogues to deplete hepatocytic glutathione was evaluated in isolated mouse hepatocytes prepared as described in section 3.4.1. The following formamides were exposed for 4h to suspended hepatocytes isolated from Balb/C mice: NMF (0.1, 1, 5, 10 and 100mM), NEF (5mM), F (10mM), DMF (10mM), NMA (10mM) and $^{2}$H-NMF (5mM). The level of intracellular glutathione was determined 2, 3 and 4h after addition of the amides to the incubation medium and expressed as a percentage of the level of glutathione in hepatocytes not exposed to formamides.

Depletion of intracellular glutathione was achieved by incubating mouse hepatocytes with diethylmaleate (DEM, 0.02% v/v) for 15min.

### 3.5.3 Inhibition of GSH biosynthesis

GSH biosynthesis in the livers of Balb/C mice was inhibited with DL-buthionine-[S,R]-sulphoximine (BSO). BSO was dissolved in 0.1N NaOH and then adjusted to pH 7.4 with 0.1N HCl to provide a dose of 1600mg/kg in a volume of 500µl (Drew and Miners, 1984). The dose was administered as a single i.p. injection given 4h before isolation of hepatocytes.

## 3.6 Studies of the generation of carbamoylating metabolites from formamides by isolated hepatocytes and liver fractions.

### 3.6.1 Determination of N-alkylcarbamoylating metabolites

The method used here is a modification of the procedure described by Mraz and Turecek (1987) for the determination of N-acetyl-S-(N-methylcarbamoyl)cysteine (SMM) in urine samples. A sample of the incubate (320µl) was diluted with distilled water (80µl) and mixed in a glass stoppered tube with propanol (0.8ml) containing quinoline (500µM) as internal standard. Powdered anhydrous potassium carbonate ($K_2CO_3$, 0.6g) was added and the mixture was shaken for 2min. The principle of the method is encapsulated in the following equation:

$$H_3C(CH_2)_nNHCOR + C_3H_7OH \text{ --------> } H_3C(CH_2)_nNHCOOC_3H_7$$

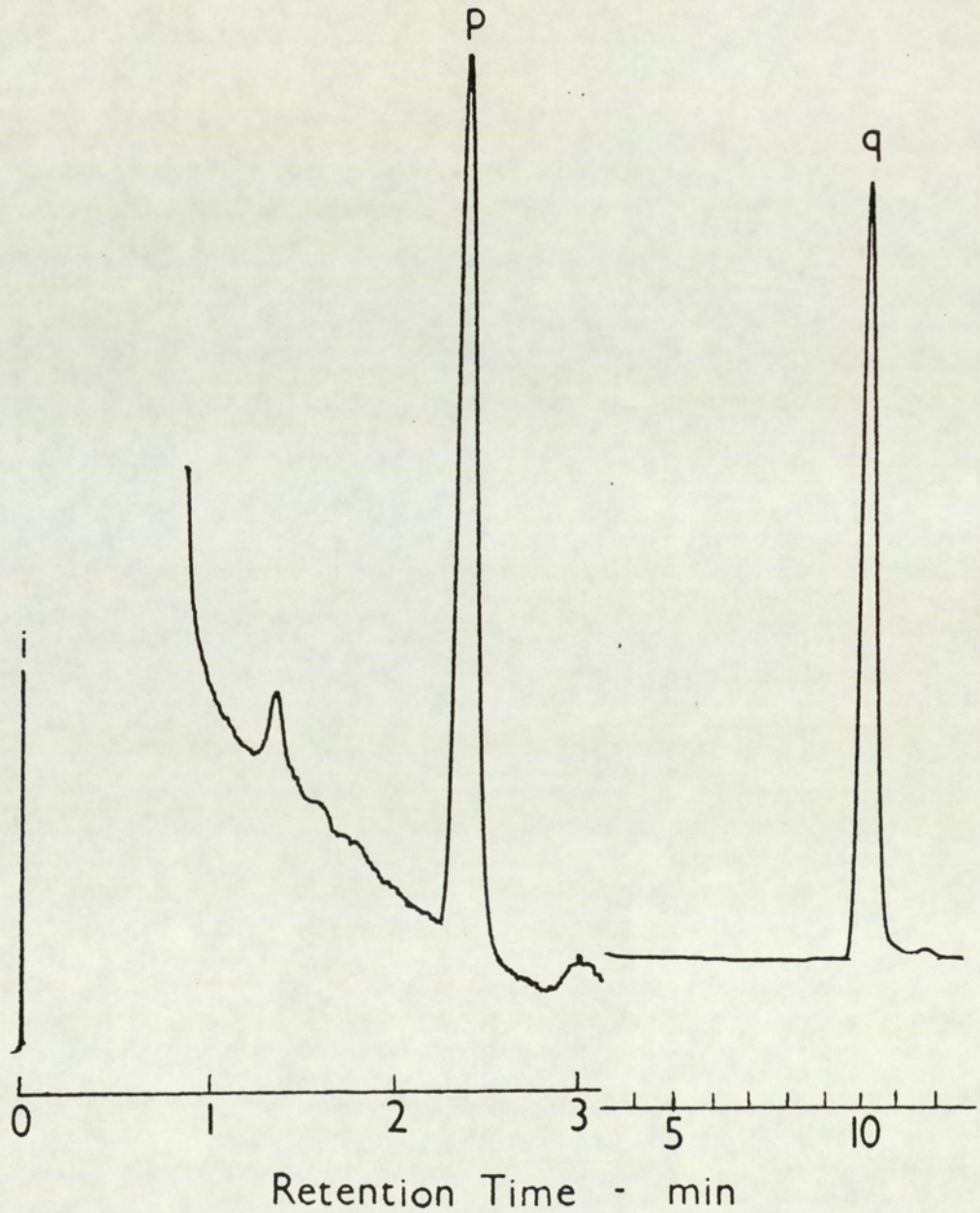
Any N-alkylcarbamoylating species ($H_3C(CH_2)_nNHCOR$) such as SMG (n = 0, R = glutathionyl) thus reacted to give propyl N-alkylcarbamate. The mixture was centrifuged at 3000rpm for 2min in a Heraeus labofuge 6000 centrifuge. An aliquot of the organic layer (1µl) was analysed by gas-liquid chromatography (GLC) using a glass column (1.5m x 2mm) packed with 5% potassium hydroxide and 10% Carbowax 20M on silanised Chromosorb W, 80-100 mesh, in a Pye Unicam series 204 chromatograph. The temperature of the injection port, column and detector were 200, 170 and 250°C respectively. Nitrogen was used as the carrier gas (50ml/min). Detection

was achieved by a nitrogen sensitive detector and displayed on a Gallenkamp pen recorder. The retention times of propyl N-alkylcarbamate and quinoline (internal standard) varied little as long as the flow rate of the carrier gas was constant (fig 12). The retention time of propyl N-alkylcarbamate was 142.5 $\pm$ 3.4s (n = 14). In the original method (Mraz and Turecek, 1987) ethanol was used as derivatising agent but control cells treated in this way displayed a peak on GLC analysis which cochromatographed with ethyl N-methylcarbamate (N-methylurethane).

Calibration curves (0-100µM) were constructed using SMG and SEG. The generation of propyl N-alkylcarbamate from these standards was influenced in an adverse manner by the presence of incubate. The dilution of incubate with distilled water enhanced the detection of propyl N-alkylcarbamate. Therefore the calibration curves were constructed using the same dilutions as used in the case of the samples. The authentic standards, diluted in distilled water (80µl), were added to control incubate (320µl), derivatised with propanol and analysed by GLC (figs 13 and 14). Initially SMM was used instead of SMG as standard. This procedure proved to be unsatisfactory as the rate of conversion of SMM to propyl N-methylcarbamate was different to the carbamoylation of propanol by SMG or SEG. The amount of propyl N-alkylcarbamate generated by SMG and SEG was 77.2 $\pm$ 12.4% (n = 12) and 165.1 $\pm$ 31.7% (n = 9) respectively of that generated by SMM.

Figure 12

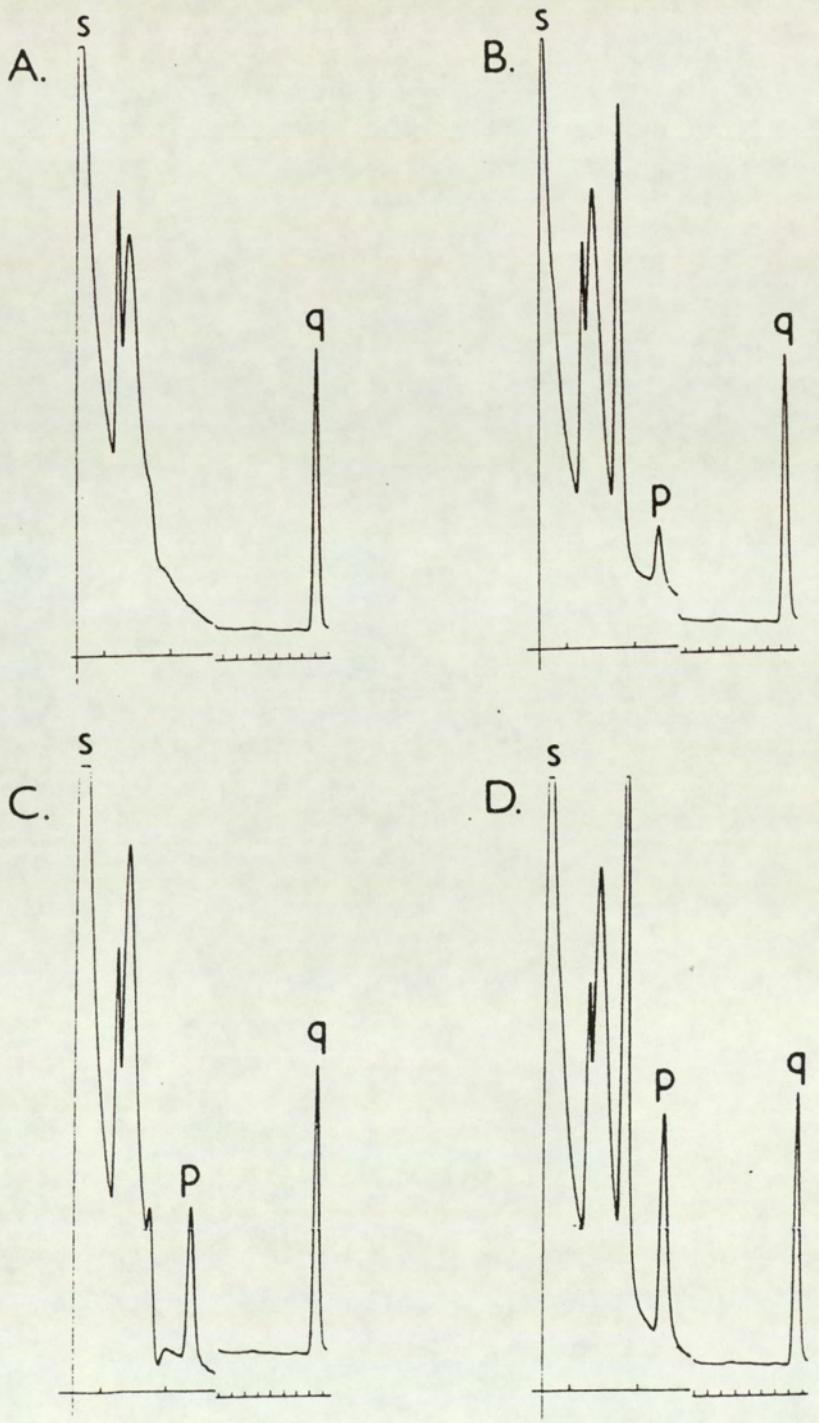
Gas-liquid chromatogram of a derivatised sample of human hepatocytes containing SMG (100μM).




Ionisation amplifier was set at 8 (0-3min) or 320 (3-12min). Abbrev: i, injection of sample; p, propyl-N-methylcarbamate; q, quinoline.

Figure 13

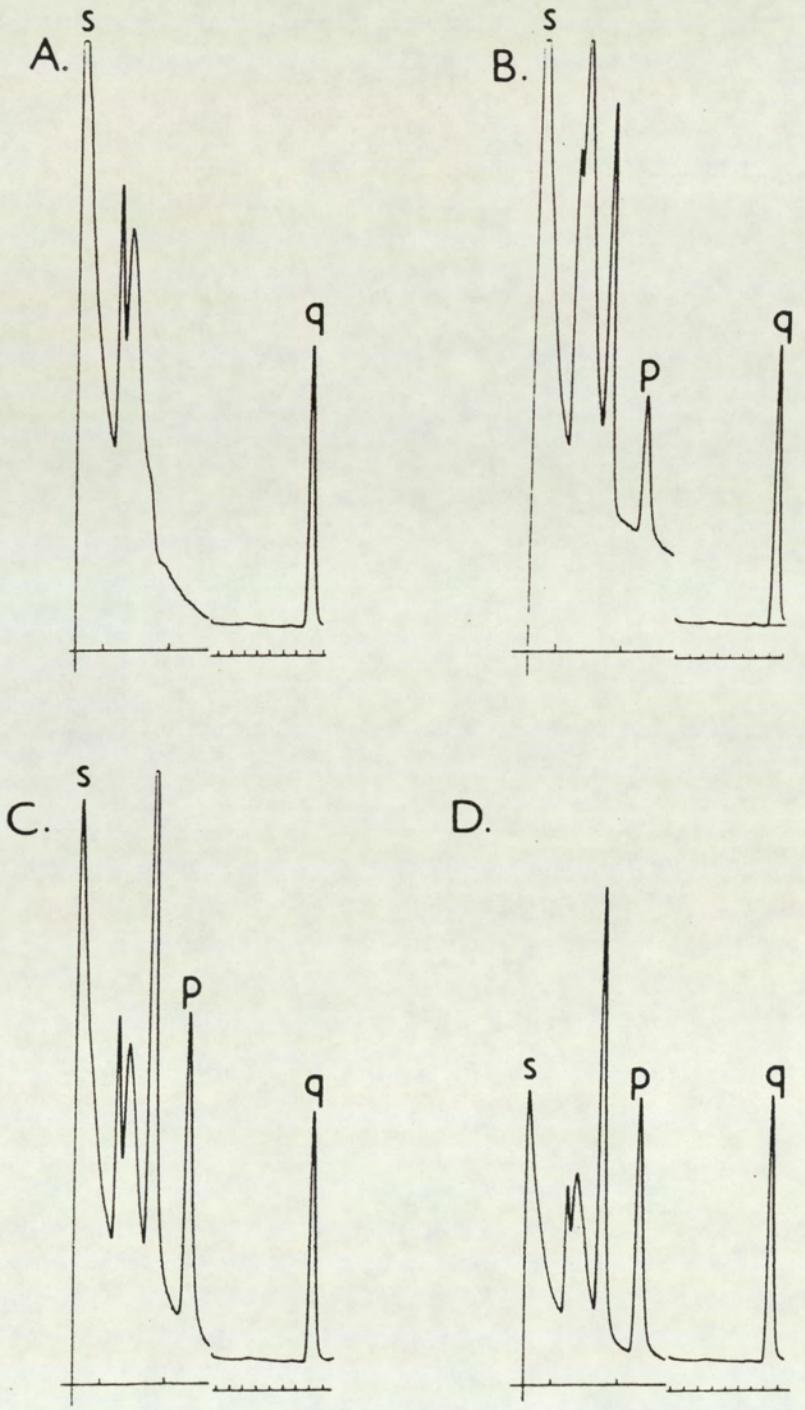
**Gas-liquid chromatograms of derivatised samples of mouse hepatocytes containing SMG (A, control; B, 20μM; C, 60μM; D, 100μM).**




Ionisation amplifier was set at 8 (0-3min) or 160 (3-12min).
Abbrev: s, solvent front; p, propyl-N-methylcarbamate; q, quinoline.

Figure 14

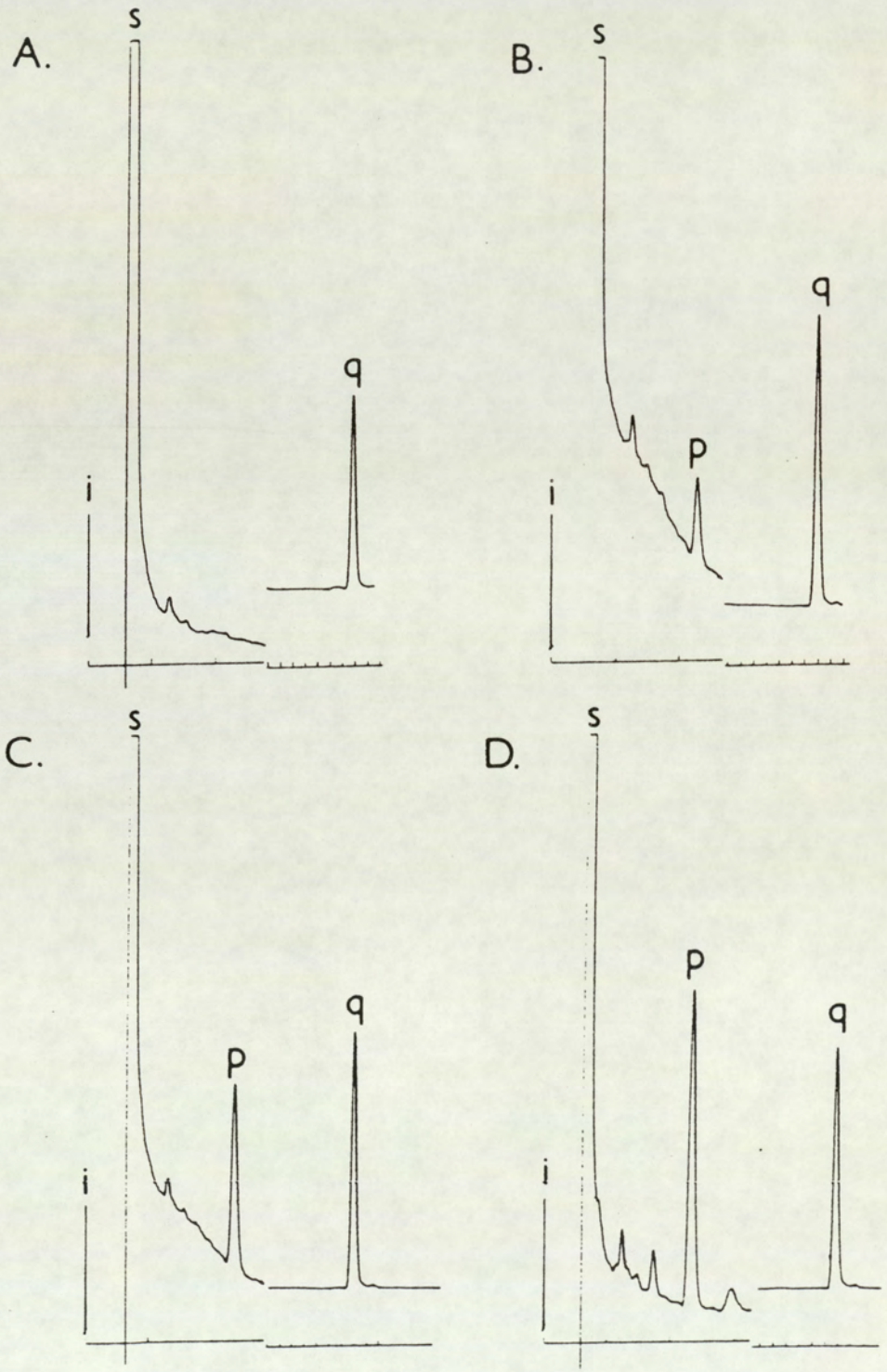
**Gas-liquid chromatograms of derivatised samples of mouse hepatocytes containing SEG (A, control; B, 20μM; C, 60μM; D, 100μM).**




Ionisation amplifier was set at 8 (0-3min) and 160 (3-12min) except for D where it was set at 16 (0-3min) and 160 (3-12min).
Abbrev: s, solvent front; p, propyl-N-ethylcarbamate; q, quinoline.

Figure 15

**Gas-liquid chromatograms of derivatised samples of human liver microsomes containing SMG (A, control; B, 20μM; C, 60μM; D, 100μM).**




Ionisation amplifier was set at 8 (0-3min) and 320 (3-12min).
Abbrev: i, injection of sample; s, solvent front; p, propyl-N-methylcarbamate; q, quinoline.

The limit of detection for the thiocarbamate by the method described was 2µM (approximately 2nmol/$10^6$ cells). In order to detect lower concentrations of carbamoylating metabolites, in particular in human hepatocytes and liver fractions, the following modification was made to the above procedure: The mixture of incubate and propanol was centrifuged at 3000rpm for 2min to remove the precipitated protein, and $K_2CO_3$ (0.6g) was added to the supernatant. After vigorous shaking for 2min the mixture was recentrifuged and the organic layer removed. The organic layer was evaporated at 10-20°C to a volume of 100µl in a Howe Gyrovap sample concentrator connected to a Savant RT-100A refrigerated condensation trap. To this concentrated sample was added 1ml of distilled water and 1ml of ethyl acetate. After shaking, the organic layer was removed and carefully concentrated by evaporation until a volume of 100-200µl remained. A sample of this extract (1µl) was analysed by GLC using the conditions described in this section. The resulting chromatogram (figs 12 and 15) was "cleaner" and the detection limit for the thiocarbamate was improved to 0.5µM.

The method described for the detection of thiocarbamate was characterised by the following coefficient of variation values calculated for 6 samples prepared with two concentrations of SMM (20 and 100µM): 6.70% for 20µM and 4.52% for 100µM obtained within one day and 7.79% for 20µM and 3.94% for 100µM obtained between 6 days. It must be emphasised that this indirect method measured all N-alkylcarbamoylating compounds and not specifically SMG or

SEG.

### 3.6.2 Studies of metabolism using isolated hepatocytes

Balb/C mouse and human hepatocytes were isolated as previously described in sections 3.4.1 and 3.4.3. Incubations (3.5ml, 1.4 x $10^6$ cells/ml) were performed for 6h after the addition of substrate diluted with incubation media (100µl). The following concentrations of formamides were incubated with isolated mouse hepatocytes: NMF (0.1, 1, 5, 10 and 100mM), NEF (5mM), $^2$H-NMF (5mM), F (10 and 50mM), DMF (10 and 50mM) and NMA (10 and 50mM). Isolated human hepatocytes were exposed to NMF (0.1, 1, 5 and 10mM), DMF (10mM) and HMMF (10mM). Aliquots (320µl) were removed for derivatisation and GLC analysis throughout the incubation period. The retention times of the formamides were approximately as follows: NMF, $^2$H-NMF and HMMF 175s, NEF 179s, F 286s, DMF 80s and NMA 160s. They did not chromatograph with propyl N-alkylcarbamate.

Mouse hepatocytes were sonicated (wave amplitude = 20µm) for 10 or 20s in a MSE sonicator before incubation with NMF (5mM) to investigate whether cell viability was required for metabolism. Incubations were also performed with hepatocytes isolated from Balb/C mice pretreated with BSO (1600mg/kg) and with mouse hepatocytes in the presence of DEM (0.02% v/v) to evaluate the importance of intracellular glutathione levels for the metabolism of NMF to N-methylcarbamoylating metabolites.

3.6.3 Studies of metabolism using liver fractions

Balb/C mouse liver post-mitochondrial supernatant and microsomal fractions were prepared as described in section 3.2.1. Incubations were performed with NMF (10mM) in the presence of NAD (1mM), NADP (1mM) or an NADPH-generating system to try to ascertain the nature of the enzymatic conversion of NMF to the N-methylcarbamoylating species.

Human liver post-mitochondrial supernatant was prepared from the liver of a 20 month old male accident victim. After weighing, the liver sample was cut into small pieces and homogenised in ice-cold phosphate buffer (50mM, pH 7.4) with 8 return strokes of a Camlab 563C homogeniser (speed 6) fitted with a teflon pestle. The 25% w/v homogenate was centrifuged at 10000g for 20min at 4°C in a MSE Pegasus ultracentrifuge. The post-mitochondrial supernatant was removed and aliquots (750µl) added immediately to 20ml flasks containing phosphate buffer and the following cofactors: G6P (5mM), G6PDH (2 i.u./ml), NADP (1mM) and $MgCl_2$ (5mM). The flasks were left for 5min in a shaking incubator (Mickle Laboratory Engineering Co., Gomshall, U.K.) at 37°C before addition of NMF, DMF or HMMF to give a concentration of 10 or 50mM in a final volume of 2ml. Incubations were performed for 1h after which aliquots (320µl) were removed for propanol derivatisation and GLC analysis.

The generation of N-methylcarbamoylating metabolites of NMF (10mM) by human liver post-mitochondrial supernatant was measured after incubation with GSH (10mM) or disulfiram

(50μM) in an attempt to further elucidate the metabolic process involved.

## 3.7 Determination of the kinetic deuterium isotope effect on the formation of the NMF metabolite SMG by isolated mouse hepatocytes

### 3.7.1 Incubation of hepatocytes

Hepatocytes were isolated from 2 Balb/C mice as described in section 3.4.1. The cells were pooled and had a viability of > 90% as determined by the exclusion of trypan blue (section 3.4.2). Four aliquots (2.5ml each) of the cell suspension (8 x $10^6$ cells/ml) were each added to 50ml round bottomed flasks each containing 7.8ml of Krebs-Henseleit buffer (pH 7.4). The incubation volume was designed to maximise the production of SMG. Likewise, horse serum and BSA were omitted to minimise the possible transfer of the N-methylcarbamoyl moiety of SMG to other thiol containing nucleophiles. An equimolar mixture of $^2$H-NMF and $^2H_3$-NMF (0.2ml) was added to each of 2 flasks while the other 2 each received an equimolar mixture of $^2$H-NMF and $^{13}C_2{}^{15}$N-NMF (0.2ml). These mixtures also contained methyl-$^{14}$C-NMF (< 5% of total substrate) as a radiotracer. The final concentration of NMF in each incubation was 5mM with a specific activity of 48µCi/mmol as determined by scintillation counting (section 3.3). The flasks were rotated gently in an incubator kept at 37°C and were gassed with 95% $O_2$: 5% $CO_2$ throughout the 4h incubation.

3.7.2 Derivatisation of SMG

The derivatisation procedure employed was based on that used by Threadgill et al (1987) to purify SMG for MS analysis. In the original methodology SMG was reacted with ethyl chloroformate ($CH_3CH_2OCOCl$) to produce a N-ethoxy-carbonyl derivative which was then subject to HPLC purification. Here benzyl chloroformate (BCF, $C_6H_5CH_2OCOCl$) replaced ethyl chloroformate as the resulting N-benzyloxy-carbonyl derivative (BCF-SMG, fig 35) had two advantages over its ethoxy analogue: (i) BCF-SMG is more lipophilic and so was eluted more slowly during HPLC analysis. This aided the purification of the derivatised conjugate. (ii) BCF-SMG contains a chromophore and was therefore detectable at 254nm as it was eluted from the HPLC column.

Incubations were terminated by the addition of acetone (20ml) to precipitate the protein. After centrifugation at 500g for 5min the supernatant was removed and the acetone removed from it by gentle evaporation at 25°C under reduced pressure in a Buchi rotavapor R110. The aqueous liquid remaining was added to a separating funnel followed by 25ml of carbonate buffer (pH 9). To this alkaline mixture was added 1ml of BCF. After gentle shaking for 10min the unreacted BCF was removed by the addition of 30ml of methylene chloride ($CH_2Cl_2$) and removal of the organic layer. The aqueous solution remaining was acidified to pH 2 by the addition of acetic acid and was applied to a pre-washed $C_{18}$ Sep-Pak cartridge (Waters associates, Milford, Mass., U.S.A.). The cartridge was rinsed with

water (5ml) and BCF-SMG was eluted with high grade methanol (4ml). The methanol was evaporated under a stream of nitrogen and the dry residue dissolved in high grade methanol (1ml) for HPLC analysis (see section 3.7.3). Fraction 12 collected, which possessed the greatest radioactivity, was processed on a second Sep-pak cartridge as described above. The post Sep-Pak sample containing the benzyloxycarbonyl derivatives and > 95% of the radioactivity eluted in fraction 12 was evaporated to dryness with nitrogen. The dried sample was treated with methanolic HCl (3ml, pH < 2) at room temperature for 2h in order to esterify the acidic groups of BCF-SMG (fig 35). The esterified sample was evaporated to dryness, redissolved in 500µl of high grade methanol and subjected to HPLC analysis (see section 3.7.3). Fractions 15 and 16 collected possessed the greatest radioactivity and so were pooled and the methanol removed by evaporation in a stream of nitrogen. The remaining aqueous sample was processed on a third Sep-pak cartridge as described above. The sample was evaporated to dryness and used for fast atom bombardment mass spectrometric (FAB-MS) analysis (see section 3.7.4). Solutions of authentic SMG were derivatised in an identical fashion as described above for cell incubates.

### 3.7.3 HPLC analysis of SMG derivatives

HPLC purification of BCF-SMG and its dimethylester (BCF-SMG-E) was performed using a Beckman model 342 dual pump instrument equipped with a reverse phase Novapak ODS

column (15cm x 4.6mm internal diameter: Waters Associates). The mobile phase consisted of a methanol/water mixture with 1% acetic acid which was pumped through the column as a linear gradient increasing the methanol content from 20% to 70% within 20min. The mobile phase was maintained at 70% methanol for a further 5min before reversing the gradient to 20% methanol over an additional 10min period. The flow rate was held at 1ml/min and 1min fractions were collected. Aliquots (0.5ml) of these fractions were added to 4ml of scintillation cocktail and used for liquid scintillation counting (section 3.3). The recovery of radioactivity in the HPLC eluate was > 95% of that applied to the column.

The elution of authentic BCF-SMG and BCF-SMG-E, 12 and 15-16min respectively after injection on to the column, was followed using a fixed-wavelength (254nm) Beckman model 160 ultraviolet detector.

### 3.7.4 Mass spectrometry (MS)

The isotopic composition of $^{2}H$-NMF, $^{2}H_3$-NMF and $^{13}C_2{}^{15}N$-NMF or of approximately equimolar mixtures of $^{2}H$-NMF and $^{2}H_3$-NMF or of $^{2}H$-NMF and $^{13}C_2{}^{15}N$-NMF were determined by Dr P.G. Pearson at the University of Washington, Seattle, U.S.A. using a VG 70-70H double focussing electron impact (EI) mass spectrometer (VG Analytical Ltd., Manchester, U.K.). Samples (1µl) were injected via a ballested reference inlet and spectra were recorded using a VG 2035 data system. The scan rate was 2s/decade and the instrument resolution was 2000. The spectra in figures 16-18 represent

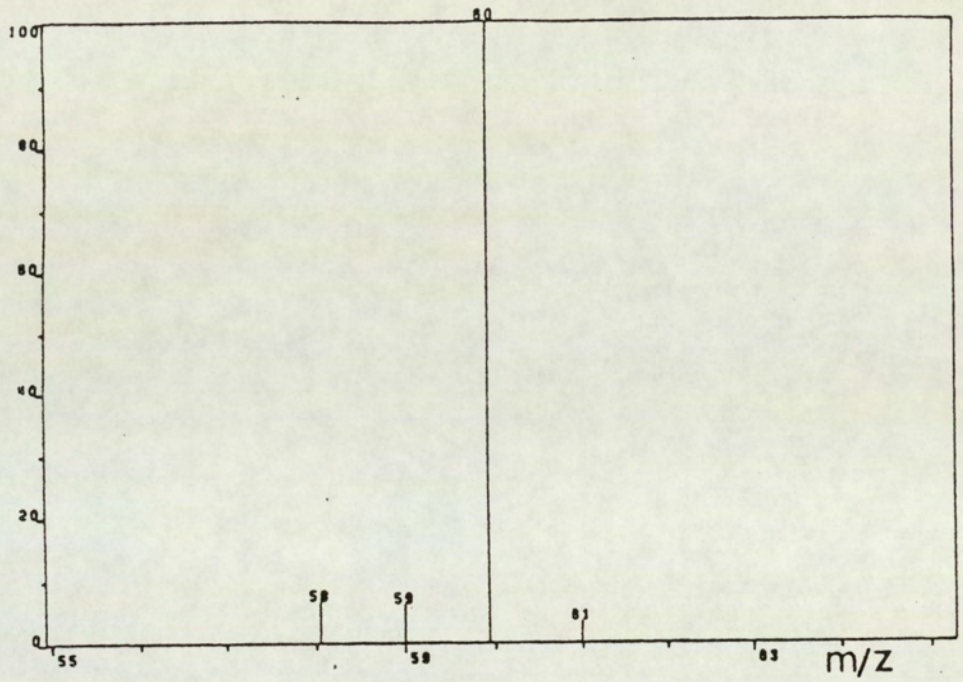
an average of 20 scans.

FAB-MS analysis of BCF-SMG-E and its isotopomers was performed by Drs W.N. Howald and P.G. Pearson at the University of Washington using a VG 70SEQ hybrid tandem instrument of EBQQ geometry (VG Analytical Ltd.) equipped with an Ion Tech fast atom gun and VG 11/250 data system. Samples (2-6µg) dissolved in methanol (3µl) were placed on a FAB target with glycerol (3µl) and 1µl of either 0.1N HCl, 0.1N NaCl or 0.1N KCl. Ionisation was achieved following bombardment with xenon (6keV) as a primary beam and FAB spectra were recorded, at an accelerating potential of 8kV, via the data system. Each sample was analysed 3 times and 5 scans (m/z 100-600) were averaged from each run to provide the peak intensities for the isotope effect calculation.

Figure 16

**EI-mass spectra of the deuterated analogues of NMF.**

(a) $^{2}H$-NMF




(b) $^{2}H_3$-NMF

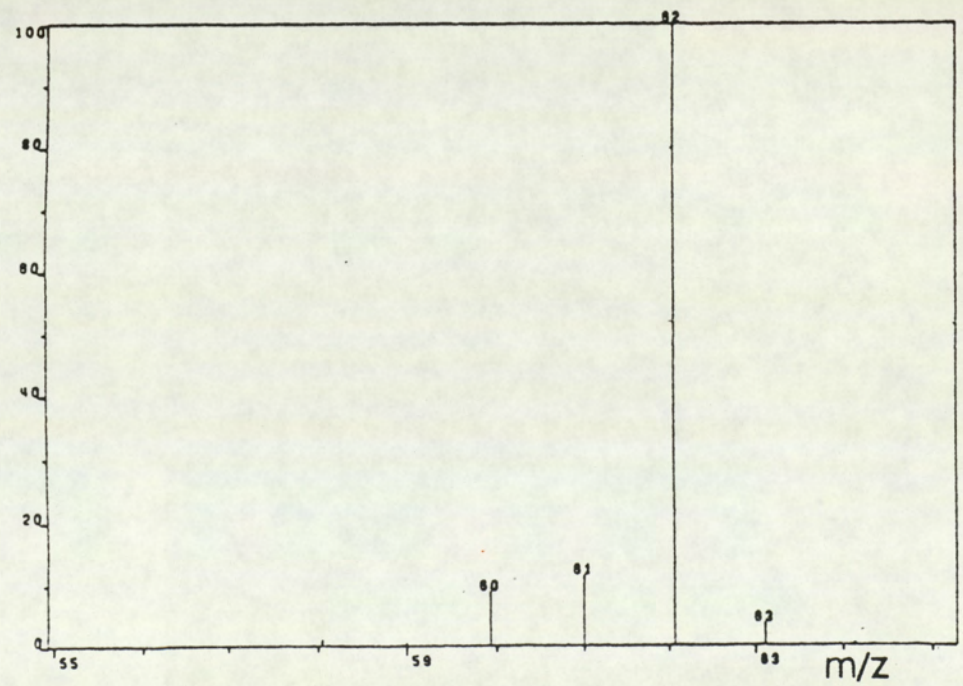


Figure 17

# EI-mass spectra of $^{13}C_2{}^{15}N$-NMF.

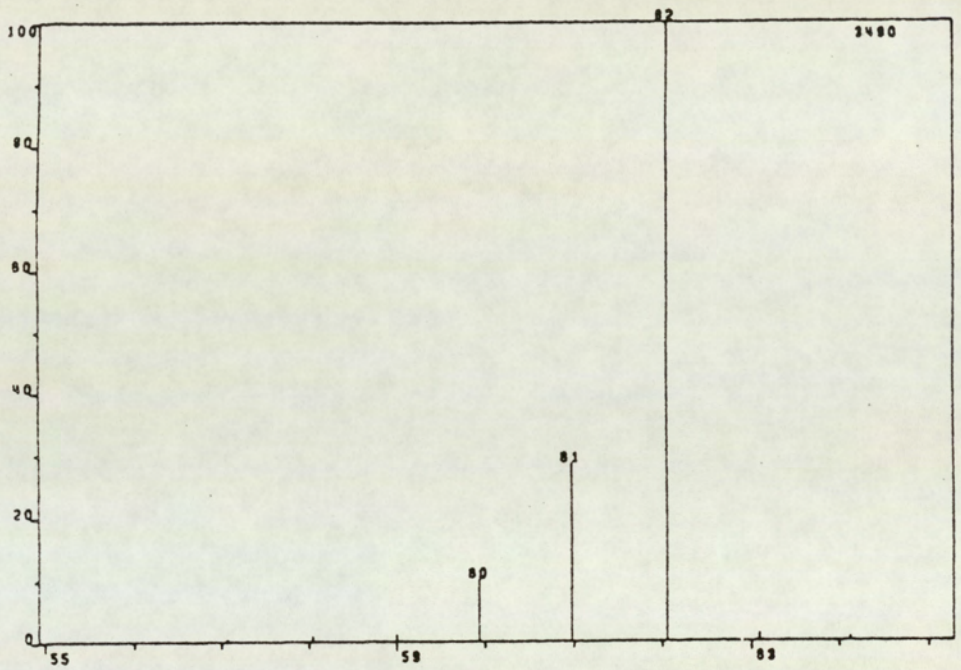
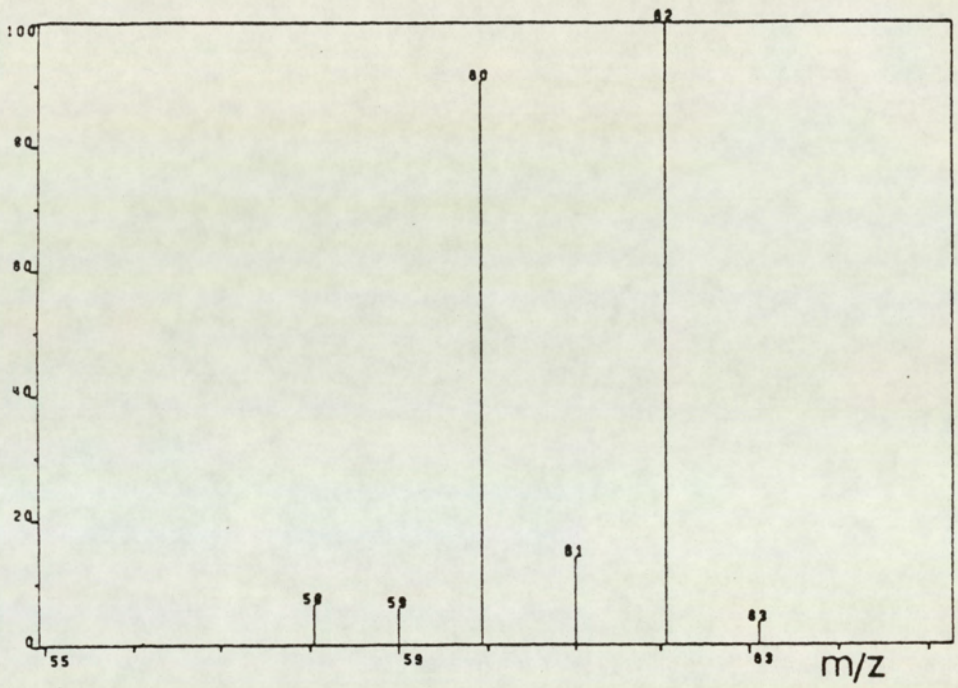


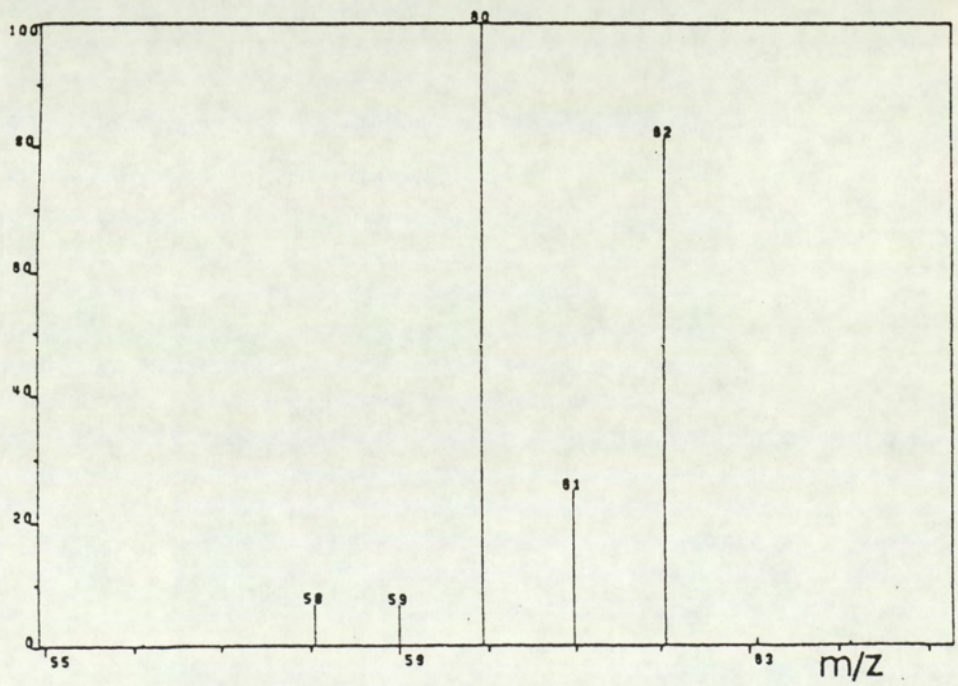
Figure 18

**EI-mass spectra of mixtures of stable isotope labelled analogues of NMF.**

(a) $^{2}H$-NMF: $^{2}H_3$-NMF




(b) $^{2}H$-NMF: $^{13}C_2{}^{15}N$-NMF



## 3.8 Determination of $^{18}O$ incorporation into SMG formed by isolated hepatocytes incubated with NMF and $^{18}O_2$

### 3.8.1 Incubation of hepatocytes with $^{18}O_2$

Hepatocytes were isolated from 2 Balb/C mice as described in section 3.4.1. The cells were pooled and had a viability of > 90% as determined by the exclusion of trypan blue (section 3.4.2). Four aliquots (1.4ml) of the cell suspension (8.5 x $10^6$cells/ml) were each added to 20ml pear-shaped flasks containing ice-cold Krebs-Henseleit buffer (3.4ml, pH 7.4). Bicarbonate, due to its ability to form gaseous $CO_2$, was omitted from the buffer and the concentration of HEPES was raised from 10 to 25mM. Horse serum and BSA were also omitted from the incubation media to prevent the possible loss of SMG by its transcarbamoylation of other thiol groups. Each flask was equipped with a small side arm containing the substrate dissolved in buffer (0.2ml). In 3 flasks the substrate was methyl-$^{14}$C-NMF (125mM, specific activity = 119µCi/mmol) while the fourth contained acetanilide (125mM). The flasks were connected by air-tight seals to a custom-made glass manifold which permitted the 4 separate incubations to be run simultaneously under identical conditions. By way of a three way valve the apparatus was connected to a vacuum pump, a cylinder of $N_2$ and a 500ml flask of $^{18}O_2$. The apparatus was evacuated to less than 10torr in 1min and then immediately filled with $N_2$ to restore atmospheric pressure. This procedure of evacuation and flushing was repeated 2

more times to remove any gaseous $O_2$ from the flasks. The apparatus was evacuated a fourth time and $^{18}O_2$ introduced to atmospheric pressure. All evacuations were conducted with the flasks on ice and pressures inside the glass manifold were monitored by an attached mercury manometer. A preliminary control experiment indicated that the evacuation and flushing procedure did not unduly affect the viability of the hepatocytes. The incubations were initiated by addition of substrate to the cell suspension by tilting the flasks. The cells were incubated for 4h in a gently shaking incubator at 37°C.

### 3.8.2 Analysis of SMG

Any SMG produced in the incubations containing methyl-$^{14}$C-NMF was converted to BCF-SMG-E and purified by reverse phase HPLC as described in sections 3.7.2 and 3.7.3. The dimethylester derivative was analysed by FAB-MS as described in section 3.7.4 except that the data system was inoperative and so spectra were recorded using a u.v. oscillograph.

### 3.8.3 Derivatisation of paracetamol (APAP)

The hepatocyte incubation containing acetanilide was terminated by the addition of acetone (10ml) to precipitate the protein. After centrifugation at 500g for 5min the supernatant was extracted and the acetone removed from it by gentle evaporation at 25°C under reduced pressure. The

aqueous liquid remaining was acidified with one drop of concentrated HCl and the acetanilide metabolite, APAP, was extracted with ethyl acetate (20ml). The organic extract was dried with anhydrous $MgSO_4$ and concentrated to dryness under a stream of dry nitrogen. A volatile trimethylsilyl (TMS) derivative of APAP was prepared by addition of bis(trimethylsilyl)trifluoroacetamide (BSTFA) to the dried residue and reaction at 70°C for 2h. A standard solution of APAP (12.5mM) in ethyl acetate was treated similarly. The mixtures were cooled and concentrated to dryness under a stream of $N_2$. In each case the remaining solid was resuspended in ethyl acetate (40µl) and analysed by gas chromatographic mass spectrometry (GC-MS) as described in section 3.8.4.

### 3.8.4 GC-MS analysis of the TMS derivative of APAP

GC-MS analysis of the TMS derivative of APAP was performed by Dr P.G. Pearson at the University of Washington using a VG 70-70H mass spectrometer equipped with a Hewlett-Packard Model 5710A gas chromatograph. The GC column was a 30m x 0.32mm internal diameter fused silica column coated with DB-5 as the stationary phase (J and W Scientific, U.S.A.). GC analysis of the TMS derivative of APAP was performed using the following conditions: carrier gas: Helium (head pressure 20psi); injection volume = 1µl; injector temperature = 250°C; temperature program; injection at oven temperature of 40°C then temperature raised rapidly to 90°C and programmed linearly to 250°C at 10°C/min. MS

analysis was performed in the EI mode at a scan rate of 1s/decade and spectra were recorded using a VG 2035 model data system.

# SECTION 4

# RESULTS AND DISCUSSION

## 4.1 Studies of the hepatotoxicity of formamides in mice

### 4.1.1 Introduction

During the phase I and phase II clinical evaluation of the potentially therapeutic antineoplastic activity of NMF, liver damage has been a major toxicity of the drug (section 1.3.1). Likewise, workers occupationally exposed to DMF have exhibited symptoms of liver and pancreatic damage (section 1.3.2) as well as alcohol intolerance (section 1.1.2). The toxicity of NMF and DMF has been studied in various species though primarily in rodents where the hepatotoxic potential of NMF appeared to be significantly greater than that of DMF (section 1.3). A study in three strains of mice showed a strain difference in the hepatotoxic potential of NMF with the Balb/C mouse proving the most susceptible (Langdon et al, 1985a).

In the present study the hepatotoxic potential of four structural analogues of NMF, namely DMF, F, NEF and NMA have been evaluated by histopathological examination of liver sections and measurement of liver-associated enzymes in the plasma to try and establish a structure-toxicity relationship for this group of compounds. Three liver-associated enzymes, SDH, ALT and AST were measured in the plasma of mice 24h after administration of the amides. The serum activity of these enzymes has previously been reported to be elevated in mice after administration of hepatotoxic doses of NMF and reached a maximum 24h after dosing (Whitby et al, 1984b; Langdon et al, 1985a; Pearson et al, 1986).

In healthy animals SDH activity is highest in the liver with levels three and five times greater than that in the prostate and kidney respectively, the only other organs with significant SDH activity (Gerlach and Hiby, 1974). As such measurement of SDH activity in the plasma is a relatively specific indicator of acute hepatic necrosis especially as SDH, unlike ALT and AST, is not present in erythrocytes and so its activity in the plasma is not influenced by the haemolysis of red blood cells (Rose and Henderson, 1975). ALT, like SDH, is present almost exclusively in the liver and is a good indicator of liver damage. Activity of AST is significant not only in hepatocytes but also in skeletal and cardiac muscle (Woodman et al, 1981). Consequently plasma levels of AST may be raised after necrosis in liver, muscle or heart tissue. The assessment of hepatotoxicity by the measurement of a panel of liver-associated enzymes in the plasma is the best determination of liver damage. Histopathological examination compliments these biochemical parameters and allows a more probing insight into the locus and nature of the damage.

The identification of the bilary metabolite SMG and corresponding urinary mercapturate SMM in mice administered NMF suggests that the bioactivation of this amide involves oxidation or dehydrogenation of the formyl moiety (section 1.1.1). In order to assess the importance of this reaction in the formation of a toxic intermediate the hepatotoxic potential of NMF was compared against two deuterated analogues, namely $^{2}$H-NMF ($^{2}$HOCHNCH$_3$) and $^{2}$H$_3$-NMF (HOCNHC$^{2}$H$_3$). In this experiment the hypothesis was tested

that NMF-induced toxicity is subject to a kinetic deuterium isotope effect with respect to the formyl carbon-hydrogen bond. Primary kinetic deuterium isotope effects on chemical-induced toxicity have previously been described: Deuterochloroform ($C^2HCl_3$) is 50-70% less toxic in rodents than the protium form indicating that metabolic cleavage of the carbon-hydrogen bond is involved in the formation of the ultimate toxic derivative (Branchflower et al, 1984). Conversely, 1,2-dibromoethane is less genotoxic than tetradeutero-1,2-dibromoethane ($BrC^2H_2C^2H_2Br$) showing that the deuterated analogue retards a competing detoxification pathway (White et al, 1983). If the cleavage of the formyl or methyl hydrogens is an integral part of the toxification or detoxification of NMF, a difference in the hepatotoxic potential of the protium and deuterium forms may be seen.

The possible role of the mixed function oxidase system in the metabolic activation of NMF was investigated in a preliminary fashion by treating Balb/C mice with the inhibitor SKF525A (proadifen) 1h prior to the administration of hepatotoxic and non-hepatotoxic doses of NMF.

## 4.1.2 Results

The hepatotoxic potential in CBA/CA mice of NMF and four structurally related analogues, namely F, NEF, DMF and NMA, were investigated by measuring the elevation of plasma activities of the enzymes ALT, AST and SDH 24h after drug administration. The results are presented graphically in figures 19-21 and are tabulated in appendix 2. The livers

from these mice were prepared for histopathological examination by Ms Rebecca Holt. Examinations were performed by Dr Iona Pratt of University College, Dublin and the findings are discussed below and tabulated in appendix 3.

The threshold dose for NMF-induced hepatotoxicity in the CBA/CA mouse, as indicated by elevation of the plasma activities of ALT, AST and SDH was approximately 200mg/kg (3.4mmol/kg, figs 19-21). Livers extracted from mice which had received 200mg/kg or greater doses of NMF were often pale, sometimes with dense red patches on one or more lobes. No enlargement was evident.

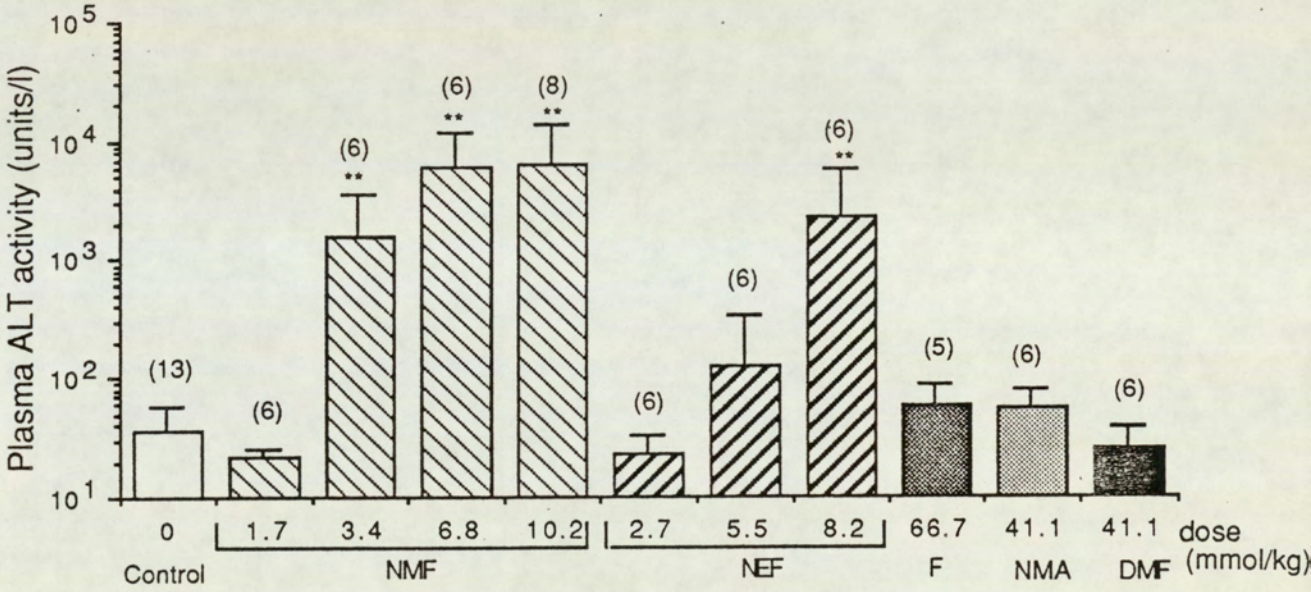
A characteristic of mice after administration of NMF was a marked accumulation of glycogen in zone 1 (periportal) hepatocytes. This was apparent after administration of 100mg/kg NMF, a dose which did not raise plasma levels of the three monitored hepatic enzymes. Glycogen accumulation may therefore constitute one of the preliminary pathological changes associated with NMF-induced hepatotoxicity.

Another characteristic of mice after administration of 100 or 200mg/kg NMF, but not of control animals, was an increase in eosinophilia of the zone 3 (centrilobular) cells. Hepatotoxic doses of 200mg/kg or greater caused extensive haemorrhagic necrosis originating in the zone 3 liver cells. Necrosis was patchy and varied between liver lobes taken from the same animal. In 2 animals haemosiderin deposits were observed in zone 3 sinusoids. Doses of 400 and 600mg/kg NMF caused minor damage to the gall bladder mucosa accompanied by submucosal oedema.

The threshold dose for NEF-induced hepatotoxicity in

Figure 19

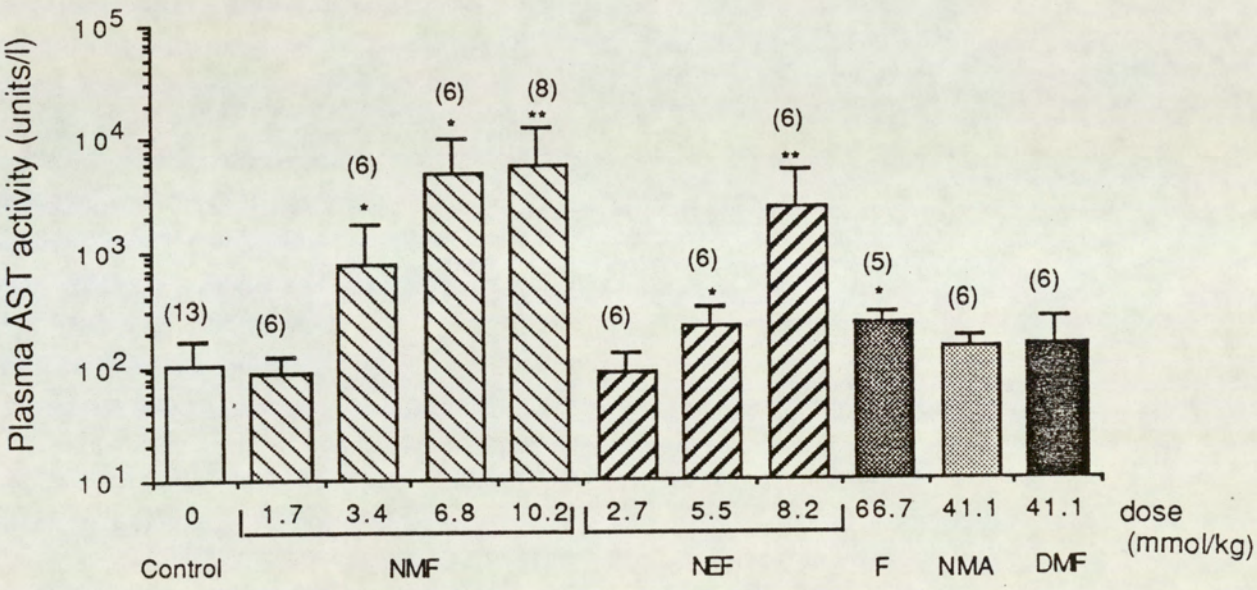
## Plasma levels of ALT in CBA/CA mice 24h after administration of NMF and four structural analogues.




Number of animals given in brackets and bars denote S.D.
Stars indicate significant difference from control (Mann-Whitney U test)
**$p<0.001$

Figure 20

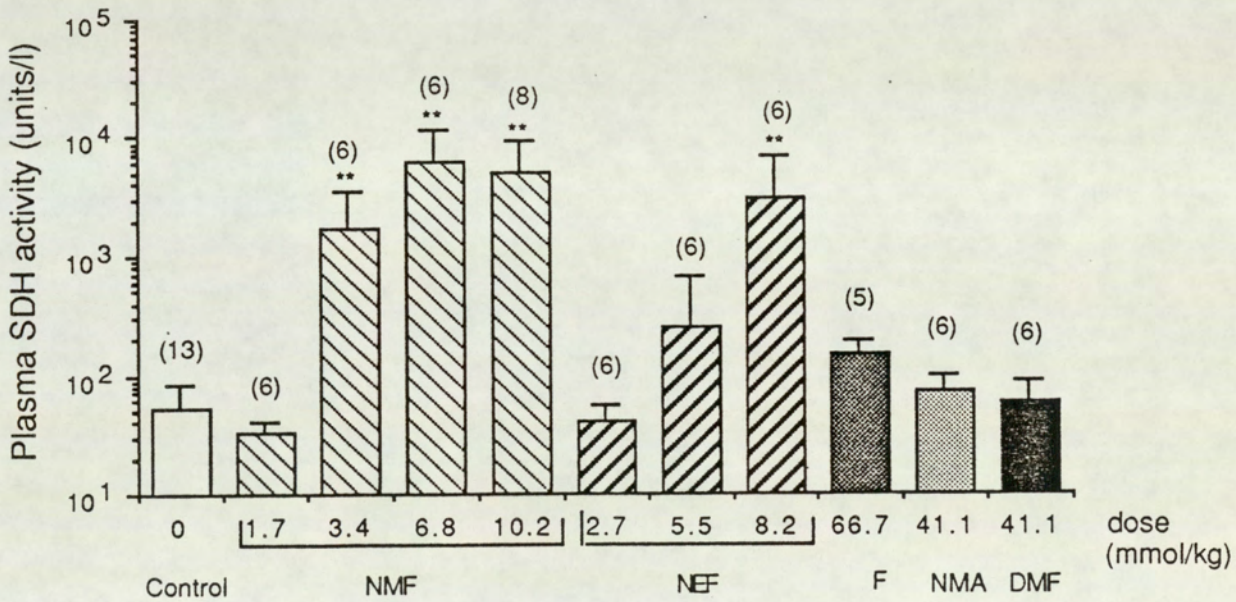
## Plasma levels of AST in CBA/CA mice 24h after administration of NMF and four structural analogues.




Number of animals given in brackets and bars denote S.D.
Stars indicate significant difference from control (Mann-Whitney U test)
*p<0.005, **p<0.001

Figure 21

**Plasma levels of SDH in CBA/CA mice 24h after administration of NMF and four structural analogues.**




Number of animals given in brackets and bars denote S.D.
Stars indicate significant difference from control (Mann-Whitney U test)
**p<0.001

the CBA/CA mouse, as indicated by the elevation of the hepatic enzymes ALT, AST and SDH in the plasma 24h after dosing was between 400 and 600mg/kg (5.5-8.2mmol/kg, figs 19-21). The livers dissected from mice 24h after receiving a hepatotoxic dose of NEF often appeared pale but rarely displayed the dark red patching observed after administering NMF. No enlargement was noticeable.

Histopathological examination of livers from mice administered 200 or 400mg/kg NEF revealed few if any abnormalities. Glycogen accumulation in zone 1 hepatocytes did occur as it did after low doses of NMF. One mouse given a dose of 400mg/kg NEF did exhibit degenerative changes in the centrilobular cells accompanied by an increase in eosinophilia in the same region. However, haemorrhagic necrosis was only observed in mice after a dose of 600mg/kg NEF.

The gall bladder appeared particularly sensitive to NEF administration. On examination 24h after mice had received 200 or 400mg/kg NEF, submucosal haemorrhage and oedema were occasionally apparent. Doses of 600mg/kg NEF elicited submucosal haemorrhage and erosion and necrosis of the mucosa.

CBA/CA mice administered hepatotoxic doses of NEF suffered significant weight loss 24h after dosing (table 2). The loss in weight was greater than that measured in mice after administration of similar doses of NMF of which those that received low hepatotoxic doses displayed no weight loss. This may indicate that the N-alkylformamides are toxic to organs other than the liver.

Table 2

**Weight changes in CBA/CA mice 24h after administration of NMF and four structural analogues.**

| Amide | Dose(mmol/kg) | No of animals | Weight change (%body wt ± S.D.) |
|---|---|---|---|
| Control | - | 14 | +1.5±2.2 |
| NMF | 1.7 | 7 | +2.4±1.4 |
| | 3.4 | 7 | +0.7±2.3 |
| | 6.8 | 9 | -6.4±2.7** |
| | 10.2 | 10 | -6.2±2.6** |
| NEF | 2.7 | 6 | -2.0±1.6* |
| | 5.5 | 6 | -8.0±1.2** |
| | 8.2 | 7 | -9.2±2.9** |
| F | 66.7 | 7 | -6.2±1.0** |
| DMF | 41.1 | 6 | -4.0±2.2* |
| NMA | 41.1 | 6 | -1.7±4.0 |

Stars indicate significant difference from control, (Mann-Whitney U test) * $p<0.005$, ** $p<0.001$.

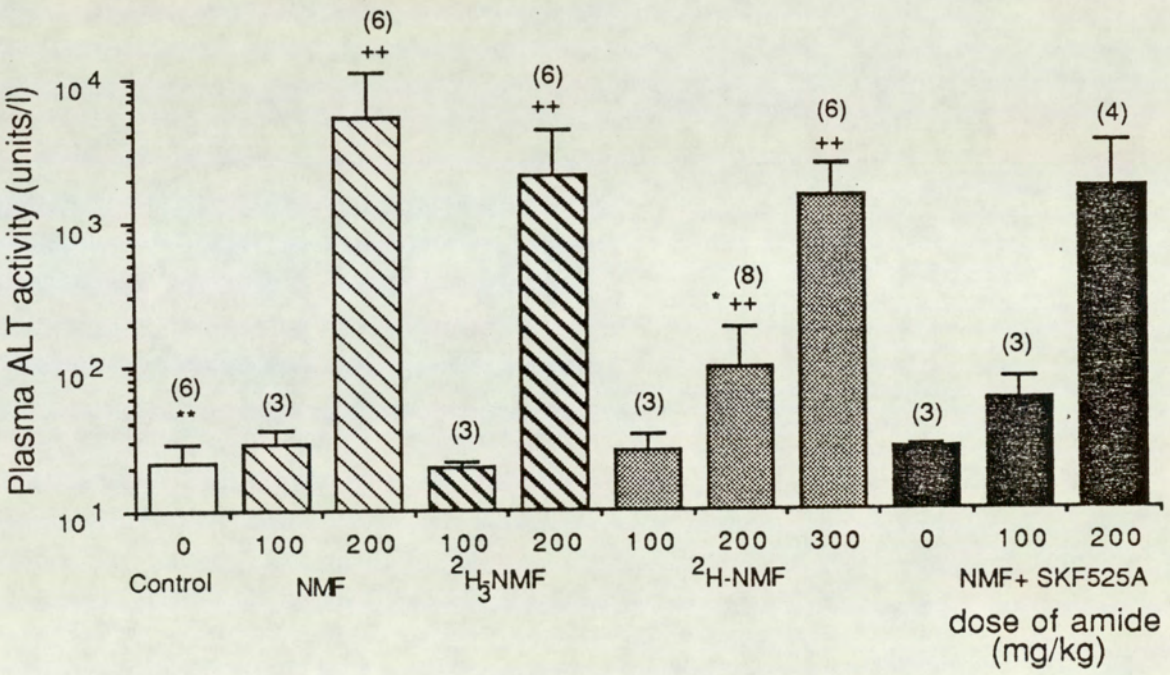
High doses (3g/kg, 41.1mmol/kg) of DMF and NMA administered to CBA/CA mice did not produce an elevation in plasma enzyme activities of ALT, AST and SDH above controls (figs 19-21). Administration of 3g/kg (66.7mmol/kg) F did raise plasma enzyme levels of SDH three fold and AST two fold but failed to significantly alter ALT levels (figs 19-21). Significant weight loss was measured 24h after dosing mice with F and DMF but not after administration of NMA (table 2).

Livers of mice which had received high doses of F, DMF or NMA appeared healthy with uniform colouration and no obvious enlargement. Histopathological examination of these livers revealed periportal glycogen accumulation similar to that observed for the 2 monoalkylformamides. No necrosis occurred in either the liver or gall bladder though an increase in eosinophilia in the centrilobular hepatocytes and pooling of blood in the sinusoidal cells was witnessed.

A marked strain difference in susceptibility to NMF-induced hepatotoxicity between CBA/CA and Balb/C mice was shown by elevation of serum hepatic enzyme activities (figs 19-24, appendix 2). Balb/C mice were more susceptible to the liver toxicity induced by NMF. The hepatotoxic dose threshold in this mouse strain was between 100 and 200mg/kg. A dose of 300mg/kg NMF was often fatal in Balb/C mice whereas CBA/CA mice tolerated doses of 600mg/kg. The hepatotoxic potential of $^{2}H_{3}$-NMF in Balb/C mice as determined by elevation of hepatic enzymes in the plasma was very similar to NMF (figs 22-24). The hepatotoxic dose threshold of $^{2}H$-NMF in the same mouse strain was appreciably

Figure 22

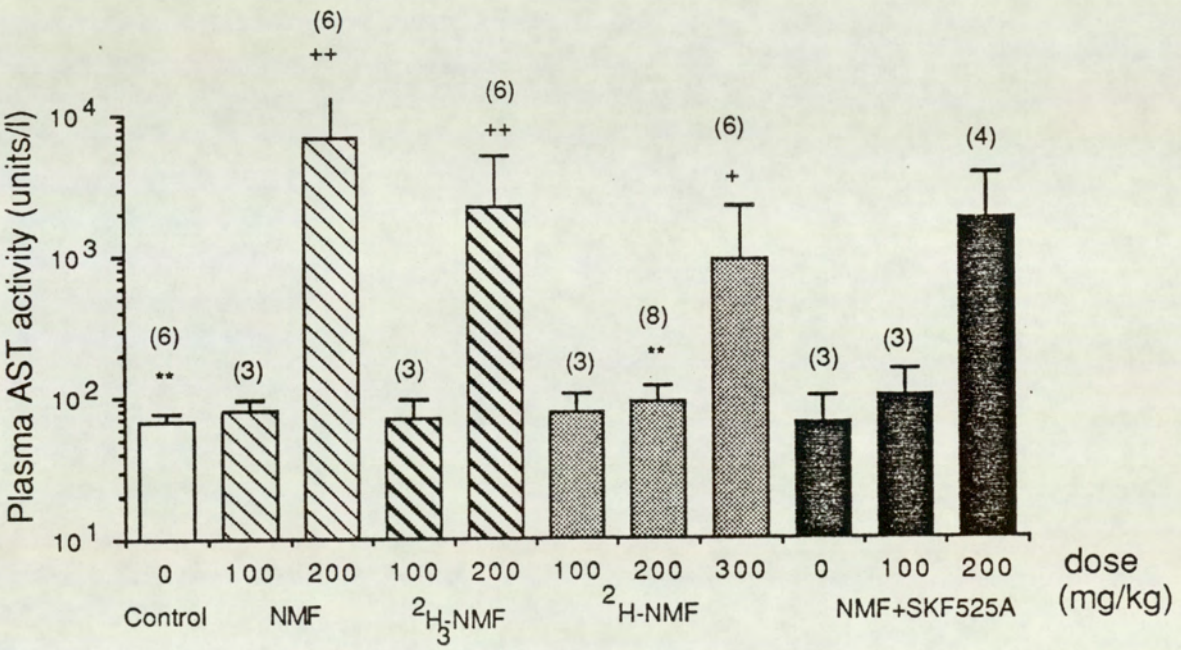
**Plasma levels of ALT in Balb/C mice 24h after administration of NMF, $^2H$ -NMF and $^2H_3$-NMF and after pretreatment with SKF525A**




Number of animals given in brackets and bars denote S.D.
Crosses indicate significant difference from control ,++p<0.002.
Stars indicate significant difference from 200mg/kg NMF,*p<0.005, **p<0.002

Figure 23

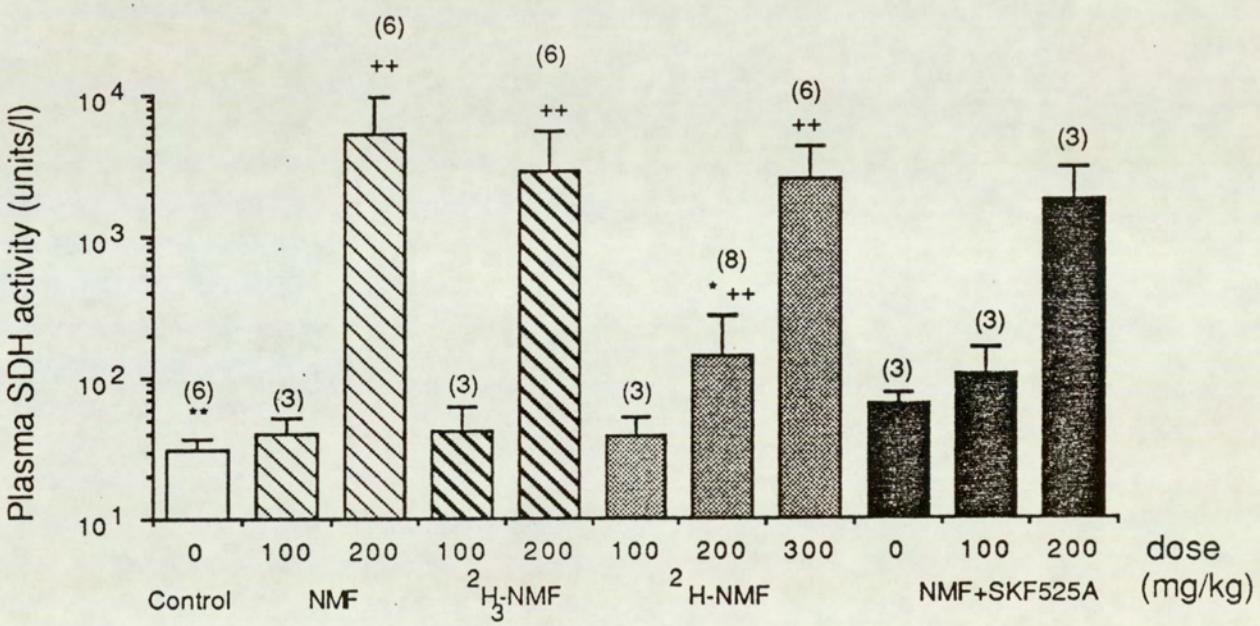
## Plasma levels of AST in Balb/C mice 24h after administration of NMF, $^{2}H$-NMF and $^{2}H_3$-NMF and after pretreatment with SKF525A




Number of animals given in brackets and bars denote S.D.
Crosses indicate significant difference from control, +p<0.005, ++p<0.002
Stars indicate significant difference from 200 mg/kg NMF,
**p<0.002.

Figure 24

**Plasma levels of SDH in Balb/C mice 24h after administration of NMF, $^{2}H$-NMF and $^{2}H_3$-NMF and after pretreatment with SKF525A.**




Number of animals given in brackets and bars denote S.D.
Crosses indicate significant difference from control, ++ $p<0.002$.
Stars indicate significant difference from 200 mg/kg NMF,
* $p<0.005$, ** $p<0.002$.

higher than that of NMF: 200mg/kg NMF caused a significantly greater elevation of the three monitored hepatic enzymes in the plasma than the same dose of the isotopomer (figs 22-24).

Administration of 60mg/kg SKF525A to Balb/C mice 1h before dosing with 100 or 200mg/kg NMF did not significantly alter the elevation in plasma enzyme levels of ALT, AST or SDH (figs 22-24).

### 4.1.3 Discussion

The two N-alkylformamides NMF and NEF appear to be equipotent hepatotoxins in CBA/CA mice. The other three amides investigated in this mouse strain, namely F, DMF and NMA seem to be non-hepatotoxic. Of these five amides only NMF possessed highly significant antineoplastic activity in a range of murine tumours (section 1.2): NEF and DMF displayed marginal antitumour activity each in one mouse model and F and NMA completely lacked antineoplastic activity. The biochemical mechanisms responsible for antitumour activity and toxicity appear distinct.

The characteristic features of livers damaged by NMF and NEF were extensive haemorrhagic necrosis of the centrilobular hepatocytes and erosion (vacuolation) and necrosis of the gall bladder mucosa. Other pathological features e.g. vacuolation and glycogen accumulation in the periportal hepatocytes were observed after administration of low doses of NMF and NEF which did not raise plasma levels of hepatic enzymes. As such they may manifest the first

pathological changes associated with N-alkylformamide-induced hepatotoxicity, however their occurrence after administration of the "non-hepatotoxic" amides suggests they are unrelated to the cell death caused by NMF and NEF. The lack of correlation between liver toxicity and weight loss indicates that the amides may be toxic elsewhere in the animals.

DMF is hepatotoxic in humans occupationally exposed to high concentrations of this industrial solvent (section 1.3.2) and SMM has been identified as a major metabolite of DMF in the urine of man (section 1.1.2). The absence of liver damage and the low levels of mercapturate in the urine of mice administered DMF (Mraz et al, 1988) indicates a species difference in toxicity. It has been postulated that the hepatotoxicity of DMF may be due to metabolism to NMF (Kimmerle and Eben, 1975a,b). It has been shown that NMF is not a major metabolite of DMF (section 1.1.2). However this does not discount the possible metabolic formation of NMF as any NMF produced in the liver is likely to be further metabolised. The generation of SMM requires the N-demethylation of either DMF or a metabolite along the toxification pathway. The ability to perform this metabolic reaction may vary between species and be responsible for the difference in susceptibility of species towards DMF-induced hepatotoxicity.

Deuteration of the formyl moiety significantly reduced the hepatotoxicity caused by NMF in Balb/C mice. No such effect was evident for the trideuteromethyl analogue. Carbon-deuterium bonds have a higher activation energy for

cleavage than do carbon-hydrogen bonds owing to the lower zero point energy of the former (Alder et al, 1971). The bioactivation of NMF to a necrogenic species therefore appears to involve the cleavage of the formyl hydrogen as a rate determining step. The rate of production of the urinary metabolites methylamine and SMM and of the biliary metabolite SMG are reported to undergo a primary kinetic deuterium isotope effect in that their production is significantly slowed by the replacement of the formyl hydrogen with deuterium (Threadgill et al, 1987). The absence or presence of only low levels of alkylamines and mercapturates in the urine of mice administered the non-hepatotoxins F, DMF and NMA (Kestell et al, 1987; Mraz et al, 1988) suggests that these metabolites, like the necrogenic species, lie downstream of the rate-limiting step in the same metabolic pathway. As the mercapturate is unlikely to be generated in the liver and methylamine is ubiquitous in biological tissues, they are unlikely to be the hepatotoxic species. The biliary excretion of the glutathione conjugate of NMF (Pearson et al, 1988a) and NEF (Pearson and Baillie, unpub.) may result in the necrotic damage to the gall bladder observed in CBA/CA mice after administration of these N-alkylformamides. However, these conjugates are unlikely to be the ultimate hepatotoxic species as the depletion and protection of hepatic glutathione levels has been shown to exacerbate and alleviate respectively the toxicity of NMF (Pearson et al, 1987a). The protective role of glutathione suggests that it is conjugated with the ultimate hepatotoxic intermediate

generated either directly or downstream from the oxidation/dehydrogenation of the formyl moiety of NMF and NEF.

Pretreatment of Balb/C mice with SKF525A, an inhibitor of the cytochrome P450 monooxygenases, did not significantly decrease liver damage caused by NMF. In a similar study (Pearson, 1985) SKF525A pretreatment appeared to exacerbate NMF-induced hepatotoxicity. Masuda et al (1986) have prevented hepatic necrosis caused by NMF in Balb/C and ddY strain mice by oral administration of diethyldithiocarbamate (DTC). DTC is known to inhibit a number of microsomal enzymes including the cytochrome P450 monooxygenases (Masuda and Nakayama, 1982). DTC markedly delayed the *in vivo* metabolism of NMF and cotreatment appeared to decrease antitumour activity against Ehrlich ascites tumours and Sarcoma 180. Whether these effects were due to the inhibition of the mixed function oxidase system, inhibition of another enzyme system or to the increase in levels of reactive thiols caused by DTC is unknown. However a role for the mixed function oxidase system in the toxicity and antitumour activity of NMF can not be discounted. Their possible role was further investigated in experiments described in section 4.2.

## 4.2 In vitro covalent binding of N-alkylformamides to microsomes.

### 4.2.1 Introduction

The hepatotoxicity of NMF is thought to be mediated via the generation of a reactive species (section 1.3.1). Various chemically diverse hepatotoxins including paracetamol (APAP, appendix 1), 1-naphthol (D'Arcy Doherty and Cohen, 1984), furosemide (Mitchell et al, 1973a) and bromobenzene (Mitchell et al, 1971) are thought to exert their toxicity by forming reactive metabolites. Such metabolites are assumed to bind covalently to nucleophilic macromolecules, either proteins, nucleic acids or polysaccharides, and modify their biological properties in a manner detrimental to the cell (Bridges et al, 1983). The extent of liver necrosis caused by these xenobiotics is often paralleled by the magnitude of *in vivo* and *in vitro* covalent binding of radiolabelled species derived from these hepatotoxins to hepatic macromolecules. For all these compounds a further insight into their bioactivation has been achieved by the use of inhibitors and inducers of drug metabolising enzymes to modulate the amount of reactive species formed or by performing microsomal incubations in the presence of nucleophiles such as reduced glutathione (GSH). Treatments such as these alter the amount of binding, not only to target macromolecules, but also to numerous other cellular constituents presumably in a parallel fashion. Consequently the involvement of

chemically reactive metabolites in the toxicity of a compound may be determined without the knowledge of the exact identity of either the reactive metabolite(s) or the cellular target(s).

Caution is required when interpreting the data from covalent binding studies as some evidence suggests that covalent binding may not be causally related to toxicity. For example, 3-hydroxyacetanilide (3-HAA), a non-hepatotoxic, positional isomer of APAP, has been found to bind more extensively to microsomal and soluble proteins than APAP (Streeter et al, 1984). In addition, GSH and ascorbic acid abolish the binding of the reactive metabolites of 3-HAA and APAP to protein in a similar manner. 3-HAA appears to be metabolised to a quinone or semiquinone species with similar chemical properties to the reactive metabolite of APAP, NAPQI. However the reactive metabolite(s) of 3-HAA appears innocuous. Two possible explanations for this are that (i) the reactive species of 3-HAA may be bound solely to macromolecules that are not essential to the life of the cell and thus deleterious effects do not ensue, or (ii), the 3-HAA metabolite-macromolecule complexes may readily undergo repair by cellular repair mechanisms (Pohl and Blanchflower, 1981).

Covalent binding of metabolites of $^{14}$C-NMF to mouse hepatic proteins has previously been studied by Pearson et al (1987b): Radioactivity was found to be associated with liver, and to a lesser extent kidney proteins, after i.p. injection of $^{14}$C-NMF into Balb/C mice. Association was higher in this mouse strain than in CBA/CA or $BDF_1$ mice.

Depletion of hepatic glutathione by treatment of mice with BSO or DEM prior to administration of formyl-$^{14}$C-NMF enhanced the association of label with hepatic proteins. Pretreatment of mice with SKF525A decreased the amount of label associated with the liver after administration of methyl-$^{14}$C-NMF but increased it after administration of formyl-$^{14}$C-NMF. *In vitro* binding of $^{14}$C-NMF to hepatic microsomes was found to be NADPH-dependent and was abolished in the presence of GSH. SKF525A quadrupled microsomal binding of formyl-$^{14}$C-NMF.

In section 4.1, NMF and NEF were shown to be equipotent hepatotoxins and it was proposed that these two N-alkylformamides were biotransformed to necrogenic species by the same metabolic pathway. If the covalent binding and hepatotoxicity of NMF are causally related, ethyl-$^{14}$C-NEF should not only generate species capable of covalently binding to microsomal protein but should bind in a quantitatively similar manner to methyl-$^{14}$C-NMF metabolites. Such a quantitative relationship has previously been demonstrated between chlorobenzene, bromobenzene, iodobenzene and o-dichlorobenzene in the rat after administration of 1mmol/kg of these hepatotoxic halogenated benzenes (Gillette, 1974).

Microsomes contain several phase I and II drug metabolising enzymes including the NADPH-dependent cytochrome P450 monooxygenases. The role of this enzyme system, if indeed there is one, in the liver toxicity induced by N-alkylformamides is uncertain at present. The strain difference observed *in vivo* in covalent binding and

toxicity of NMF may help shed some light on this matter. If the strain difference in binding is paralleled by a difference in the ability of microsomes to perform cytochrome P450 catalysed reactions, this finding could be considered to be indirect evidence for the involvement of the mixed function oxidase system in the toxicity of N-alkylformamides.

In conclusion, the two main aims of the experiments described in this section are to investigate further the relationship between toxicity and covalent binding of N-alkylformamide metabolites to microsomal protein, and to determine whether cytochrome P450 has a role in the bioactivation of NMF and NEF.

4.2.2 Results

The metabolising capacity of the microsomal preparations was assessed by the determination of formaldehyde (HCHO) as a product of the cytochrome P450 monooxygenase-dependent N-demethylation of aminopyrine (5mM). Metabolism was dependent on the presence of NADPH and the rate did not vary significantly between microsomes prepared from Balb/C or $BDF_1$ mice (table 3) or from Sprague-Dawley rats as reported by Scailteur et al (3.01 $\pm$ 0.56nmol/mg protein/min; n = 6; 1984). HCHO production was reduced by > 60% in the presence of SKF525A (1mM; fig 25). Pretreatment of mice with phenobarbitone (50mg/kg/day for 4 days) prior to isolation of the microsomes did not increase the rate of metabolism of aminopyrine (1.24 $\pm$ 0.26nmol

HCHO/mg protein/min; n = 3).

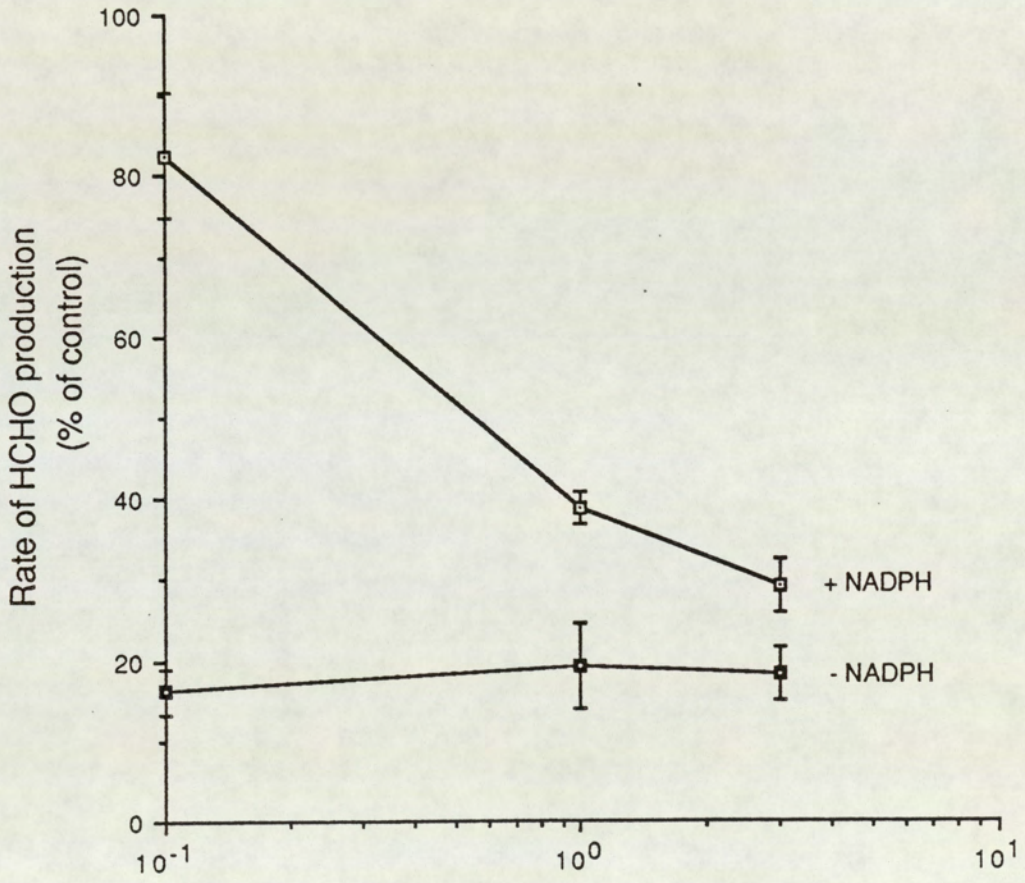
In vitro association of methyl-$^{14}$C-NMF derived radioactivity to microsomal protein was dependent on time (fig 26) and the presence of a NADPH-generating system (table 4). Heat inactivation of microsomes prior to incubation with methyl-$^{14}$C-NMF (7mM) caused a dramatic reduction in covalent binding (0.34nmol methyl-$^{14}$C-NMF equivalents/mg protein/h; n = 2). Increasing the incubation volume also appeared to reduce binding (2.11nmol methyl-$^{14}$C-NMF equivalents/mg protein/h; n = 2) possibly due to a decrease in oxygen permeating the suspension.

Covalent binding of methyl-$^{14}$C-NMF metabolites to microsomes isolated from Balb/C mice was significantly greater than binding to microsomes isolated from $BDF_1$ mice (table 4). Binding was not significantly reduced in the presence of SKF525A (1mM; table 5) but was nearly abolished in the presence of GSH (10mM; table 6).

Radioactivity derived from ethyl-$^{14}$C-NEF was bound to microsomal protein in a manner quantitatively similar to that observed with the $^{14}$C-labelled methyl analogue. Binding was NADPH-dependent and not significantly reduced in the presence of SKF525A (1mM; table 7).

Figure 25

**Effect of inhibition of cytochrome P450 on the metabolism of aminopyrine.**

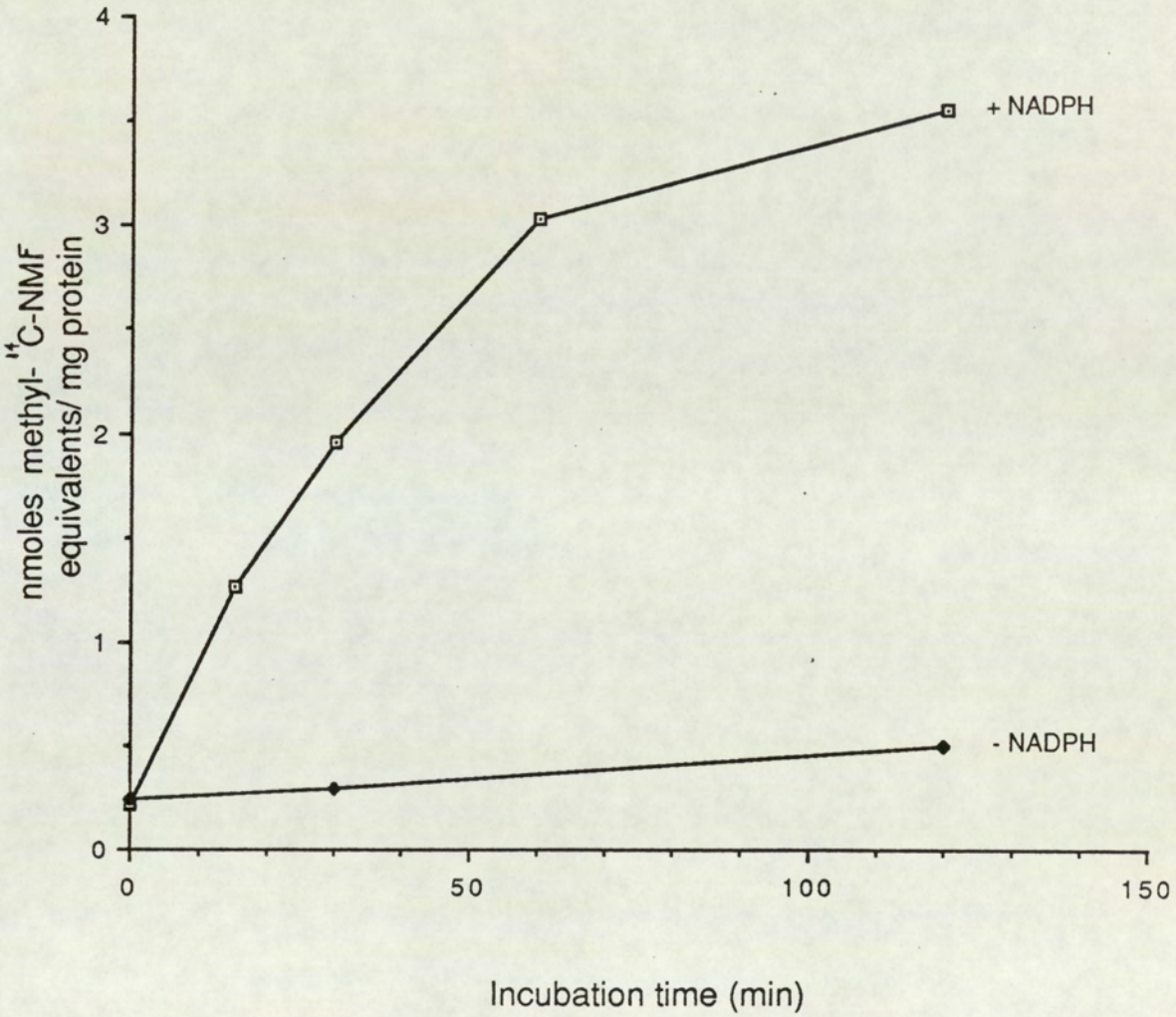



Microsomes were prepared from livers of Balb/C mice.

Each point represents the mean ± S.D. of four separate incubations.

Figure 26

**Effect of NADPH on the covalent binding of methyl-$^{14}$C-NMF metabolites to microsomal protein.**




Microsomes were prepared from the livers of Balb/C mice.

Each point represents the mean of two incubations.

All values were within 9% of the calculated mean.

Table 3

**In vitro metabolism of aminopyrine by mouse liver microsomes.**

| Mouse strain | Incubation conditions | Production of HCHO(a) (nmolHCHO/mgprot/min) |
|---|---|---|
| Balb/C | +NADPH | 1.39±0.27 (10)** |
| Balb/C | -NADPH | 0.25±0.10 (10) |
| $BDF_1$ | +NADPH | 1.18±0.28 (6)* |
| $BDF_1$ | -NADPH | 0.24±0.11 (6) |

(a) Measured by the method of Nash (1953).

Numbers in brackets indicate the number of individual incubations performed.
Stars indicate significant difference from -NADPH.
*$p<0.005$,**$p<0.001$

Table 4

**In vitro covalent binding of methyl-$^{14}$C-NMF derived radioactivity to microsomal protein.**

| Mouse strain | Incubation conditions | Covalent binding (nmoles methyl-$^{14}$C-NMF equiv /mg prot/h). |
|---|---|---|
| Balb/C | +NADPH | 2.88±0.52(14)** |
| Balb/C | -NADPH | 0.21±0.09(6) |
| $BDF_1$ | +NADPH | 1.97±0.07(6)*++ |
| $BDF_1$ | -NADPH | 0.34±0.06(3) |

Numbers in brackets indicate the number of individual incubations performed for 1h.
Stars indicate significant difference from -NADPH, *p<0.005,**p<0.001.
Crosses indicate significant difference from Balb/C +NADPH, ++p<0.001.

Table 5

**Effect of inhibition of cytochrome P450 on the covalent binding of methyl-$^{14}$C-NMF metabolites to microsomal protein.**

| Conc of SKF525A (mM) | Incubation conditions | Covalent binding (nmol methyl-$^{14}$C-NMF equiv/mg protein/h) | % of control |
|---|---|---|---|
| 0.0 | +NADPH | 2.88±0.52(14) | 100 |
| 0.0 | -NADPH | 0.21±0.09(6) | 7 |
| 1.0 | +NADPH | 2.11±0.58(3) | 73 |
| 1.0 | -NADPH | 0.10±0.03(3) | 3 |
| 0.1 | +NADPH | 2.74±0.38(a) | - |

(a) Results from literature (Pearson, 1985).

Microsomes were prepared from the livers of Balb/C mice.

Figures in brackets indicate the number of individual incubations performed for one hour.

Table 6

**Effect of GSH (10mM) on the covalent binding of methyl-$^{14}$C-NMF metabolites to microsomal protein.**

| Incubation conditions | Covalent binding (nmol methyl-$^{14}$C-NMF equiv/mg protein/h) |
|---|---|
| +NADPH | 2.88±0.52 (14) |
| +NADPH, +GSH | 0.37±0.14 (3)* |
| -NADPH, +GSH | 0.10 (2) |

Microsomes were prepared from the livers of Balb/C mice.

Figures in brackets indicate the number of individual incubations.
* Indicates significant difference from +NADPH, $p<0.005$.

Table 7

## Effect of inhibition of cytochrome P450 on the covalent binding of ethyl-$^{14}$C-NEF metabolites to microsomal protein.

| Conc of SKF525A (mM) | Incubation conditions | Covalent binding (nmol ethyl-$^{14}$C-NEF equiv/mg protein/h) | % of control |
|---|---|---|---|
| 0.0 | +NADPH | 3.31±0.60 (3) | 100 |
| 0.0 | -NADPH | 0.08±0.07 (3) | 2 |
| 1.0 | +NADPH | 2.80±0.84 (3) | 85 |
| 1.0 | -NADPH | 0.08±0.10 (3) | 2 |

Microsomes were prepared from the livers of Balb/C mice.

Figures in brackets indicate the number of individual incubations performed for one hour.

4.2.3 Discussion

The results indicate that NMF and NEF are metabolically activated to covalently bound species by the same NADPH-requiring pathway. As the pathway is heat labile the involvement of one or more enzymes is likely. The extent of covalent binding of methyl-$^{14}$C-NMF (2.88nmol/mg protein) and ethyl-$^{14}$C-NEF (3.31nmol/mg protein) represents approximately 0.2% biotransformation of the parent compound which is greater than previously reported (0.058%; Pearson, 1985). As it has hitherto proved impossible to detect metabolites of NMF *in vitro* (Gescher et al, 1982) the measurement of covalent binding represents the first indication that N-alkylformamide metabolism occurs *in vitro*.

Microsomes isolated from Balb/C mice generated more NMF derived species bound to protein than microsomes isolated from $BDF_1$ mice. Strain differences in mice after administration of NMF have been reported in the following four biological properties related to the N-alkylformamide: (i) the *in vivo* association of $^{14}$C-NMF derived radioactivity with hepatic protein is greater in Balb/C mice than in CBA/CA and $BDF_1$ mice (Pearson et al, 1987b), (ii) the order of susceptibility towards NMF-induced hepatotoxicity in mouse strains is Balb/C > CBA/CA >> $BDF_1$ (section 4.1; Langdon et al, 1985a), (iii) Balb/C mice metabolise NMF to a greater extent than do CBA/CA mice (Gescher et al, 1985), and (iv) NMF depletes hepatic glutathione in Balb/C mice more dramatically than in CBA/CA mice (Pearson et al, 1987a). Studies comparing Balb/C mice and Sprague-Dawley

rats have displayed a species difference where the former metabolises NMF more extensively and is more acutely sensitive to NMF-induced liver damage and depletion of non-protein thiols (Tulip et al, 1985). These strain and species differences suggest a link between the hepatotoxicity of NMF and the generation of a species capable of binding to cellular macromolecules and of depleting hepatic glutathione. The ability of Balb/C mice to bioactivate NMF more extensively than other strains of mice is presumably due to a quantitative difference in the toxification or detoxification of the parent compound. As no difference was apparent in the ability of microsomes isolated from either Balb/C or $BDF_1$ mice to N-demethylate aminopyrine and as SKF525A failed to significantly decrease the binding of $^{14}C$-labelled N-alkylformamides to microsomal protein, it appears unlikely that the cytochrome P450 monooxygenases play a part in the bioactivation of NMF and NEF. On the contrary, the report that SKF525A markedly stimulates the *in vivo* and *in vitro* association of formyl-$^{14}C$-NMF to hepatic protein (Pearson et al, 1985) suggests a role for cytochrome P450 in the detoxification of N-alkylformamides. Induction and/or inhibition of different cytochrome P450 isoenzymes, for example with aroclor 1254 (Alvares and Kappas, 1977), may be useful in the future to establish more fully the importance of this enzyme system in the toxicity of N-alkylformamides.

Addition of GSH to the incubation media almost abolished the covalent binding of methyl-$^{14}C$-NMF. This demonstrates that GSH is capable of reacting with

metabolite(s) generated from the parent compound by mouse liver microsomes. The nature of these metabolite(s) is presently unknown. In section 4.1 a H/D isotope effect on NMF-induced hepatotoxicity was demonstrated where the replacement of the formyl hydrogen with deuterium markedly decreased the liver necrosis caused by NMF. This implies that the biotransformation of NMF to a necrogenic species requires the dehydrogenation or oxidation of the formyl group. A similar H/D isotope effect on the bilary production of SMG (section 1.1.1) suggests that GSH conjugates to a metabolite downstream of the cleavage of the formyl hydrogen. As GSH appears to protect against the toxicity of NMF (Pearson et al, 1987a) and prevents covalent binding, it is reasonable to assume that it conjugates with either the ultimate toxic species or a precursor of it. Two possible candidates are methyl isocyanate ($OCNCH_3$) and N-methylcarbamic acid ($HOOCNHCH_3$), both of which are highly reactive and could conceivably be hydrolysed to generate methylamine. Methylamine is a major urinary metabolite in mice after administration of NMF which displays a metabolic H/D isotope effect similar to that of SMG (section 1.1.1).

Whereas NMF is metabolised *in vitro* only to a very minor extent by liver homogenates (Brindley et al, 1982) it undergoes extensive *in vivo* biotransformation in rodents to produce methylamine, HMF and SMM as metabolites in the urine (Kestell et al, 1986b). The further elucidation of the link between the metabolism and hepatotoxicity of N-alkyl-formamides would be difficult *in vivo* because of the inaccessibility of the organ towards the investigation of

biochemical changes. However a satisfactory *in vitro* model of N-alkylformamide metabolism has yet to be found. In the experiments in section 4.3 the hypothesis is tested that suspended and cultured hepatocytes are such *in vitro* systems suitable for the study of the metabolism and cytotoxicity of formamides.

## 4.3 Studies of the cytotoxicity of formamides in isolated hepatocytes.

### 4.3.1 Introduction

The hepatotoxic potential of NMF and structural analogues measured in CBA/CA mice in section 4.1 correlated well with the urinary excretion of alkylamines and mercapturates in this mouse strain (Kestell et al, 1987). Also, the kinetic deuterium isotope effect on NMF-induced hepatotoxicity observed in Balb/C mice (section 4.1) was matched by a similar effect in this mouse strain on the rate of metabolism of NMF to methylamine, SMG and SMM (Threadgill et al, 1987). These findings link the hepatotoxicity of N-alkylformamides to their metabolism. Unfortunately the usefulness of the in vivo system, especially in the study of reactive, short-lived metabolites, is limited by the inaccessibility of the organ towards biochemical monitoring and is complicated by the nature of the regulatory processes, involving endogenous and exogenous factors, exerted on hepatic metabolism.

A major obstacle to the in vitro elucidation of the link between the metabolism and toxicity of N-alkylformamides has been the failure to detect any metabolites of formamides in such systems (Brindley et al, 1982). In section 4.2 and in previous reports (Pearson et al, 1985; 1987b) N-alkylformamides were bioactivated by microsomal preparations to metabolites capable of covalently binding to hepatic macromolecules. These findings, and the

abolition of *in vitro* binding in the presence of GSH, again indicates that metabolism is intrinsic in the toxicity of formamides. However the extent of *in vitro* biotransformation represented only 0.2% conversion of the parent compound which is very small compared to the extensive *in vivo* metabolism of formamides reported (Kestell et al, 1985a; Mraz et al, 1988). Thus liver homogenates do not appear to mimic the *in vivo* metabolism of formamides and furthermore do not give any information about the effect these compounds have on cell viability. In the following study the hypothesis has been tested that mouse hepatocytes in suspension and in culture are a valid model for the study of N-alkylformamide-induced cytotoxicity, glutathione depletion (see section 4.4) and metabolism (see section 4.5) and are thus suitable for the further elucidation of the mechanism of formamide toxicity (see section 4.6).

Isolated and cultured hepatocytes have been increasingly used over the past 15 years for pharmacological and toxicological studies. Isolated hepatocytes express most of the functional activities of the intact liver and are therefore suitable for investigating xenobiotic metabolism, cytotoxicity and the effects of drugs on cellular metabolism (Guillouzo, 1986). Isolated hepatocytes have been widely used as an experimental model for toxicity assessment (Klassen and Stacey, 1982; Hayes and Pickering, 1985). Cytotoxicity is usually evaluated by the measurement of trypan blue exclusion, morphological changes and/or cytosolic enzyme leakage of which LDH is considered the most suitable (Guillouzo, 1986).

Hepatotoxins which require metabolic activation to exert their toxic effects often induce similar toxicity in suspensions of hepatocytes as they do in the intact animal. Examples include aflatoxin $B_1$ (Hayes and Pickering, 1985), bromobenzene (Thor and Orrenius, 1980) and paracetamol (Moldeus, 1978). The latter two require the depletion of cellular GSH before cellular damage is provoked. Tyson et al (1980) tested a number of compounds and found that all but two correlated to *in vivo* results. Species differences in toxicity are also detected *in vitro*: Isolated mouse hepatocytes were more susceptible to paracetamol than rat hepatocytes thus paralleling observations *in vivo* (Moldeus, 1978).

The viability of hepatocytes in suspension is short-lived which prevents their use for studies of chronic effects of hepatotoxins. Such chronic investigations can be carried out with primary cultures of hepatocytes however the activities of drug metabolising enzymes, including cytochrome P450 monooxygenases, rapidly decline during culture and do not necessarily reflect the *in vivo* state (Guillouzo, 1986).

Parenchymal hepatic cells were prepared by the methods of Berry and Friend (1969) and Seglen (1973) and adjusted by the method of Klaunig et al (1981) for the particular problems posed by mouse livers (section 3.4.1). Balb/C mice were chosen for this study because of their susceptibility to N-alkylformamide toxicity and because of the considerable amount of *in vivo* data accumulated on formamide metabolism and toxicity in this mouse strain. Incubations of suspended

hepatocytes were performed in Krebs-Henseleit buffer containing HEPES (10mM), BSA (0.2% w/v) and horse serum (10% v/v), the latter being included to try and prevent loss of intracellular GSH (Hogberg and Kristoferson, 1977). Hepatocyte cultures were performed by the method of Hayes and Pickering (1985).

The aim of the work in this section is to evaluate the cytotoxicity of NMF and a number of structurally related analogues in isolated hepatocytes. By comparison to the *in vivo* hepatotoxicity results reported in section 4.1, the suitability of this *in vitro* model for the further investigation of the toxicity of formamides will be evaluated.

### 4.3.2 Results

The cytotoxic potential of the formamides was assessed by measurement of both release of LDH from and uptake of trypan blue by cultured or suspended Balb/C mouse hepatocytes. In preliminary experiments both methods gave almost identical cytotoxicity values (fig 27), therefore, in subsequent experiments, only release of enzyme was determined. Suspended hepatocytes appeared more sensitive than cultured cells towards NMF-induced cytotoxicity: Exposure to NMF (10mM) caused 76% cell death of suspended hepatocytes after 6h incubation (fig 27) while only 37% cell death of cultured cells after 22h incubation (fig 28). As the cytotoxic potential of NMF was more readily apparent in suspended hepatocytes, and because of the greater

complexities involved in culturing hepatocytes, suspensions of mouse liver cells were chosen for a detailed assessment of the cytotoxic potential of NMF and a number of structural analogues.

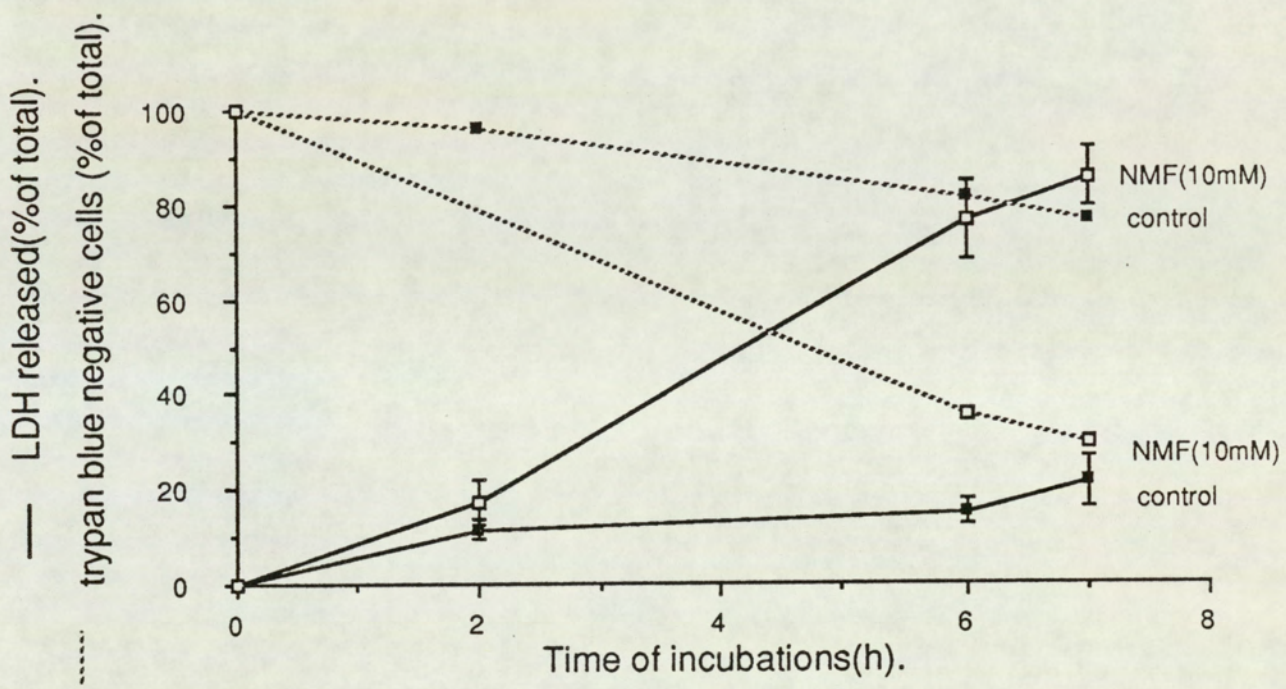
Preliminary hepatocyte suspensions of > 2 x $10^6$cells/ml appeared to have a lower cell viability than incubations of fewer cells. Also the cytotoxic potential of NMF appeared to fluctuate depending on cell density, in general being more toxic in suspensions containing small cell numbers. Consequently a constant medium hepatocyte density of 1.4 $\pm$ 0.1 x $10^6$cells/ml was used for all studies of formamide-induced cytotoxicity, glutathione depletion (see section 4.4) and metabolite formation (see section 4.5). Hepatocyte isolations of < 80% viability were discarded.

Cytotoxicity was only apparent beyond 2h of exposure of suspended hepatocytes to NMF (fig 29). Cytotoxicity was dependent on substrate concentration: At a concentration of 0.1mM, NMF was innocuous whereas concentrations of 5mM or greater caused maximal toxicity. Concentrations above 50mM NMF (and other formamides) tended to interfere with the assay for LDH and so for such high concentrations toxicity was assessed by trypan blue exclusion.

Of the analogues of NMF studied, only NEF was cytotoxic at concentrations < 10mM (fig 30). NMF and NEF appeared equipotent as cytotoxins. F, DMF and NMA did not reduce cell viability below controls unless 50 or 100mM was used and at these concentrations cell death after 6h was never greater than 50%. $^2$H-NMF was markedly less cytotoxic towards hepatocytes than NMF. After incubation of liver

Figure 27

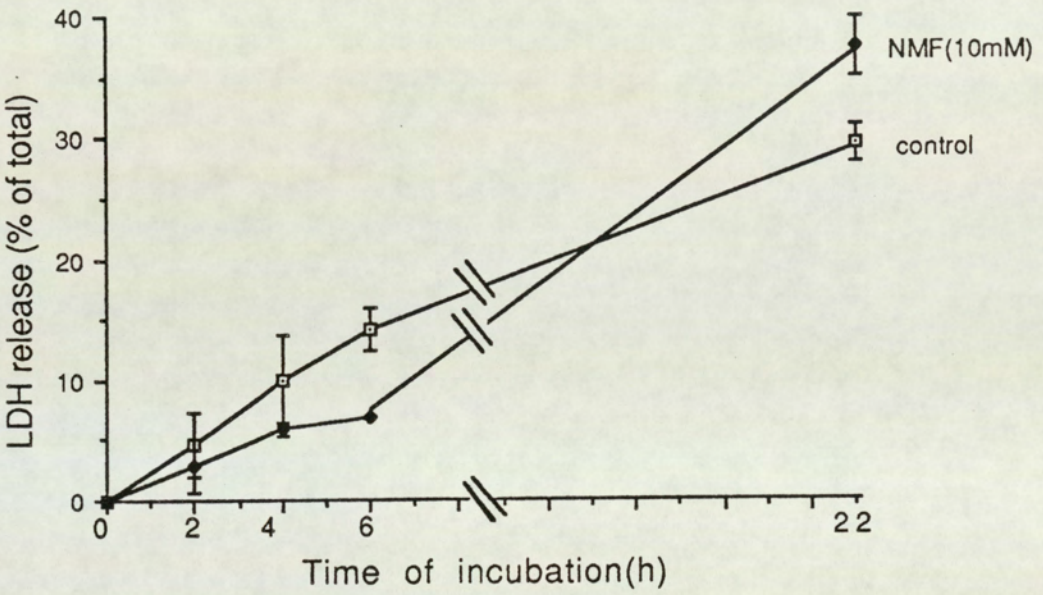
**Effect of NMF (10mM) on the viability of suspended mouse hepatocytes.**




LDH values are the mean ± S.D. of four separate incubations.

Figure 28

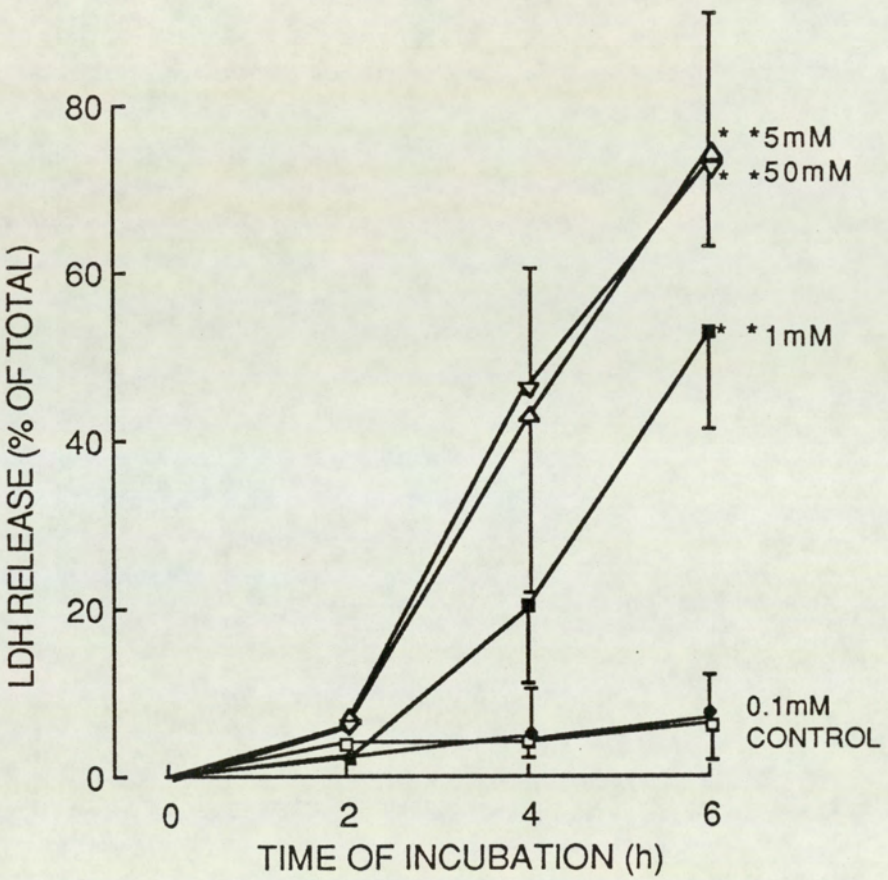
**Effect of NMF(10mM) on the viability of cultured mouse hepatocytes.**




Values represent the mean ± S.D. of 3 to 6 separate hepatocyte cultures.

Figure 29

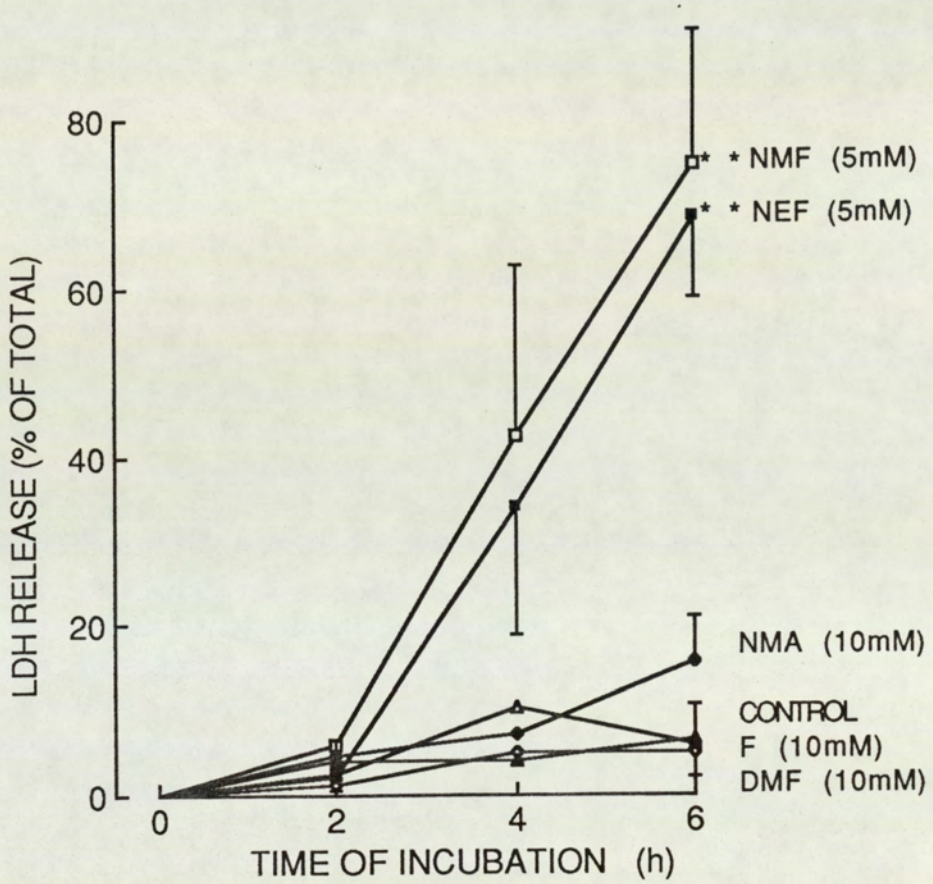
**Effect of concentration of NMF on the viability of suspended mouse hepatocytes.**




Values represent the mean ± S.D. of 4 to 15 separate preparations of hepatocytes.
** indicate significant differences from 6hr control, $p<0.001$.
For the sake of clarity, the error bars have been omitted for the values obtained at 2h where the S.D. did not exceed 52% of the mean.

Figure 30

**Effect of NMF and structural analogues on the viability of suspended mouse hepatocytes.**




Values represent the mean ± S.D. of 4 to 15 separate preparations of mouse hepatocytes.

**indicates significant difference from 6h control, $p<0.001$.

Error bars on some of the points have been excluded for the sake of clarity. In these cases, the S.D. did not exceed 65% (at 2h), 60% (4h) or 31% (6h) of the mean.

cells with 5mM $^2$H-NMF for 6h, release of LDH was only 7.3 ± 1.6% (n = 5) and was thus indistinguishable from controls.

Treatment of mice with BSO (1600mg/kg) 4h before isolation of hepatocytes markedly exacerbated the cytotoxicity of NMF: The onset of toxicity occurred within 2h of exposure of the cells to NMF (10mM) and 100% cell kill was apparent in 6 separate incubations after 4h. LDH release after 4h in controls containing pretreated cells was 17.3 ± 1.9% (n = 4).

A preliminary experiment with human hepatocytes in suspension ( > 80% viable) isolated from a 4 year old male displayed < 20% cell kill after 6h incubation with up to 10mM NMF. This may indicate a greater resistance of human liver cells towards NMF-induced cytotoxicity compared to Balb/C mouse hepatocytes, however the high cell density (6 x $10^6$ cells/ml) used may have significantly influenced this result.

### 4.3.3 Discussion

NMF and NEF were potently cytotoxic towards isolated hepatocytes, $^2$H-NMF was markedly less toxic and the structural analogues F, DMF and NMA did not elicit damage to liver cells at high concentrations. Thus in summary there is excellent agreement concerning the structure-activity relationship of formamides in toxicity reported here and reported *in vivo* in section 4.1. This characterises mouse hepatocytes in suspension as a suitable model *in vitro* system for the further investigation of the metabolism and

the biochemical changes associated with the hepatotoxicity of this class of compound.

NMF is cytotoxic towards isolated hepatocytes at concentrations of 1mM or above. The peak plasma concentration in mice administered a single i.p. hepatotoxic dose of NMF (400 mg/kg) was 7mM (Brindley et al, 1982). The concentration of NMF measured in the plasma of patients who received the drug in a phase I clinical trial did not exceed 2mM (Griffiths et al, 1983) though patients on NMF frequently exhibited liver damage (section 1.3.1). Thus the in vitro cytotoxic concentration threshold of NMF agrees well with in vivo findings. As a concentration of 0.5M NMF (and NEF) was required to kill cultured TLX5 tumour cells (Gescher et al, 1982), metabolism would appear necessary in the toxicity of N-alkylformamides.

NMF-induced hepatotoxicity in vivo has been shown to develop after a lag period of at least 10h (Whitby et al, 1984a) even though peak plasma concentrations of the parent compound occurred within 1h of dosing (Brindley et al, 1982). A similar lag phase occurred in cultured cells exposed to NMF (10mM), however cytotoxicity in suspended hepatocytes was apparent within 2-4h of exposure. The difference in the time lag preceding the emergence of toxic manifestations between the whole liver and cultured hepatocytes and as observed in suspended liver cells may be due to an increased loss of GSH in the latter. Hepatocytes are known to lose some of their original GSH during isolation and incubation (Vina et al, 1978). Suspension in Krebs-Henseleit buffer containing 2% albumin results in the

continuous loss of GSH from the cells while incubation in a culture medium containing amino acids and horse serum results in increased levels suggesting active synthesis (Hogberg and Kristoferson, 1977). Thus the combined loss of GSH and decreased efficiency in GSH synthetic ability may make the suspended hepatocytes more susceptible to NMF-induced cytotoxicity. This is further supported by the exacerbation of NMF toxicity by the prior depletion of hepatocellular GSH with BSO observed here *in vitro* and by Pearson et al (1987a) *in vivo*. The ability of NMF and structural analogues to deplete GSH *in vitro* will be investigated in section 4.4.

## 4.4 Studies of formamide-induced glutathione depletion in suspended hepatocytes

### 4.4.1 Introduction

Depletion of hepatic glutathione is a prerequisite to the hepatotoxicity exerted by numerous toxins. The metabolic activation of hepatotoxins such as paracetamol and bromobenzene, and their ability to deplete hepatocytic glutathione, has been demonstrated in suspended hepatocytes (Moldeus, 1978; Thor and Orrenius, 1980). Conjugation of glutathione with the reactive, potentially toxic metabolites of such xenobiotics is generally a detoxification process resulting in the excretion of innocuous mercapturates in the urine. Exhaustion of the supply of intracellular glutathione, though not in itself lethal (Hogberg and Kristoferson, 1977), leaves the cell vulnerable to the toxicity of the reactive metabolites.

NMF is thought to require metabolic activation and glutathione is believed to play a protective role in N-alkylformamide toxicity (section 1.3.1): Of three mouse strains, depletion of hepatic glutathione after administration of a hepatotoxic dose of NMF was greatest in Balb/C mice, the strain most susceptible to NMF toxicity (Pearson et al, 1987a). Likewise the depletion of hepatic non-protein sulphydryls was significantly greater in Balb/C mice than in rats which were resistant to hepatotoxicity caused by NMF (Tulip et al, 1985). Pretreatment of Balb/C mice with BSO prior to administration of NMF reduced the

hepatotoxic dose threshold of the N-alkylformamide (Pearson et al, 1987a). Thus the maintenance of intracellular glutathione levels appears to be crucial for the protection of the cell against damage elicited by NMF.

The aim of the work described in this section is to assess the ability of NMF and structural analogues, both toxic and non-toxic, to deplete intracellular glutathione in suspended hepatocytes. As such these experiments question the contention that glutathione depletion and N-alkylformamide-induced cytotoxicity are mechanistically linked.

## 4.4.2 Results

The concentration of intracellular glutathione (GSSG and GSH) as measured by the method of Tietze (1969) was 44 $\pm$ 5.3nmol glutathione/$10^6$ hepatocytes (n = 9). This value is in agreement with the glutathione content previously reported in rodent hepatocytes, namely 44 $\pm$ 3nmol glutathione/$10^6$ cells (Hogberg and Kristoferson, 1977). Studies in which the experimental design was developed showed that the concentration of hepatocytic glutathione in control cells seemed to be stabilised by the presence of 10% horse serum and it did not decrease by more than 10% during the initial 4h incubation period. Addition of DEM (0.02% v/v) to the incubation medium or pretreatment of mice with BSO (1600mg/kg) 4h before cell isolation reduced levels of glutathione to 11.8 $\pm$ 4.8% (n = 4) and 14.6 $\pm$ 9.8% (n = 10) of control values respectively. The extent of glutathione depletion by BSO and DEM agrees with previous reports

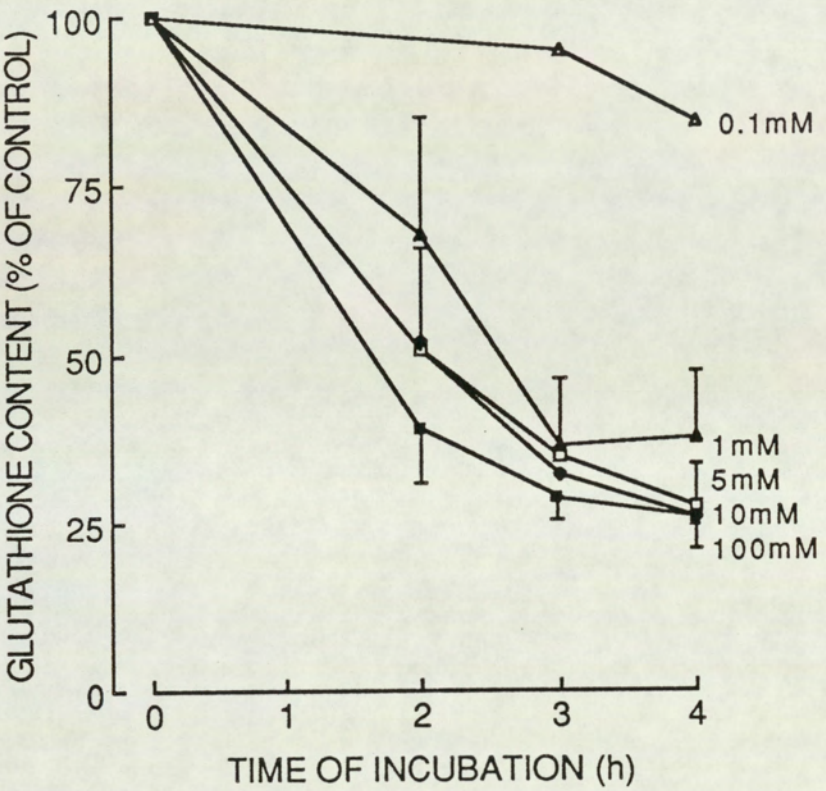
(Hogberg and Kristoferson, 1977; Pearson et al, 1987a).

NMF concentrations of 1mM or above markedly depleted levels of intracellular glutathione in suspended Balb/C mouse hepatocytes (fig 31). Glutathione levels tended to reach a minimum after 3-4h incubation and maximal depletion to 26% of control values occurred after 4h exposure to NMF concentrations of 5mM or above. Assuming a low rate of GSH resynthesis this percentage constitutes a loss of 32.7nmol glutathione/$10^6$ cells.

NEF (5mM) decreased hepatocytic glutathione levels down to 12.2% of control values after 4h incubation (fig 32). This depletion was significantly greater than observed for the same concentration of NMF. $^2$H-NMF was also able to affect levels of glutathione but not as markedly as the protomeric analogue (fig 32). Neither F, DMF or NMA (10mM) caused significant glutathione depletion (fig 32).

Figure 31

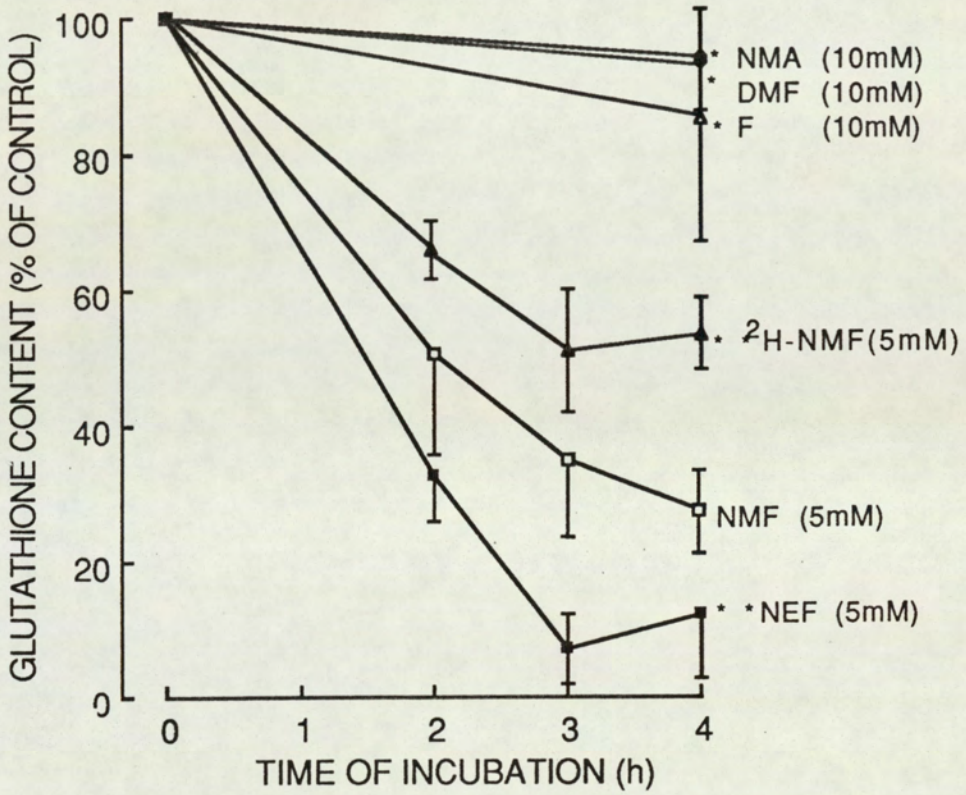
**Effect of concentration of NMF on the loss of intracellular glutathione from suspended mouse hepatocytes.**




Values are the mean of 2 (in case of 0.1 and 10mM NMF)
or the mean ± S.D. of 3 to 11 separate preparations of hepatocytes.

Figure 32

**Effect of NMF and structural analogues on the loss of intracellular glutathione from suspended mouse hepatocytes.**




Values are the mean±S.D. of 3 to11 separate preparations of hepatocytes.

Stars indicate significant difference from 5mM NMF after 4h,

*p<0.01,**P<0.005.

4.4.3 Discussion

The results presented here provide further evidence for the contention that N-alkylformamide-induced liver damage and glutathione depletion are mechanistically linked. Only the formamides that displayed toxicity *in vivo* (section 4.1) and *in vitro* (section 4.3) were capable of depleting hepatocytic glutathione. Furthermore, prior depletion of glutathione by pretreatment with BSO exacerbated the cytotoxicity of NMF (section 4.3). Concentrations of NMF that elicited maximal toxicity depleted cellular glutathione to a nadir of 26% of control values within 4h of incubation. This time course of depletion closely resembles that measured *in vivo* (Pearson et al, 1987a) and further confirms the suitability of suspended hepatocytes as a model of NMF-induced liver damage. As NMF-induced cell death of isolated hepatocytes commenced only after a lag period of 2-4h (section 4.3) it seems reasonable to assume that the level of glutathione reached after this time was below the threshold required for protection of the cells. Hepatocytes pretreated with BSO or exposed to DEM did not lose their viability during the incubation period (section 4.3) though glutathione was depleted to 15% and 12% of control values respectively. Thus cell death does not seem to be a direct consequence of glutathione depletion but is presumably the result of the interactions of reactive metabolites of NMF with unprotected target macromolecules.

It is puzzling that NEF depleted glutathione to a larger extent than did NMF. Glutathione depletion caused by

NMF formed a plateau at 26% of control values whereas NEF caused a depletion down to 7% of control values. This suggests that the necrogenic metabolite of NEF influences glutathione pools unaffected by the analogous metabolite of NMF. This may be a result of greater bioactivation of the ethyl analogue which is reported to undergo more metabolism than the methyl form (Kestell et al, 1987). Whatever the reason, it appears to have little consequence for cell survival as the toxic potentials of these two N-alkylformamides are very similar both *in vivo* (section 4.1) and *in vitro* (section 4.3).

The deuterium isotope effect on NMF-induced toxicity observed *in vivo* (section 4.1) and *in vitro* (section 4.3) is matched by a similar effect on glutathione depletion. Deuteration of the formyl moiety of NMF reduced both the cytotoxicity and the ability to deplete glutathione suggesting that both events occur downstream of the metabolic cleavage of the formyl hydrogen. As the production of SMG is also subject to a primary kinetic H/D isotope effect (Threadgill et al, 1987) it is likely that most, if not all of the glutathione lost by the hepatocytes, is conjugated to the reactive metabolite. This hypothesis is investigated in the next section by quantifying the production of such N-alkylcarbamoylating metabolites in isolated mouse hepatocytes.

## 4.5 Studies of the metabolism of formamides to carbamoylating species by isolated hepatocytes.

### 4.5.1 Introduction

The results of *in vivo* and *in vitro* experiments described in sections 4.1-4.4 suggest that N-alkylformamides require bioactivation in order to exert toxicity and that glutathione protects against N-alkylformamide-induced toxicity by conjugating with the ultimate toxic species and/or a reactive precursor of it. SMG and SMM have been identified as metabolites in the bile and urine respectively of mice after administration of NMF (section 1.1.1) however metabolites formed via this pathway have so far not been detected *in vitro*. Isolated hepatocytes combine the advantage of an *in vitro* metabolising system with most properties of the intact liver and have proven useful in studies of drug metabolism including studies of mixed function oxidation reactions mediated by cytochrome P450 (Moldeus et al, 1974) and of conjugation reactions (Moldeus et al, 1976). It has been described in sections 4.3 and 4.4 that the cytotoxicity of NMF and NEF was only apparent after substantial depletion of hepatocytic glutathione levels. This loss of glutathione is presumably due to the conjugation of the reduced tripeptide with one or more reactive metabolites of the N-alkylformamides. The formation of glutathione conjugates of paracetamol by isolated mouse and rat hepatocytes has been reported to be directly correlated with the loss of intracellular

glutathione (Moldeus, 1978). Similarly, if the depletion of hepatocytic glutathione caused by formamides is solely due to conjugation with reactive metabolites of N-alkylformamides, a direct relationship between glutathione loss and metabolite production should be expected in hepatocytes exposed to NMF and NEF.

The aims of the experiments described in this section were to determine whether isolated hepatocytes exposed to formamides generated carbamoylating metabolites and to determine whether metabolite production accounted for the loss of intracellular glutathione witnessed in liver cells exposed to N-alkylformamides. The method used for the detection of glutathione conjugates of oxidised formamides was a modification of a procedure described by Mraz and Turecek (1987) for the determination of SMM in the urine of a man exposed to NMF and DMF (section 3.6.1). N-Alkylcarbamoylating metabolites were reacted with propanol under alkaline conditions and measured by GLC as propyl N-alkylcarbamate. The amount of metabolite present was estimated from calibration curves constructed by the derivatisation of the authentic standards SMG and SEG and measurement by GLC.

### 4.5.2 Results

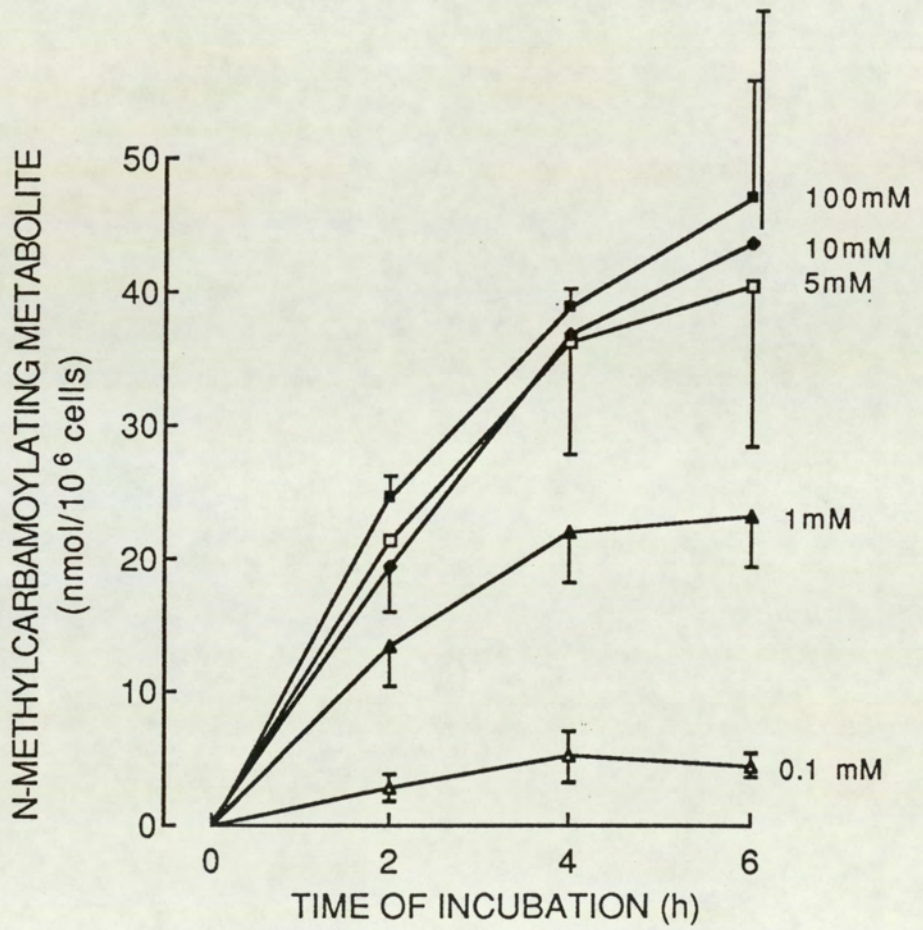
The formation of N-methylcarbamoylating metabolites by isolated mouse hepatocytes incubated with NMF was observed during the whole of the 6h incubation period but it decreased with time (fig 33). This decrease in metabolite

production was presumably the result of a combination of loss of enzyme function (Guillouzo, 1986) and cell death (section 4.3). The rate of metabolism was dependent on concentrations of NMF up to 5mM and formed a plateau at higher concentrations (fig 34). Cell viability was essential for metabolism: Sonication of the cells for 10s prior to incubation, which reduced cell viability by 20% as established by trypan blue exclusion, reduced metabolite production by 30 $\pm$ 7% (n = 3). Sonication for 20s, which caused > 96% cell death, abolished the production of detectable levels of metabolite. Depletion of hepatocytic glutathione, either by pretreating animals with BSO (1600mg/kg) or by incubating hepatocytes with DEM (0.02% v/v) dramatically reduced the formation of N-methyl-carbamoylating species (table 8).

Hepatocytes isolated from the liver of a 4 year old boy generated a N-methylcarbamoylating metabolite on incubation with concentrations of NMF of 0.1mM and higher (table 9). The rate of metabolite production was less than that observed in preparations of mouse hepatocytes. Upon derivatisation with propanol, the metabolite generated by the human liver cells chromatographed with the same retention time as the derivatisation products of either the N-methylcarbamoylating metabolite produced by isolated mouse hepatocytes or the authentic SMG. No carbamoylating metabolite was detected after incubation of human hepatocytes with DMF or HMMF (10mM).

Figure 33

**Time course of the formation of the N-methyl-carbamoylating metabolite of NMF by suspended mouse hepatocytes.**

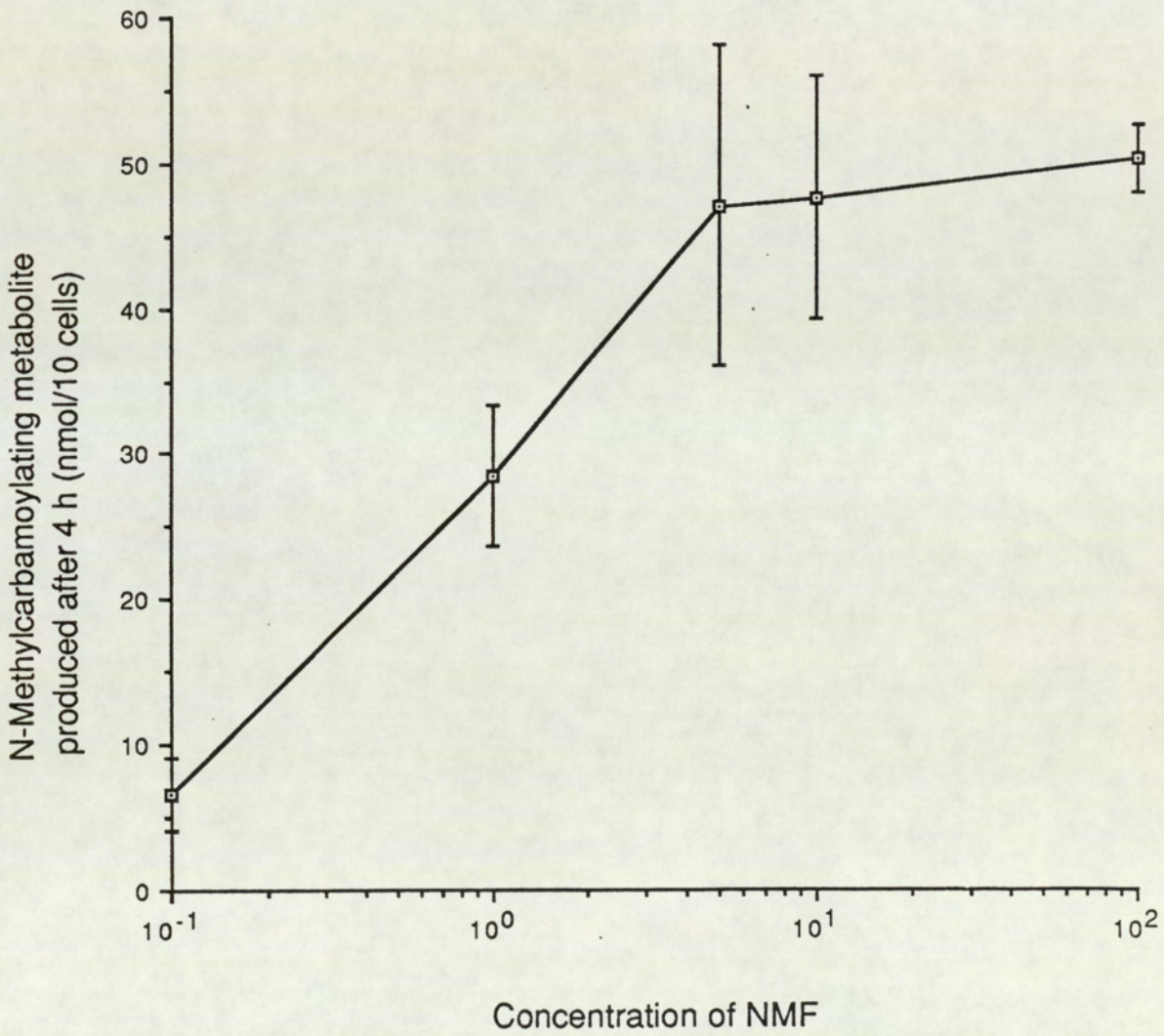



Values represent the mean ± S.D. of 3 to 6 separate preparations of hepatocytes.

Error bars on certain points representing formation of metabolites for 5 or 10mM NMF have been omitted for the sake of clarity. At these points the S.D. was below 30%.

Figure 34

**Formation of the N-methylcarbamoylating metabolite by isolated mouse hepatocytes after 4 h incubation with a range of concentrations of NMF.**




Values represent the mean ± S.D. of 3 to 6 separate preparations of hepatocytes.

Table 8

**Formation of the N-alkylcarbamoylating metabolite by isolated mouse hepatocytes incubated with N-alkylformamides and after depletion of glutathione.**

| Amide | Incubation conditions | % metabolite formed after 4h incubation compared to incubations containing NMF (5mM). |
|---|---|---|
| NMF(5mM) | - | 100 (6) a |
| NMF(5mM) | DEM(0.02%v/v) | 9.4,10.6 b |
| NMF(5mM) | BSO pretreated cells | 41.0±17.2 (6) |
| NEF(5mM) | - | 94.8±9.6 (4) |
| $^{2}$H-NMF(5mM) | - | 35.1±2.9 (5) |

a. 100% value is equivalent to 47nmol metabolite/$10^{6}$ cells.

b. Results from two hepatocyte preparations.

Numbers in brackets indicate the number of separa preparations of hepatocytes.

Values represent the mean ± S.D.

Table 9

**Formation of the N-methylcarbamoylating metabolite by isolated human hepatocytes(a) incubated with NMF for 4h.**

| Concentration of NMF(mM) | nmol metabolite /$10^6$ cells(b) |
|---|---|
| 0.1 | 0.5 |
| 1 | 2.6 |
| 5 | 3.0 |
| 10 | 3.8 |

(a) Hepatocytes isolated from a 4 yr old male.

(b) Results are the mean of duplicates.

The biotransformation of NEF by isolated mouse hepatocytes to a N-ethylcarbamoylating metabolite was quantitatively indistinguishable from the biotransformation of NMF (table 8). On incubation of hepatocytes with F, DMF or NMA (10 and 50mM) carbamoylating metabolites were not detected. $^{2}$H-NMF (5mM) was also metabolised to a N-methyl-carbamoylating species though the yield after 4h incubation was substantially less than that measured in the presence of 5mM NMF (table 8).

4.5.3 Discussion

The results presented here show for the first time that the hepatotoxins NMF and NEF are metabolised by hepatocytes *in vitro* to N-alkylcarbamoylating species. Furthermore, the rates at which these two equipotent toxins are metabolised are very similar. There is good agreement with previous observations *in vivo* concerning the metabolism of formamides. The NMF analogues F, DMF and NMA, which have been shown to be devoid of toxicity (sections 4.1 and 4.3) and not to undergo metabolism to detectable amounts of S-(N-alkylcarbamoyl)mercapturates *in vivo* (Kestell et al, 1987), do not generate measurable amounts of N-alkyl-carbamoylating metabolites in hepatocyte preparations. These results provide further evidence for the contention that formamide-induced toxicity and formation of N-alkylcarbamoylating metabolites are mechanistically linked.

The toxicity of NMF' and its biotransformation to SMM

and SMG are subject to a kinetic deuterium isotope effect when the molecule is deuterated in the formyl moiety: $^{2}$H-NMF was significantly less toxic *in vivo* (section 4.1) and *in vitro* (section 4.3) and its biotransformation occurred at a slower rate *in vivo* (section 1.1.1) than that observed for NMF. Likewise in mouse hepatocytes, $^{2}$H-NMF was metabolised to a N-methylcarbamoylating metabolite at a slower rate than the protium form. This result suggests that the carbamoylating metabolite is a product of the biotransformation route which affords the hepatotoxic species. The cleavage of the formyl hydrogen appears to be a rate limiting step in this pathway. Consequently the production of N-methylcarbamoylating species can be assumed to quantitatively reflect the oxidation or dehydrogenation of the parent compound. As metabolism was only saturated at concentrations of 5mM NMF or above it appears that NMF has a low affinity for this toxification pathway.

The rate of production of N-methylcarbamoylating species from NMF in human hepatocytes was less than that in mouse liver cells. In section 4.3 it was reported from the results of a preliminary experiment that human hepatocytes might be less susceptible to the cytotoxicity of NMF compared to mouse liver cells. These results if confirmed suggest that a species difference exists between human and mouse whereby the former is less able to bioactivate NMF to the toxic species.

Isolated human hepatocytes exposed to DMF or its major *in vivo* urinary metabolite HMMF (section 1.1.2) did not generate detectable levels of carbamoylating metabolites.

In man both DMF and NMF undergo metabolism to SMM, however in the case of the dimethyl analogue a marked delay in the excretion of the mercapturate has been observed (Mraz and Turecek, 1987): SMM was only detectable in the urine of a man 50h after the initial exposure to DMF as opposed to 2-4h after exposure to similar amounts of NMF. N-Alkylformamide-induced liver damage is delayed after the administration of DMF, but not NMF, to rats (Lundberg et al, 1981). Thus both formation of the mercapturate and toxicity of DMF may be the result of the slow generation of NMF in the liver, either directly from the parent compound, or via a metabolite such as HMMF. It is possible that this slow production of NMF from DMF makes hepatocyte suspensions unsuitable for the assessment of the toxicity and metabolism of DMF. Ideally cultured hepatocytes should be used for the elucidation of the toxicity of DMF. As rodents appear to be unsatisfactory animal models for the prediction of the metabolism and toxicity of DMF (Mraz et al, 1988) cultures of human hepatocytes may be the experimental model of choice for the further investigation of the mechanism of DMF-induced toxicity.

The method used for metabolite detection measured without discrimination all species derived from N-alkylformamides capable of N-alkylcarbamoylating propanol. Therefore it can not be excluded that hepatocytes produced a mixture of such species. However, allowing for a modicum of GSH resynthesis, the comparison of the amount of metabolite(s) generated from NMF with the amount of glutathione which was lost by the cells during exposure to

NMF shows a good congruity (table 10). This, and the dramatic reduction in metabolite formation after depletion of hepatocytic glutathione, suggests strongly that the metabolic species detected after derivatisation as propyl N-methylcarbamate was primarily SMG. Glutathione conjugates of paracetamol have been reported to undergo little if any metabolism to cysteine or N-acetylcysteine derivatives in isolated rat or mouse hepatocytes (Moldeus, 1978). Thus it is likely that the SMG formed is not further metabolised. The identification of this metabolite produced by isolated mouse hepatocytes was one of the aims of the experiments described in section 4.6.

Table 10

**Comparison of amounts of N-methylcarbamoylating metabolite formed in hepatocytes and of glutathione removed from hepatocytes on incubation with NMF.**

| Concentration of NMF(mM) | Incubation time(h) | Amount of metabolite[a] formed (nmol/$10^6$cell) | Amount of glutathione[b] depleted (nmol/$10^6$cell) |
|---|---|---|---|
| 0.1 | 4 | 6.7±2.5 | 2.0 |
| 1 | 2 | 17.4±3.8 | 14.3±7.7 |
| 1 | 4 | 28.5±4.8 | 27.6±4.4 |
| 5 | 2 | 27.7±7.9 | 21.7±6.5 |
| 5 | 4 | 47.0±11.0 | 32.0±2.7 |
| 10 | 2 | 25.1±4.4 | 21.8 |
| 10 | 4 | 47.5±8.3 | 32.7±1.4 |
| 100 | 2 | 31.9±1.9 | 27.0±3.5 |
| 100 | 4 | 50.1±2.3 | 32.7±1.8 |

(a) Values are the mean ± S.D. of 3 to 6 separate hepatocyte preparations.

(b) Values are the mean of 2 or the mean ± S.D. of 3 to 11 separate hepatocyte preparations.

## 4.6 Studies of the bioactivation of NMF in vitro using stable isotopes

### 4.6.1 Introduction

Stable isotopes of hydrogen, carbon, nitrogen and oxygen have been increasingly used since the early 1970s in pharmacological research. This is largely due to the refinement in the analysis of such isotopes, particularly with the introduction of GC-MS, as well as a growing demand for the development of nonradioactive tracer techniques for human studies. The application of stable isotope labelling techniques in the mechanistic study of metabolic pathways has been extensively reviewed by Baillie (1981). The experiments described in this section were performed to elucidate further the mechanism of bioactivation of NMF using stable isotopes in two separate approaches:

#### 4.6.1.1 Deuterium isotope effects

Primary and secondary deuterium isotope effects have often been used in the study of the mechanism of enzyme-catalysed reactions (Baillie, 1981). Primary kinetic isotope effects refer to the cleavage of the deuterium bond (usually C-D) and are expressed as the specific rate constants, $k_H/k_D$, for the initial reaction of the proteo and deuterium-labelled compounds respectively. The primary kinetic deuterium isotope effect is due to the large relative mass difference between hydrogen and deuterium

which results in a difference of 1.2-1.5 kcal/mol between the zero point energy of a bond to deuterium versus hydrogen (Alder et al, 1971). This in turn leads to a greater energy requirement for cleavage of a bond to deuterium than to hydrogen. Thus where rupture of the C-D bond is rate-determining an isotope effect on the rate of metabolism is evident. Theoretically the maximum primary kinetic deuterium isotope effect should be in the order of 7-10 (Wiberg, 1955). In practice however $k_H/k_D$ effects are usually less than 7 as the intrinsic isotope effect is often not the sole rate-limiting factor in the enzymatic process. The observation of a large apparent kinetic deuterium effect ($k_H/k_D > 5$) on a given metabolic reaction is taken as strong evidence that the cleavage of the C-H bond in question is rate-limiting in the reaction. Smaller isotope effects ($k_H/k_D < 2$) may also be due to primary effects or to secondary effects where by the C-D bond is not broken but exerts an effect on the transition state energy of the reaction.

Threadgill et al (1987) identified SMG as the sole glutathione metabolite of NMF in the bile of mice which had been administered the amide and demonstrated a large primary $k_H/k_D$ isotope effect in its formation when the formyl hydrogen was replaced with deuterium: Bile samples were subjected to a two step reaction, firstly with ethyl chloroformate and secondly with methanolic HCl to produce an N-(ethoxycarbonyl) dimethylester derivative of the glutathione conjugate. Products of both reactions were purified by reverse phase HPLC. The dimethylester derivative

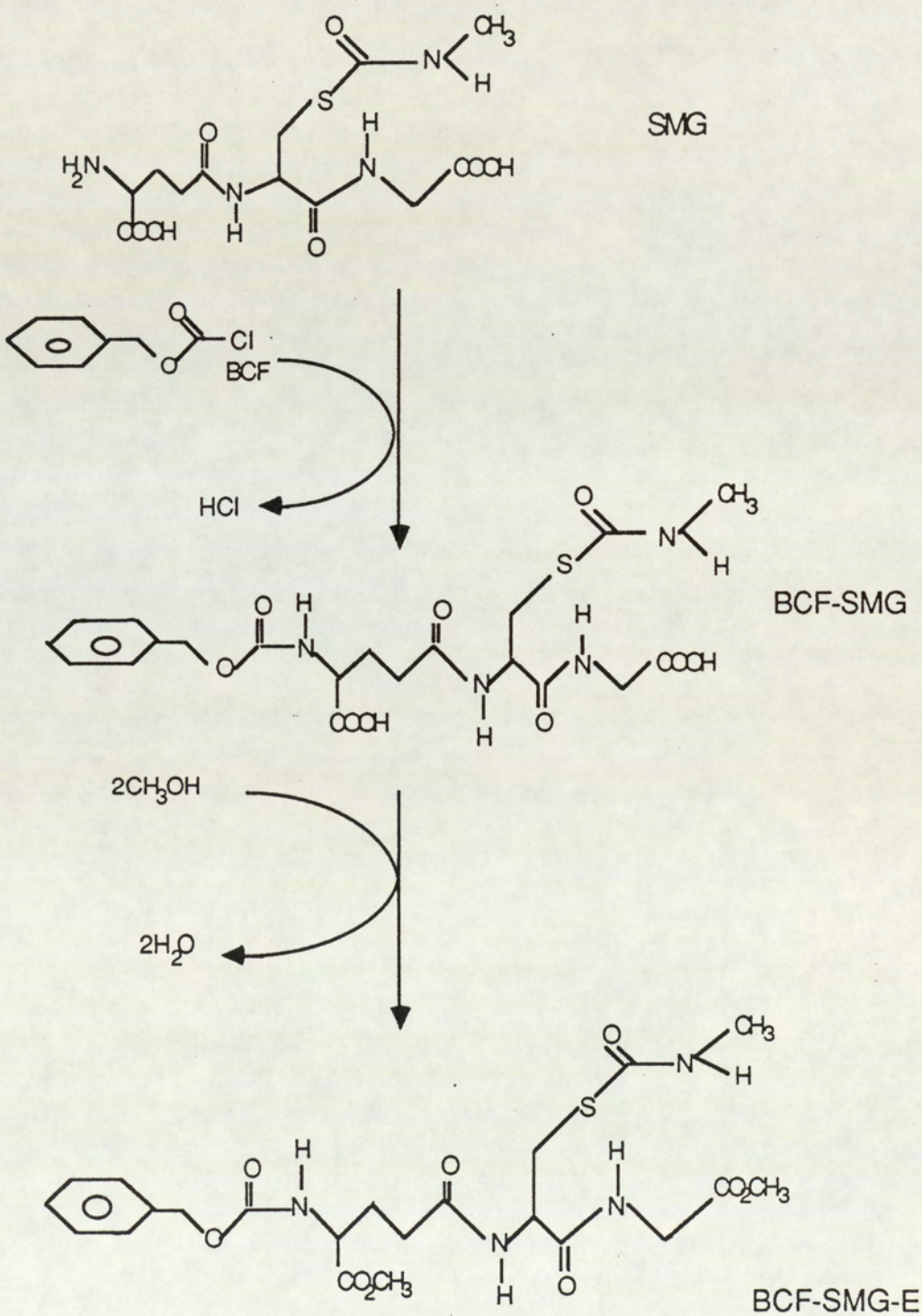
was analysed by $Cs^+$ ion LSI-MS. The resulting mass spectrum exhibited 3 prominent ions corresponding to $M+H^+$ (m/z 465), $M+Na^+$ (m/z 487) and $M+K^+$ (m/z 503). The derivative had a molecular weight of 464, the sum of NMF (59) and N-(ethoxycarbonyl)glutathione dimethylester (407) minus 2 hydrogens. A similar spectra was observed for derivatised bile samples obtained from mice which had received $^2H$-NMF. This indicated the loss of deuterium from the parent compound and that the conjugation of the NMF moiety to GSH occurred exclusively through the formyl carbon. The magnitude of the apparent primary kinetic deuterium isotope effect *in vivo* was estimated by administering a known mixture of NMF and N-(trideuteromethyl)deuteroformamide ($^2H_4$-NMF, $DOCNHCD_3$) to mice and analysing the bile as described above. Based on the relative intensities of the ions at m/z 487-490 in the LSI-MS spectrum of the derivatised conjugate, it was calculated that SMG was enriched in unlabelled over deuterated molecules relative to the starting substrate by a factor of approximately 7. It was assumed that the deuterated methyl moiety of NMF played no part in the large isotope effect observed.

Results from experiments described in sections 4.4 and 4.5 strongly indicated that monoalkylformamides were metabolised to glutathione conjugates by isolated hepatocytes. Furthermore this metabolic pathway appeared to be subject to a kinetic deuterium isotope effect in that $^2H$-NMF was less able than NMF to deplete hepatocytic glutathione or to form carbamoylating metabolites. Likewise a marked kinetic deuterium effect on hepatotoxicity was

observed *in vivo* (section 4.1) and *in vitro* (section 4.3) with $^{2}H$-NMF being less toxic than either NMF or $^{2}H_3$-NMF. In the work described in this section the hypothesis has been tested that the metabolic cleavage of the C-H bond in the formyl moiety of NMF is a rate-limiting step in the formation of SMG *in vitro*. To this end isolated mouse hepatocytes were incubated with an approximately equimolar mixture of $^{2}H$-NMF and either $^{2}H_3$-NMF or $^{13}C_2{}^{15}N$-NMF. The methodology employed for the analysis of SMG (section 3.7) was based on that used by Threadgill et al (1987) outlined above. Hepatocyte incubations were treated in turn with benzylchloroformate (BCF) and anhydrous methanolic HCl to convert any SMG formed to its N-benzyloxycarbonyl- (BCF-SMG) and N-(benzyloxycarbonyl) dimethylester (BCF-SMG-E) derivatives respectively (fig 35). The lipophilicity of these derivatives and the possession of a chromophore made them most suitable for purification by reverse phase HPLC. The isotopic composition of BCF-SMG-E was determined by FAB-MS.

Figure 35

**Derivatisation of SMG**

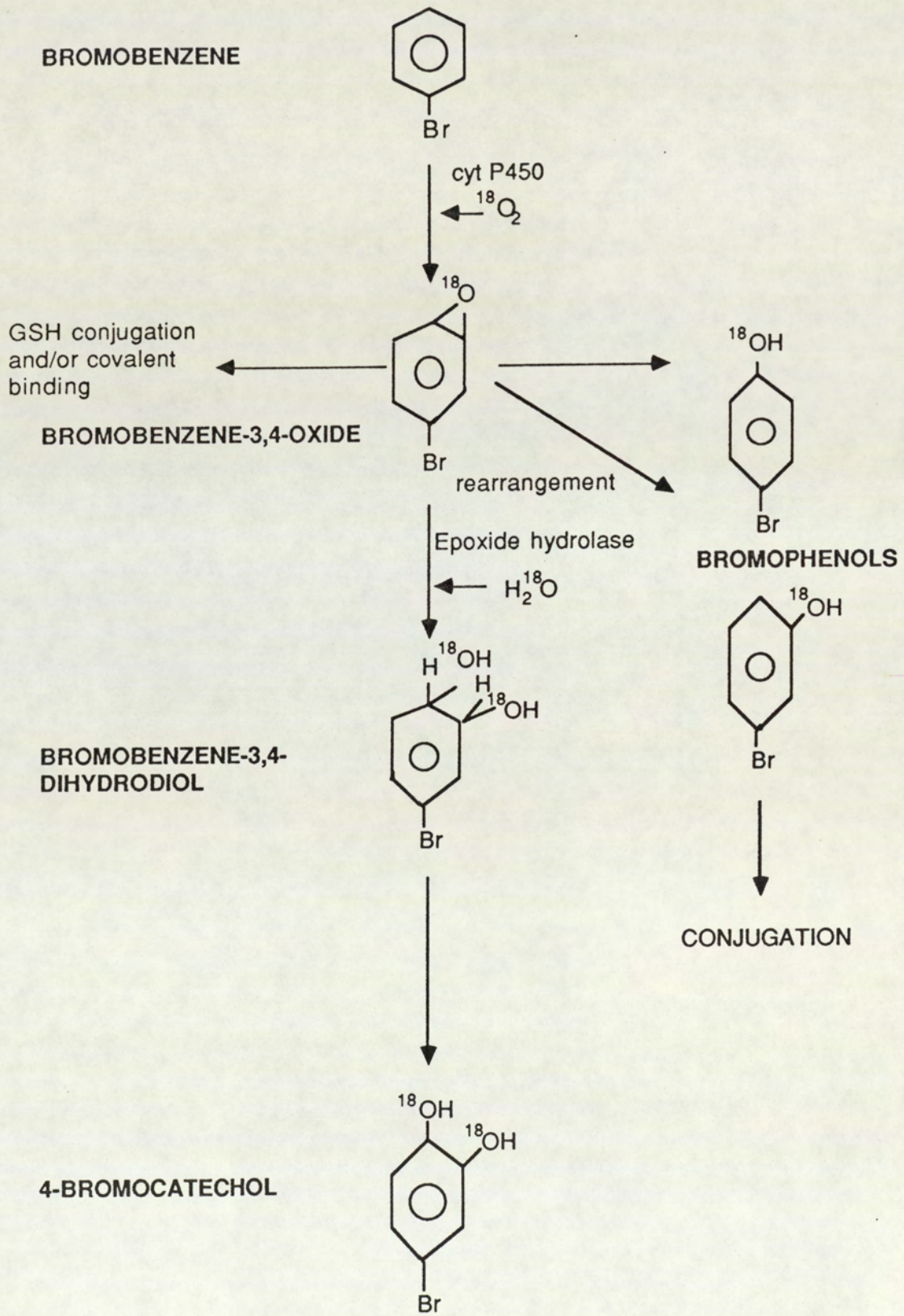


4.6.1.2 Incorporation of $^{18}O$

$^{18}O$ labelling techniques are a powerful and widely used tool for studies of the mechanism of metabolic oxidation of xenobiotics in biological systems (Pohl et al, 1977; Prickett and Baillie, 1984; Dankovic et al, 1985). Indeed present understanding of the reactions catalysed by mixed function oxidase systems have been largely due to metabolic studies carried out in the presence of $^{18}O_2$ or $H_2{}^{18}O$ (Baillie, 1981). The biological incorporation of $^{18}O$ into a drug from either of these sources not only reveals information as to the reaction chemistry but also provides clues as to the enzyme system(s) involved: The mechanism of 4-bromocatechol formation from the hepatotoxin bromobenzene (fig 36) has been investigated by examining the incorporation of $^{18}O$ from $^{18}O_2$ or $H_2{}^{18}O$ into 4-bromocatechol and its intermediates during incubations of bromobenzene with isolated rat hepatocytes (Dankovic et al, 1985). Incubation of bromobenzene with hepatocytes under an atmosphere of $^{18}O_2$ led to high levels of incorporation of $^{18}O$ into the bromophenols produced while incubations with $^{18}O$-enriched water did not. However both bromobenzene-3,4-dihydrodiol and 4-bromocatechol contained one atom of oxygen derived from the atmosphere and one atom of oxygen derived from water. Thus these results indicated that the formation of 4-bromocatechol in hepatocytes proceeded almost exclusively via the dihydrodiol presumably generated by cytochrome P450-mediated hydroxylation of the parent compound using $O_2$ followed by hydrolysis of the resulting

Figure 36

**The metabolism of bromobenzene and incorporation of $^{18}O$ into 4-bromocatechol.**



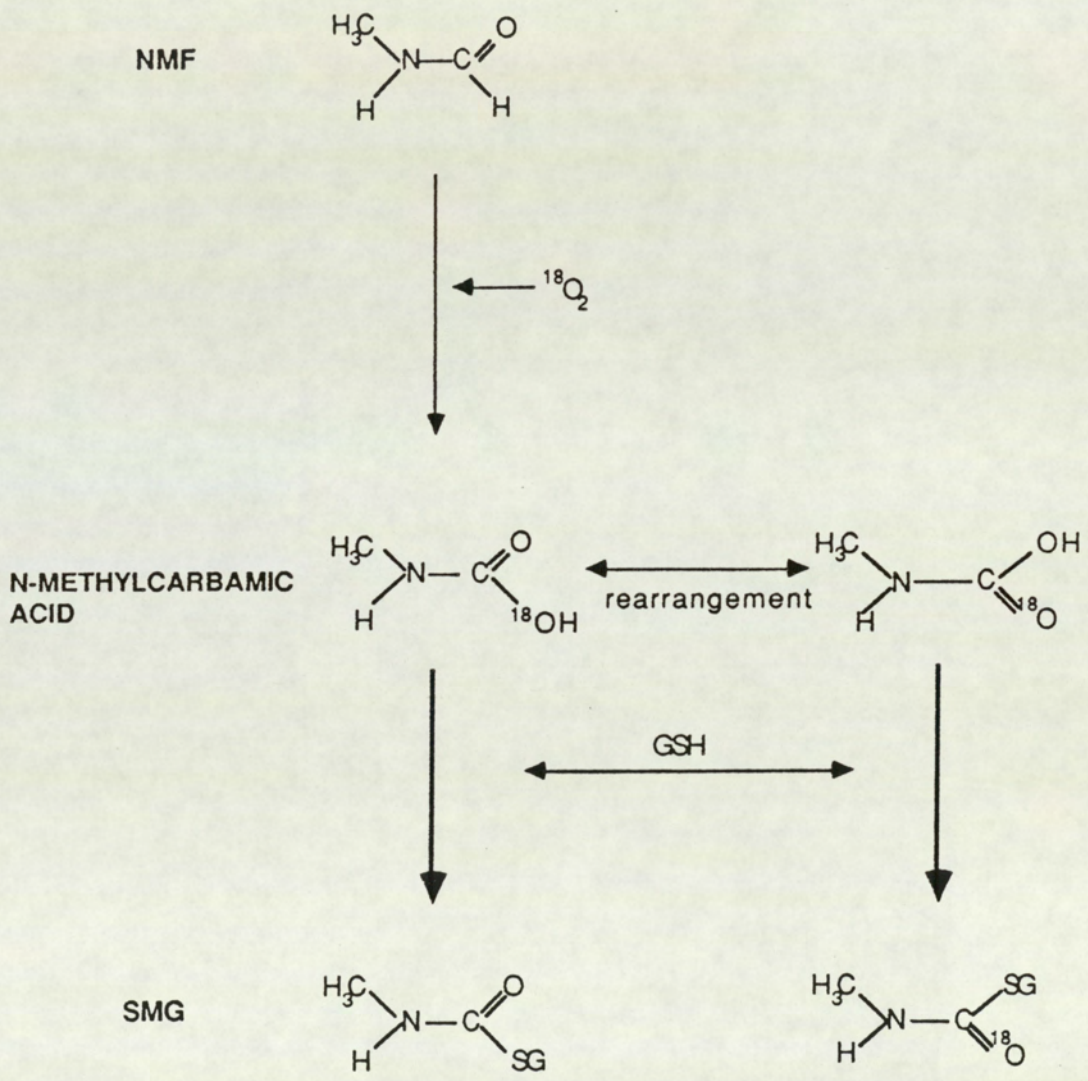
epoxide by epoxide hydrolase.

The bioactivation of NMF appears to involve the cleavage of the formyl hydrogen (section 4.6.1.1). This metabolic step may be catalysed by a mixed function oxidase system with the resulting formation of N-methylcarbamic acid. Assuming that the carbamic acid is an entity in its own right, the hydrogen in the acid group would be expected to alternate freely between the two oxygen atoms. Thus if the oxygen incorporated into the amide is $^{18}O$ and the resulting carbamic acid lives long enough, an equal ratio of metabolite labelled and unlabelled in the C=O position should be formed (fig 37). Such a species might be able to conjugate indirectly with GSH and form SMG either after formation of an ester (e.g. the phosphate ester) or after elimination of water to yield methyl isocyanate. Consequently, if N-methylcarbamic acid is labelled with $^{18}O$, the resulting glutathione conjugate will retain the label. Such a finding would indicate the formation of N-methylcarbamic acid as a possible transient intermediate in the bioactivation of NMF.

Three preparations of hepatocytes were incubated for 4h with NMF (5mM) in an atmosphere of $^{18}O_2$. Any SMG formed was derivatised to BCF-SMG-E and purified by reverse phase HPLC (section 3.7.2). Analysis of the isotopic composition of BCF-SMG-E was performed by FAB-MS (section 3.8.2). A fourth hepatocyte incubation containing acetanilide in place of NMF was also performed under the same $^{18}O_2$ atmosphere. Acetanilide is known to be efficiently metabolised by the

Figure 37

**A proposed mechanism of bioactivation of NMF and of incorporation of $^{18}O$ into SMG.**

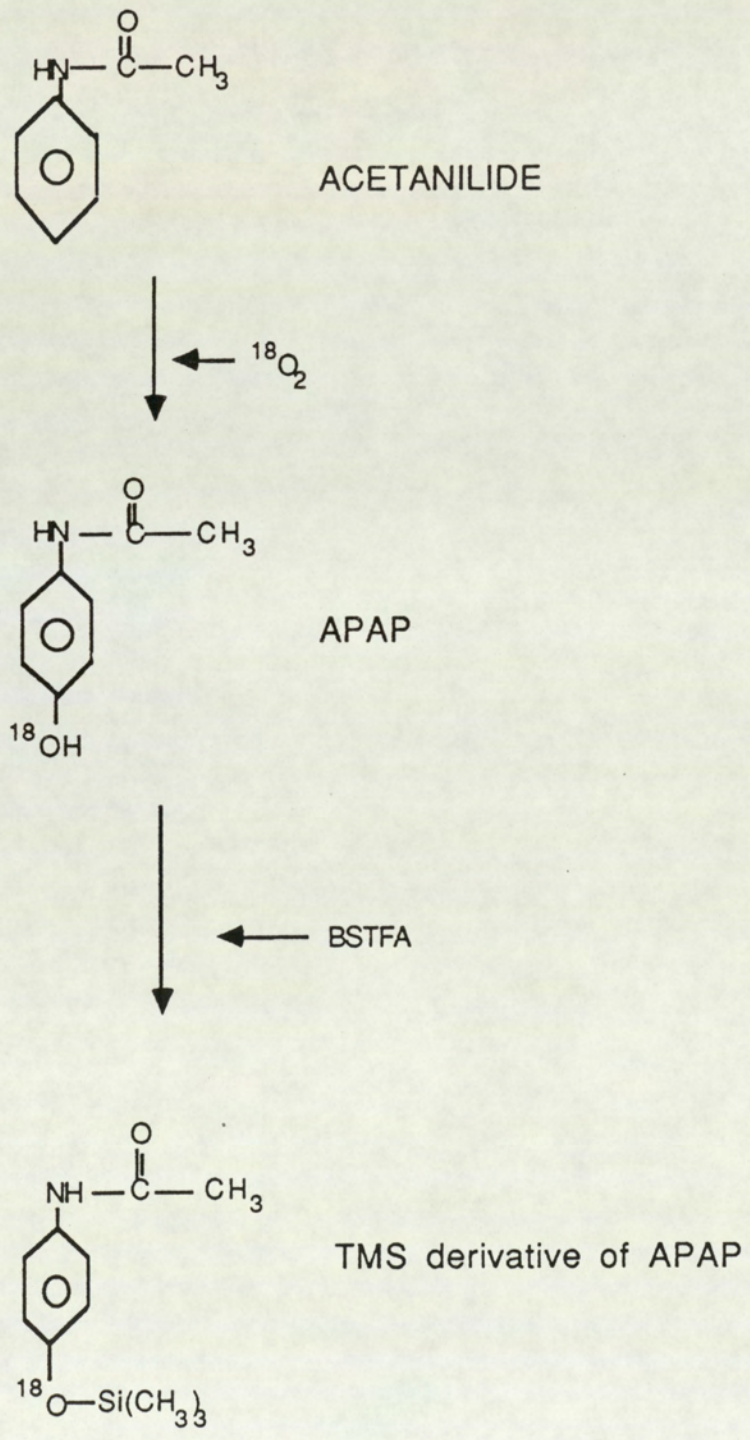


cytochrome P450 mixed function oxidase system to 4-hydroxyacetanilide (APAP). The measurement of $^{18}O$ incorporation into APAP by derivatisation to a trimethylsilyl derivative (fig 38) and GC-MS analysis (section 3.8.4) served as a positive control to indicate the isotopic enrichment of the molecular oxygen available to the hepatocytes. Thus with this knowledge, and the isotopic compositions of both authentic SMG and the glutathione conjugate formed by the metabolism of NMF under an atmosphere of $^{18}O_2$, the percentage of $^{18}O$ incorporation into SMG could be determined.

In summary, the aims of the experiments described in this section were twofold: (i) To determine whether the metabolism of NMF to SMG <u>in vitro</u> was subject to a $k_H/k_D$ isotope effect, and (ii) to determine whether this pathway involved the incorporation of atmospheric oxygen. The information obtained by these experiments should lead to a clearer understanding of the bioactivation mechanism of NMF toxicity.

Figure 38

**The formation and derivatisation of APAP.**

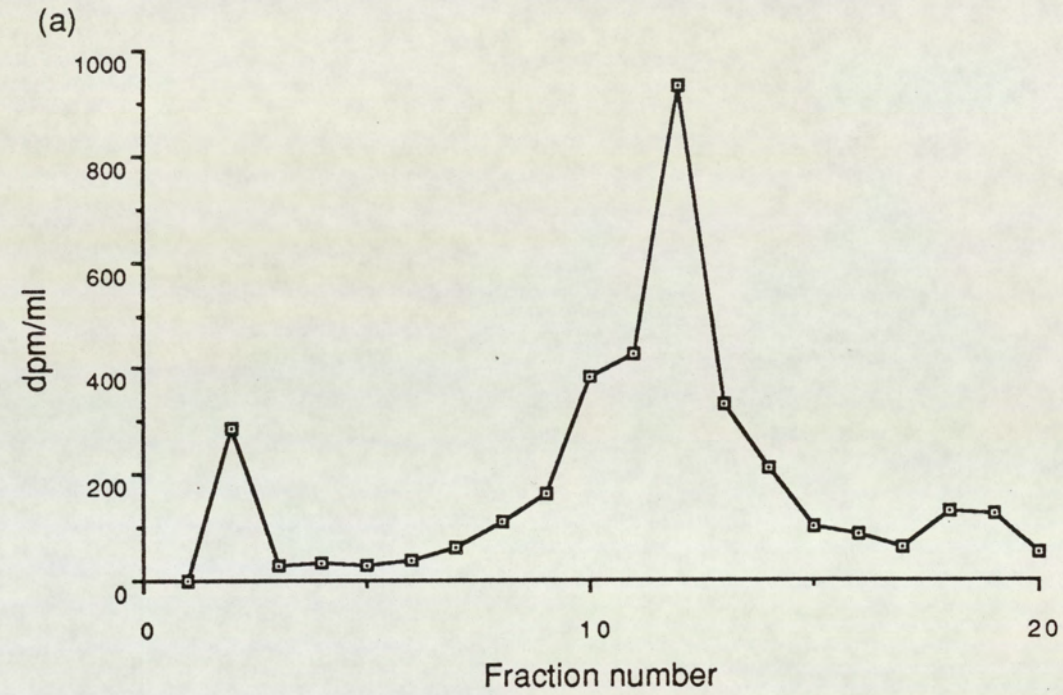


4.6.2 Results

The magnitude of the apparent primary kinetic deuterium isotope effect associated with the formation of SMG from $^{2}H$-NMF was estimated by incubating mouse hepatocytes with approximately equimolar mixtures of $^{2}H$-NMF and $^{2}H_3$-NMF or of $^{2}H$-NMF and $^{13}C_2{}^{15}N$-NMF plus a radiotracer (methyl-$^{14}C$-NMF). The exact isotopic composition of these mixtures was determined by EI-MS (section 3.7.4) and were as follows: $^{2}H$-NMF (45.02%): $^{2}H_3$-NMF (54.98%) and $^{2}H$-NMF (53.19%): $^{13}C_2{}^{15}N$-NMF (46.81%). The incubations were terminated after 4h after which time > 80% of the cells had lost their viability as determined by exclusion of trypan blue. Any SMG formed was derivatised to BCF-SMG and analysed by reverse phase HPLC. The chromatograms (fig 39) showed a strong peak of radioactivity was eluted 12min after injection of the sample on to the column. This retention time coincided with the elution of authentic BCF-SMG as determined by u.v. detection at 254nm. This fraction was esterified and analysed in the same HPLC system. The radioactive component eluted 15-16min after injection on to the column (fig 40) which coincided with the elution of authentic BCF-SMG-E as determined by u.v. detection. Fractions 15 and 16 were pooled and prepared for FAB-MS analysis. Figures 41a and 41b are FAB-MS spectra representative of the data obtained for each of the isotope effect experiments. The calculation of the isotope effect was based on the relative intensities at m/z 527 and 530 ($M+H^+$), or in samples containing $Na^+$, 0.1N NaCl was added

Figure 39

**High pressure liquid chromatograms of hepatocyte suspensions incubated with (a) $^{2}H$-NMF + $^{2}H_3$-NMF, and (b) $^{2}H$-NMF + $^{13}C^{15}N$-NMF, after treatment with BCF.**



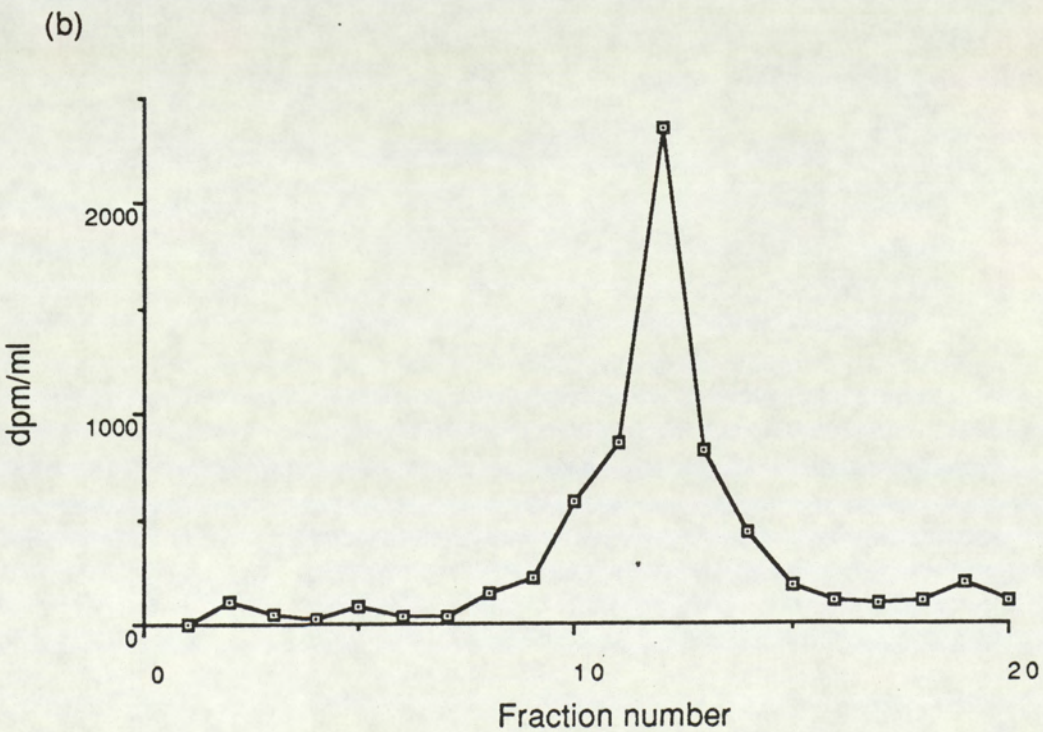
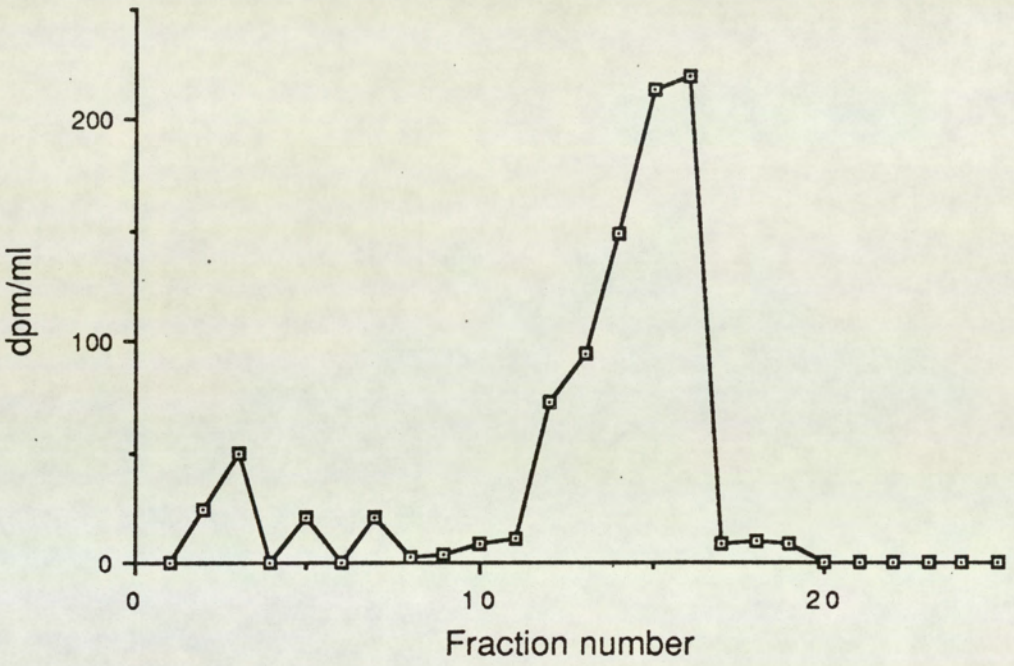



Figure 40

**High pressure liquid chromatograms of fraction 12 from (a)chromatogram 39a and (b) chromatogram 39b, after treatment with methanolic HCl.**

(a)




(b)

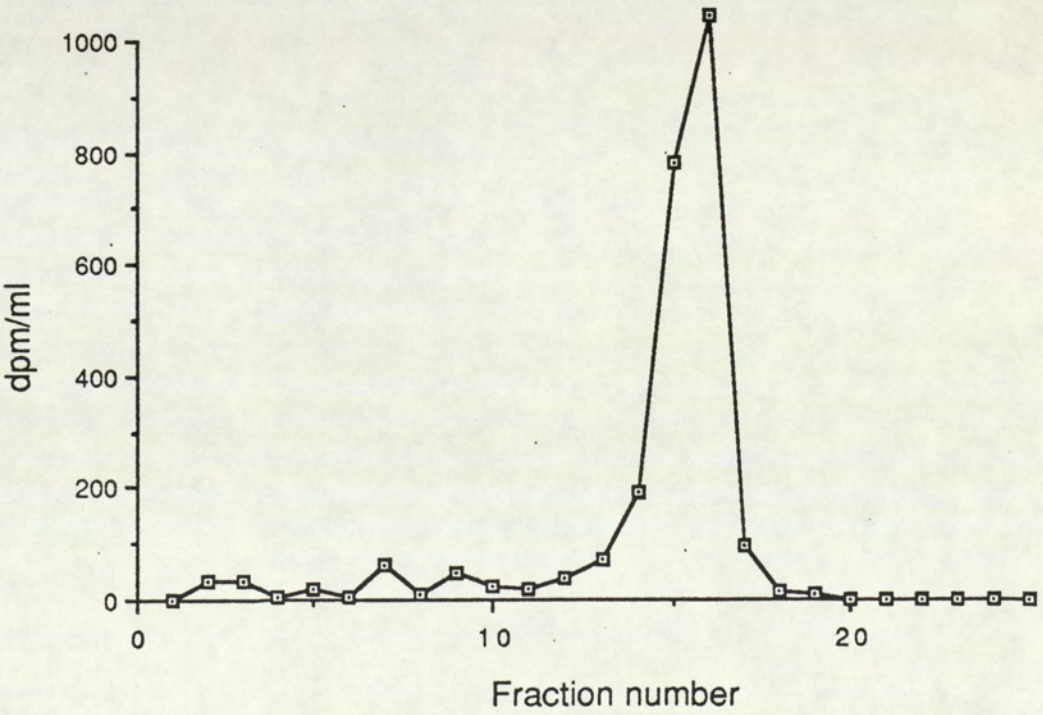
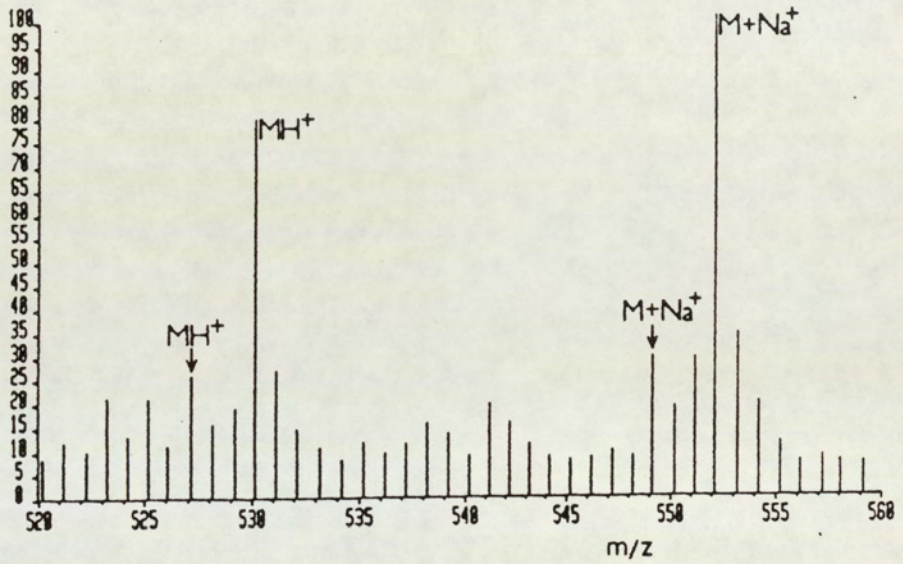


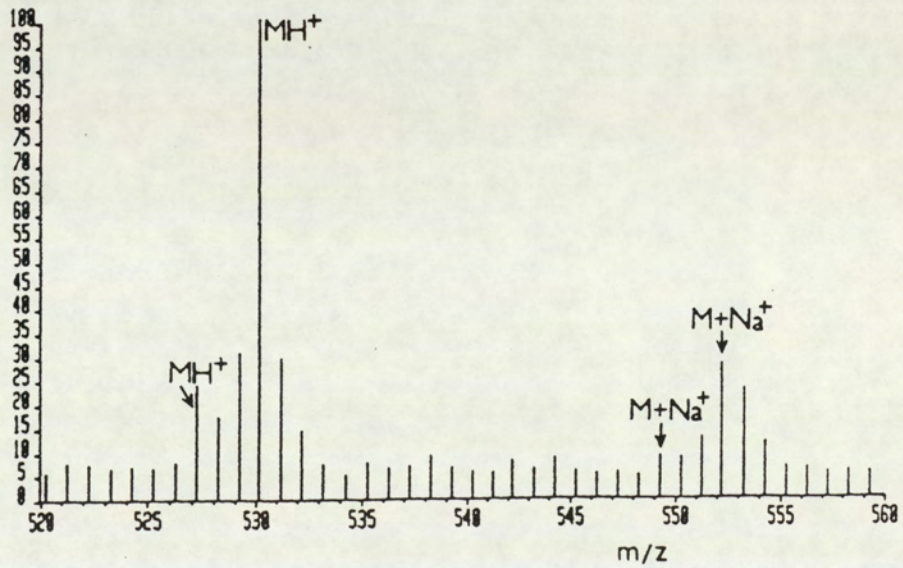
Figure 41

FAB-MS spectra of derivatised SMG obtained from isolated mouse hepatocytes incubated with approximately equimolar mixtures of (a) $^{2}H$-NMF + $^{2}H_{3}$-NMF, and (b) $^{2}H$-NMF + $^{13}C_{2}{}^{15}N$-NMF.

(a)




(b)



and the isotopic ratios at m/z 549 and 552 ($M+Na^+$) were used. The latter approach provided the best possible signal to noise ratio. The strongest peaks were at m/z 530 and 552 the molecular weight of the compound (M = 529) being equivalent to the sum of those of $^2H_3$-NMF or $^{13}C_2{}^{15}N$-NMF (62) and N-(benzyloxycarbonyl)glutathione dimethylester (469) minus two hydrogens. This indicates that a large $k_H/k_D$ isotope effect on the formation of SMG exists. The isotope effect was calculated from the knowledge of the accurate isotopic composition of both the starting substrate and the final product. From these values $k_H$ and $k_D$, and subsequently $k_H/k_D$, were deduced. The magnitude of the isotopic deuterium effect was 3.7 in the experiment using a mixture of $^2H$-NMF and $^2H_3$-NMF, and 6.2 in the experiment with $^2H$-NMF and $^{13}C_2{}^{15}N$-NMF.

The incorporation of atmospheric oxygen into the NMF metabolite SMG was investigated by incubating mouse hepatocytes with methyl-$^{14}C$-NMF in an atmosphere of $^{18}O_2$. Incubations were terminated after 4h by which time > 85% of the hepatocytes were non-viable as determined by the exclusion of trypan blue. Any SMG formed was derivatised to BCF-SMG and then to BCF-SMG-E. Both derivatives were purified by reverse phase HPLC. The resulting chromatograms were similar to those in figures 39 and 40. The isotopic composition of BCF-SMG-E was determined by FAB-MS analysis using the ion intensities at m/z 549 and 551 ($M+Na^+$) and m/z 565 and 567 ($M+K^+$)(table 11). $^{18}O$ incorporation into SMG was calculated using these values and a knowledge of the isotopic enrichment of the molecular oxygen available to the

Table 11

## Incorporation of $^{18}O$ into SMG.

The results below are the abundance of ions at $M+Na^+$ and $M+K^+$ of BCF-SMG-E. The results are expressed as the relative ion intensities of each ion based upon setting at 100% the most abundant ion of the authentic standard.

(1) Results for $M+Na^+$

| | m/z | | | $^{18}O$ incorporation (atom% excess) |
|---|---|---|---|---|
| **BCF-SMG-E** | **549** | **550** | **551** | |
| authentic standard | 100 | 32 | 17 | 0 |
| biological [$^{18}O$] sample | 100 | 37 | 48 | 24 |

(2) Results for $M+K^+$

| | m/z | | | $^{18}O$ incorporation (atom% excess) |
|---|---|---|---|---|
| **BCF-SMG-E** | **565** | **566** | **567** | |
| authentic standard | 100 | 29.5 | 20.7 | 0 |
| biological [$^{18}O$] sample | 100 | 42.3 | 185.4 | 62 |

hepatocytes. The $O_2$ atmosphere was > 95% labelled as determined by GC-MS analysis of the incorporation of $^{18}O$ into the acetanilide metabolite, APAP. The percentage incorporation of $^{18}O$ into the NMF glutathione conjugate was calculated, using the ion intensities at $M+Na^+$ and $M+K^+$, as 26% or 62% respectively. The considerable variation in values is mainly due to the large matrix contribution (background) of the mass spectra.

4.6.3 Discussion

The apparent primary kinetic deuterium isotope effect on the formation of SMG from $^2H$-NMF as compared to $^{13}C_2{}^{15}N$-NMF *in vitro* ($k_H/k_D$ = 6.2) is of similar magnitude to that measured *in vivo* for $^2H_4$-NMF compared to NMF ($k_H/k_D$ = 7 $\pm$ 2; Threadgill et al, 1987). These similar values indicate that the isotope effect is solely due to the formyl C-D bond. As such these results are strong evidence for the contention that the cleavage of the formyl hydrogen is a rate-limiting step in the bioactivation of NMF. Also these equivalent findings support the use of isolated hepatocytes as an *in vitro* model of the metabolic activation of NMF *in vivo*.

The apparent $k_H/k_D$ isotope effect of 3.7 found in the experiment with a mixture of $^2H$-NMF and $^2H_3$-NMF *in vitro*, though still large, was smaller than that measured in the experiment with $^2H$-NMF and $^{13}C_2{}^{15}N$-NMF. This discrepancy may be due to a difference in the chemical behaviour of $^2H_3$-NMF and $^{13}C_2{}^{15}N$-NMF, possibly as a result of a secondary

kinetic deuterium isotope effect exerted by the former. However this appears unlikely due to the comparable isotope effect results obtained for $^{2}H_4$-NMF and $^{13}C_2{}^{15}N$-NMF. It is more likely that this difference is a result of "experimental error" in the determination of the isotopic composition of SMG. As the isotope effect was large the matrix contribution towards the intensity of the ions at m/z 527 and 549 ($M+H^+$ and $M+Na^+$ respectively of unlabelled SMG) was significant. A "true" background subtraction could not be performed as it was not possible to design a control experiment to address this problem. As a consequence, background subtraction was based upon an average intensity for the surrounding ions which may or may not have been representative of all ion intensities. A better solution to this problem may be the employment of collisionally activated dissociation (CAD) methods in conjunction with tandem mass spectrometry as described by Pearson et al (1988) for the analysis of the isotopic ratio of daughter ions obtained from m/z 527 and 530.

The finding that $^{18}O$ was incorporated into SMG generated from NMF by isolated hepatocytes incubated in an atmosphere of $^{18}O_2$ strongly implies that the metabolic cleavage of the formyl hydrogen of NMF is a mixed function oxidation reaction (fig 37). In experiments described in section 4.2, incubations of liver microsomes with methyl-$^{14}C$-NMF were found to generate metabolites capable of covalently binding to hepatic proteins. Covalent binding was dependent on the presence of NADPH. Thus the enzyme system responsible for the oxidative activation of NMF

appears to reside in the endoplasmic reticulum of the hepatocytes. This being the case, liver microsomes ought to be able to generate carbamoylating metabolites of NMF. This hypothesis was examined in the experiments described in section 4.7.

## 4.7 Studies of the metabolism of N-alkylformamides to N-alkylcarbamoylating species by liver fractions.

### 4.7.1 Introduction

Metabolites of monoalkylformamides have not previously been detected in preparations of liver fractions (section 1.1.1). However, evidence accrued in this investigation indicates that the initial step in the bioactivation of monoalkylformamides is a microsomal mixed function oxidation in the formyl group of the parent compound: (i) Incubations of liver microsomes generated reactive species from methyl-$^{14}$C-NMF or ethyl-$^{14}$C-NEF capable of covalently binding to hepatic proteins (section 4.2). Covalent binding was dependent on the presence of NADPH and was almost abolished by the addition of GSH. (ii) The toxicity of NMF *in vivo* (section 4.1) and *in vitro* (section 4.3) and the formation of the NMF metabolite SMG by isolated hepatocytes (sections 4.4-4.6) was subject to a large kinetic deuterium isotope effect when the formyl hydrogen was replaced with deuterium. This is strong evidence that the oxidation of the formyl moiety is a rate-limiting step in the metabolic activation of the amide. (iii) A preliminary experiment described in section 4.6 suggested that the metabolism of NMF to SMG involved the incorporation of one atom of oxygen from $O_2$.

The microsomal fraction contains several enzymes involved in xenobiotic metabolism including cytochrome P450 monooxygenases, mixed function amine oxidase, epoxide

hydratase and UDP-glucuronyltransferase. Microsomal mixed function oxidations, whereby one atom of molecular oxygen is reduced to water while the other is incorporated into the substrate, are largely catalysed by a non-specific multienzyme system which has cytochrome P450 as the terminal oxidase. The aim of the experiments described in this section was to assess the ability of microsomal fractions, isolated from mouse and human liver, to bioactivate N-alkylformamides and generate carbamoylating metabolites. By doing this the suitability of liver fractions for the further investigation of N-alkylformamide metabolism was assessed.

## 4.7.2 Results

The formation of N-methylcarbamoylating metabolites by mouse liver fractions incubated with NMF (10mM) for 1h was dependent on the presence of NADPH and appeared to be greater in the post-mitochondrial supernatant than in the microsomal fraction (table 12). Metabolite formation was also observed, though to a lesser extent, when NADPH was replaced with NAD (157 $\pm$ 5 nmol/g liver/h; n = 3). Incubations of mouse hepatocytes for 2h in the presence of NMF (5mM) produced 27.7nmol metabolite/$10^6$ cells (section 4.5). As 37 $\pm$ 2 x $10^6$ hepatocytes were isolated per gram of liver (section 3.4.1), which is in accordance with a previous report (Maslansky and Williams, 1982), the rate of metabolite production was calculated as a minimum of 512nmol metabolite/g liver/h. Subsequently the rate of formation of

Table 12

**Formation of the N-methylcarbamoylating metabolite by mouse and human liver fractions incubated with NMF (10mM).**

| Species | Cell fraction | Incubation conditions | Amount of metabolite formed (nmol/g liver/h) |
|---|---|---|---|
| mouse | microsomal | +NADPH | 122±4(3) |
| | | -NADPH | 0(3) |
| mouse | post-mitochondrial supernatant | +NADPH | 204±40(4) |
| | | -NADPH | 0(3) |
| human(a) | post-mitochondrial supernatant | +NADPH | 50.1±11.7(6) |
| | | +NADPH, +GSH(10mM) | 33.1, 33.1(2) |

(a) Male, 20 months old.

Numbers in brackets indicate the number of seperate incubations performed.
Values represent the mean ± S.D.

N-methylcarbamoylating species from NMF was faster in isolated hepatocytes than in liver fractions.

The post-mitochondrial supernatant prepared from the liver of a 20 month old male human metabolised NMF (5mM) to a N-methylcarbamoylating species but at a substantially slower rate than the post-mitochondrial supernatant prepared from livers of Balb/C mice (table 12). Incubations with 50mM NMF marginally increased the rate of metabolite formation (81nmol/g liver/h; n = 2). Addition of GSH (10mM) did not increase the rate of metabolism (table 12) while addition of disulfiram (50µM), an inhibitor of various drug metabolising enzymes (Marselos et al, 1976), abolished the formation of detectable levels of metabolite. On incubation of the human liver post-mitochondrial supernatant for 1 hour with DMF or HMMF (10 and 50mM) no carbamoylating species could be detected.

### 4.7.3 Discussion

The generation of a reactive species of NMF by microsomes capable of covalently binding to hepatic proteins (section 4.2) and of forming a carbamoylating metabolite, presumably SMG, was dependent on the presence of NADPH. This suggests that covalent binding and glutathione conjugation are events downstream of a common NADPH-dependent step, most likely the microsomal mixed function oxidation of the parent compound to an electrophilic intermediate. This reaction of NMF has been proposed as a rate-limiting step in the bioactivation of NMF

and as such may explain why the addition of GSH to microsomal incubations did not enhance the production of carbamoylating metabolites.

Though the above results imply that the cytochrome P450 monooxygenases may be responsible for the metabolic activation of NMF, the total available evidence is not unequivocal. If as assumed the rate of carbamoylating metabolite production reflects the rate of oxidative activation of NMF, then the intact cell would appear more capable of performing this transformation than liver fractions. In general, the cytochrome P450 dependent oxidation of drugs, e.g. prenolol (Moldeus et al, 1974), is performed as fast if not faster in microsomal fractions containing a NADPH generating system than in isolated hepatocytes. Further, contrary to the role of cytochrome P450 in the bioactivation of NMF is the formation of carbamoylating metabolites of NMF in liver fractions containing NAD in place of NADPH. These findings suggest that another enzyme system may, at least in part, be able to oxidise NMF to reactive species and put in doubt the suitability of microsomal fractions as an in vitro model of N-alkylformamide metabolism. Possible alternative enzyme systems capable of oxidising N-alkylformamides are discussed in section 5.

Human liver fractions appear less able than their mouse counterparts to metabolise NMF to carbamoylating species. Likewise human hepatocytes seemed to be less able to metabolise NMF (section 4.5) and were more resistant to its cytotoxicity (section 4.3) than mouse hepatocytes. This may

indicate a species difference whereby humans, like rats (section 1.3.1), are less susceptible to the hepatotoxicity of NMF than mice. However caution is required when analysing such data as other factors, such as the age of the liver donor or the delay in use of the human liver after death, may have adversely influenced the metabolising ability of the tissue used.

It has been shown that there is a species difference between rodents and humans whereby the latter metabolise DMF more extensively to SMM (section 1.1.2). Metabolism of DMF to its mercapturate is thought to procede via HMMF. However, human liver fractions incubated with either DMF or HMMF (10 or 50mM) failed to generate any carbamoylating metabolites. Similarly in the work described in section 4.5, isolated human hepatocytes incubated with DMF (10mM) did not produce detectable levels of carbamoylating metabolites. The excretion of SMM in the urine of humans exposed to DMF vapours was markedly delayed in comparison to the excretion of the same mercapturate after exposure to NMF (section 1.1.2). Thus the bioactivation of DMF, possibly by way of NMF, may be substantially slower than that of NMF and so not detectable after relatively short incubations with liver fractions or hepatocytes. It may be necessary to study the metabolism of DMF in vitro using long term exposures such as with cultures of hepatocytes (section 3.4.5). The long term culturing of liver cells does have its problems, most notably the decline in activity of numerous metabolising enzymes during culture. This may be sumounted by the coculturing of human adult liver cells with rat liver

epithelial cells as the former under these conditions appear to retain high levels of cytochrome P450, and the capability to metabolise drugs by phase I and II pathways, for several days (Guillouzo, 1986).

# SECTION 5

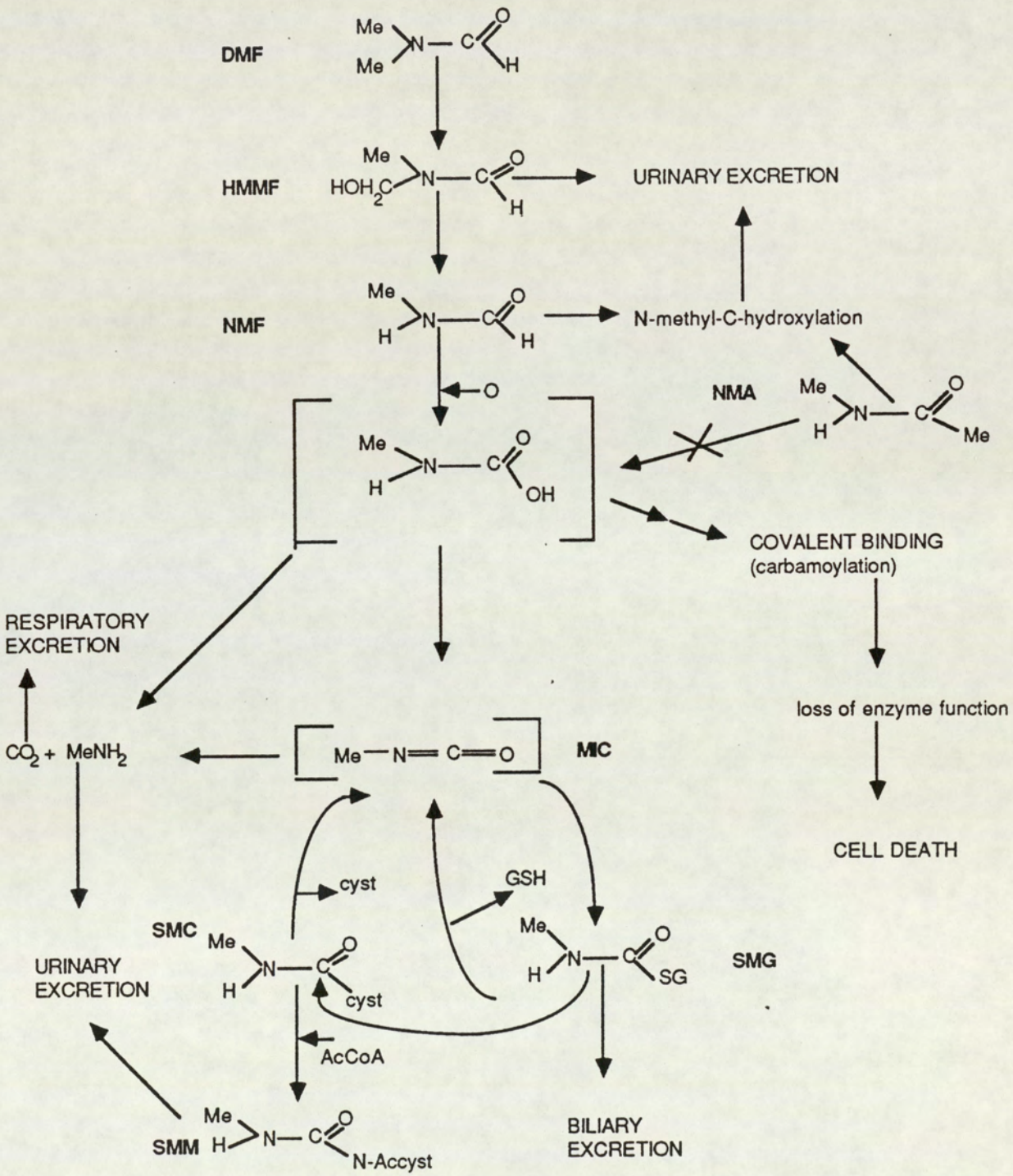
# GENERAL DISCUSSION

The aim of the work reported in this thesis was to elucidate the mechanism of hepatotoxicity of NMF and related amides. A strict relationship between the chemical structure of formamides and their toxicity both *in vivo* (section 4.1) and *in vitro* (section 4.3) was observed in that only monoalkylformamides were hepatotoxic. *In vitro* studies using liver fractions and particularly isolated hepatocytes further revealed that only these monoalkylformamides were able to generate reactive metabolites capable of covalently binding to hepatic macromolecules (section 4.2) and of depleting hepatocytic glutathione (section 4.4). Depletion of intracellular glutathione in isolated hepatocytes was quantitatively matched by the formation of carbamoylating metabolites (section 4.5) presumably S-(N-alkylcarbamoyl)glutathione (section 4.6). The discovery of these inter-relationships between structure, toxicity and metabolism has enabled conclusions to be drawn as to the mechanism of toxicity of this group of compounds. In the following these conclusions are discussed.

A proposed mechanism for the toxicity of N-alkylformamides based on the results in section 4 is summarised in figure 42. Central to this scheme is the biotransformation of the amide to one or more electrophilic, potentially toxic metabolites capable of covalently binding to hepatic protein and of forming glutathione conjugates. Evidence accrued in this investigation suggests that this toxification pathway involves the cleavage of the formyl

Figure 42

**A proposed mechanism for the metabolism and toxicity of N-alkylformamides.**




Ac = acetyl
Me = methyl
cyst = cysteine

hydrogen of the amide as a rate-limiting step: (i) Deuteration of the formyl moiety of NMF significantly reduced its hepatotoxicity both *in vivo* (section 4.1) and *in vitro* (section 4.3). (ii) $^{2}$H-NMF was less able than NMF to deplete hepatocytic glutathione (section 4.4) or form carbamoylating metabolites *in vitro* (section 4.5). (iii) SMG was tentatively identified as a metabolite of NMF *in vitro* and its formation was significantly reduced by the deuteration of the N-alkylformamide in the formyl moiety (section 4.6). (iv) Replacement of the formyl hydrogen atom of NMF with a methyl group abolished toxicity *in vivo* (section 4.1) and *in vitro* (section 4.3) and prevented the depletion of hepatocytic glutathione (section 4.4) or the formation of carbamoylating metabolites (section 4.5) in isolated hepatocyte preparations. If cleavage of the formyl hydrogen of N-alkylformamides is rate-limiting towards their biotransformation, then the production of carbamoylating metabolites should quantitatively reflect their metabolic activation. In section 4.5 the production of carbamoylating metabolites of NMF by isolated mouse hepatocytes was shown to be saturated at concentrations of NMF of 5mM or above. This result suggests that NMF has a low affinity for the enzyme(s) responsible for its bioactivation.

A plausible candidate for the electrophilic metabolite resulting from the oxidation of N-alkylformamides is alkyl isocyanate. Due to their extreme volatility and toxicity, isocyanates have been studied to a very limited extent in biological systems (Shelby et al, 1987). However, the recent leak of methyl isocyanate (MIC) at Union Carbide's

pesticide plant in Bhopal, India, which resulted in the death of more than 3000 people, has led to research into the toxicity of isocyanates (Lepkowski, 1988). MIC is also thought to be the metabolite of the investigational antitumour agent caracemide (NSC 253272) responsible for the severe central nervous system toxicity observed in patients who had received the drug (Newman and Farquhar, 1987).

Alkyl isocyanates have a half-life of approximately 2min at physiological pH being hydrolysed rapidly to alkylamines and $CO_2$ (Brown et al, 1987), both of which are metabolites of hepatotoxic N-alkylformamides. The metabolism of NMF to methylamine, like the metabolism of NMF to SMG and SMM, is subject to a large primary kinetic deuterium isotope effect in vivo and as such appears intimately linked to the toxification pathway. As methylamine is an endogenous metabolite in mammalian species it is unlikely that it itself is responsible for the biological actions of NMF.

Glutathione protects against the toxicity of NMF by reacting with the electrophilic metabolite(s) to form SMG and so presumably prevents the reactive species from binding irreversibly to essential cellular macromolecules. The sulphydryl groups of GSH and cysteine react with MIC in aqueous solution to form SMG (section 2.5) and SMC (Pearson and Han, unpublished) respectively. Furthermore, incubation of SMG with cysteine in phosphate buffer (pH 7.4) results in the formation of SMC (Pearson and Baillie, unpublished). Likewise, SMC has the ability to carbamoylate GSH at physiological pH presumably either by direct trans-

carbamoylation between the two molecules or by the release of MIC. Bruggeman et al (1986) have reported similar findings for the GSH and L-cysteine conjugates of the thio-derivatives of allyl and benzyl isocyanates (R-N=C=S): These isothiocyanate conjugates displayed considerable toxicity towards cultured rat hepatocytes. The reaction of thiols with the two isothiocyanates was readily reversible at physiological pH. Two hours after addition of L-cysteine-isothiocyanate conjugates (1mM) to medium containing GSH (5mM), 80% of the total conjugates were present as the GSH conjugate. The GSH conjugates were similarly converted to L-cysteine conjugates. Such findings suggest that SMG and its metabolites may be transport vehicles for MIC in vivo and as such may be able to carbamoylate nucleophilic sites and cause toxicity. SMC, unlike SMG, is likely to be able to enter cells and so is potentially more toxic. Related compounds such as S-carbamoylcysteine and S-ethyl-carbamoylcysteine are known to be cytotoxic (Skinner et al, 1958; Nemeth et al, 1978) and may act by irreversibly carbamoylating nucleophilic sites on cellular macromolecules. S-Carbamoylcysteine has been reported to carbamoylate the amino group of triglycine at pH 8 (Cohen and Oppenheimer, 1977). Therefore the removal of highly toxic alkyl isocyanate from hepatocytes by glutathione conjugation, though constituting a detoxifying mechanism for the liver, may pose a threat to the rest of the body. However, N-alkylformamide cytotoxicity appears to occur only after substantial depletion of intracellular glutathione (section 4.4) and so the presence of very low levels of

carbamoylating metabolites in the body may pose little specific threat except to sites where glutathione concentration is low. The necrotic damage to the gall bladder observed in mice after administration of hepatotoxic doses of NMF or NEF (section 4.1) may be a result of the concentration of S-(N-alkylcarbamoyl)glutathione in this organ.

Isocyanates are highly reactive with a variety of functional groups found on biological molecules (table 13). Hydroxyl, sulphydryl and imidazole groups readily react with isocyanates at physiological pH and are thus potential sites of reaction in vivo (Brown et al, 1987). Alkyl isocyanates might be expected to carbamoylate nucleophilic sites on cellular macromolecules, including proteins, RNA and DNA, and alter their normal function in a manner which is ultimately detrimental to the cell. MIC is capable of inducing chromosomal damage (Shelby et al, 1987). This is thought to be due to carbamoylation of either DNA or to DNA-associated proteins. Brown et al (1987) have proposed that alkyl isocyanates may cause toxicity by the random carbamoylation of proteins. This is probable when considering the short half-life of alkyl isocyanates as any nucleophilic centre will have to compete not only against the sulphydryl group of GSH but also against the fast hydrolysis of these compounds. Protein sulphydryls appear particularly susceptible to alkyl isocyanates. MIC inhibits cholinesterases (Brown et al, 1987) and butyl isocyanate inactivates yeast alcohol dehydrogenase (Twu and Wold, 1973) by carbamoylating sulphur groups on the enzymes. This may

Table 13

Reactions of isocyanates with biologically relevant functional groups.

| Reactive functional group | Product |
|---|---|
| $H_2N-R'$ | $R-NH-\overset{\overset{O}{\|}}{C}-NH-R'$ |
| $HO-R'$ | $R-NH-\overset{\overset{O}{\|}}{C}-O-R'$ |
| $HS-R'$ | $R-NH-\overset{\overset{O}{\|}}{C}-S-R'$ |
| $HO-\overset{\overset{O}{\|}}{C}-R'$ | $R-NH-\overset{\overset{O}{\|}}{C}-O-\overset{\overset{O}{\|}}{C}-R'$ |
| | $R-NH-\overset{\overset{O}{\|}}{C}-R' + CO_2$ |
| $C=C-R'$ (N, N, C) | $R-NH-\overset{\overset{O}{\|}}{C}-N(C=C-R')-N$ |
| $H_2O$ | $R-NH_2 + CO_2$ |

Reference: Brown et al (1987)

account for the inhibition of the thiol-containing enzymes alcohol and aldehyde dehydrogenase reported in rodents and humans after administration of NMF and DMF (section 1.1). However the carbamoylation of thiol groups appears to be readily reversible and it may be that the less frequent, irreversible carbamoylation of biological amino or hydroxyl groups is the initial event in the induction of cell death.

In section 4.6 a preliminary experiment indicated that the bioactivation of NMF involved the incorporation of oxygen from $O_2$ into the formyl moiety of the amide. The proposed product of this mixed function oxidation reaction, namely N-methylcarbamic acid, could be a possible precursor of MIC. Alkylcarbamic acids are highly unstable and may non-enzymatically form alkylamines and $CO_2$ *in vivo*. Furthermore, the esters of such compounds, e.g. the phosphate ester, could conceivably carbamoylate cellular macromolecules and cause cell damage.

The metabolic activation of NMF by a mixed function oxidation reaction may be catalysed by cytochrome P450. This is supported by the finding that covalent binding of radioactivity derived from methyl-$^{14}$C-NMF and ethyl-$^{14}$C-NEF to microsomal protein *in vitro* required NADPH (section 4.2). However not all the findings reported in this thesis support this view. Pretreatment of mice with SKF525A prior to the administration of NMF did not alleviate the hepatotoxicity of NMF (section 4.1). One possible explanation for this finding is that the cytochrome P450 monooxygenases may not only bioactivate NMF, but may also catalyse the detoxifying N-methyl-C-hydroxylation of the parent compound. However

the incubation of mouse liver microsomes with SKF525A (1mM) did not significantly reduce the covalent binding of methyl-$^{14}$C-NMF and ethyl-$^{14}$C-NEF metabolites to microsomal protein (section 4.2). Thus it appears that the metabolic activation of NMF is performed either by a cytochrome P450 isozyme not inhibited by SKF525A or by a different enzyme system. DTC, an inhibitor of cytochrome P450 monooxygenases, has been reported to protect against the hepatotoxicity of NMF in mice (Masuda et al, 1986). However this protection may be due to the sulphydryl group of DTC reacting with the electrophilic metabolites of NMF. The role of the microsomal mixed function oxidase system in the bioactivation of N-alkylformamides requires further investigation. One approach may be the measurement of the production of carbamoylating metabolites of these amides by microsomal fractions pretreated with various inhibitors and inducers of cytochrome P450 monooxygenases.

Biotransformation of NMF to carbamoylating metabolites by liver fractions occurred in the presence, not only of NADPH, but also NAD (section 4.7). Thus the bioactivation of NMF may be catalysed, wholly or in part, by an enzyme that uses NAD as a hydride ion acceptor. Such an enzyme is aldehyde dehydrogenase (EC 1.2.1.3) which is located in the mitochondria and cytosol of cells and is capable of oxidising a wide variety of aldehydes to their corresponding acids. These non-specific oxidations are potently inhibited by disulfiram (Hodgson and Dauterman, 1980) which also abolished the biotransformation of NMF *in vitro* (section 4.7). Inhibition of aldehyde dehydrogenase has also been

reported in rodents and humans after administration of NMF and DMF (section 1.1). However, NMF was not metabolised in vitro at physiological pH in the presence of pure aldehyde dehydrogenase extracted from bakers yeast (Shaw, unpublished result).

Aldehyde and xanthine oxidases (EC 1.2.3.1 and EC 1.2.3.2 respectively) catalyse the oxidation of a wide variety of aldehydes to their corresponding acids (Krenitsky et al, 1972). The affinity of aldehydes for xanthine oxidase is generally lower than that for aldehyde oxidase which in turn is usually much lower than for aldehyde dehydrogenase. Thus the oxidation of aldehydes does not appear to be their major role in vivo (Richert and Westerfield, 1957). These two oxidases are structurally similar flavohaemoproteins found in the liver and are capable of functioning with a wide variety of electron acceptors (Rajagopalan and Handler, 1964). The low affinity of NMF for the bioactivation pathway (section 4.5) indicates that such enzymes can not be disregarded as possible metabolic activators of N-alkylformamides.

Preliminary experiments with human hepatocytes suggest that they are not as susceptible to NMF toxicity (section 4.3) or as capable of metabolising NMF to carbamoylating metabolites (section 4.5) as mouse hepatocytes. The susceptibility of mice towards NMF hepatotoxicity is greater than that of rats and displays a marked difference between mouse strains (section 1.3.1). The strain difference is paralleled by the susceptibility of mice towards NMF-induced hepatic glutathione depletion. Thus there may be a

difference between species and strains in their ability to bioactivate NMF. This may be due to a qualitative or quantitative difference in the enzyme system responsible for the metabolic activation of NMF.

A second species difference in the hepatotoxicity of N-alkylformamides is apparent. In humans, DMF is hepatotoxic (section 1.3.2) and SMM was detected as a major urinary metabolite (section 1.1.2). However the hepatotoxic potential of DMF appears to be very low in mice (section 4.1). Incubation of up to 50mM DMF with isolated mouse hepatocytes failed to significantly reduce either cell viability (section 4.3) or levels of intracellular glutathione (section 4.4) and did not generate any carbamoylating metabolites (section 4.5). SMM has been detected as a very minor urinary metabolite of DMF in rodents the major metabolite being HMMF (Mraz et al, 1988). HMMF, like HMF, is thought to be innocuous in biological systems though this hypothesis, and the chemical stability of HMMF at physiological pH, are presently being investigated within this department. The possibility exists that HMMF may degrade slowly in vivo to NMF. At present it is not possible to separate analytically between NMF and HMMF as the latter degrades on GLC analysis to the former. The slow formation of NMF in vivo, and its subsequent metabolic activation, would account for the hepatotoxicity of DMF and for the delay in excretion of SMM witnessed in humans after exposure to DMF as compared to the excretion of the same mercapturate after exposure to NMF (Mraz and Turecek, 1987). If the formation of NMF from HMMF is

non-enzymatic, it is unlikely to give rise to the inter-species variation in susceptibility to DMF toxicity reported. Likewise, the initial metabolism of DMF to HMMF does not appear to be the source of the difference (Mraz et al, 1988). The species difference in susceptibility towards DMF hepatotoxicity appears distinct to that of NMF. As such it may be the result of the ability of different species to tolerate the slow formation of NMF in the body as opposed to the sudden assault of this amide on the liver caused by the administration of hepatotoxic doses of NMF.

In conclusion, the work presented in this thesis has helped to define more clearly the mechanism of hepatotoxicity of N-alkylformamides. This has largely been achieved by the utilisation of isolated hepatocytes as a good *in vitro* model of the *in vivo* biotransformation of this group of compounds. The future use of *in vitro* systems, be they isolated hepatocytes, microsomal fractions or probably most importantly, hepatocyte cultures, now seems essential in the further elucidation of the hepatotoxicity of NMF and structural analogues.

# APPENDICES

## Appendix 1

### Mechanisms of "paracetamol-type" toxicity

Paracetamol (acetaminophen, N-acetyl-p-aminophenol, APAP) belongs to a class of toxins of diverse structure that cause tissue damage by the generation of reactive, electrophilic metabolites. Toxicity is thought to be caused by the metabolites covalently binding to cellular macromolecules and subsequently altering their biological properties in a detrimental fashion. However the evidence to support such a role for covalent binding is largely circumstantial and based upon the persistent correlation between the extent of binding and the severity of the accompanying lesion.

The experimental work carried out to unravel the mode of toxicity of APAP and related hepatotoxins has led to three important observations (Plaa, 1980):

(a) Hepatotoxicity need not be correlated with the pharmacokinetics of the parent compound or even its major metabolites, but may be correlated with the formation of quantitatively minor, highly reactive intermediates.

(b) A threshold tissue concentration must be attained before liver injury is elicited. If it is not attained, injury does not occur.

(c) The endogenous tripeptide glutathione plays an essential role in protecting hepatocytes from injury by preventing the reactive metabolites from attaining a critical cell concentration.

Depletion of hepatic glutathione to below 30% of control levels precedes manifestations of toxicity. GSH is conjugated to the electrophilic species and generally forms a less toxic intermediate. This conjugate is further metabolised and excreted in the urine as the mercapturate. Glutathione-S-transferases catalyse the conjugation of GSH to the reactive species. At least 7 different glutathione-S-transferases have been isolated from the cytosol of rat liver (Neal, 1980). Substrates for these enzymes, as well as containing an electrophilic carbon atom, must also be hydrophobic to some degree and react non-enzymatically with GSH at some measurable rate.

### Mechanisms of APAP-induced hepatotoxicity

APAP is a widely used analgesic and antipyretic drug which in normal dosage is considered safe and nontoxic. However taken in large overdose it produces acute hepatic necrosis (Prescott et al, 1971). Given in large doses the drug produces similar lesions in laboratory animals although considerable species variation exists. For instance, the mouse is considerably more susceptible to APAP-induced hepatotoxicity than the rat (Mitchell et al, 1973b).

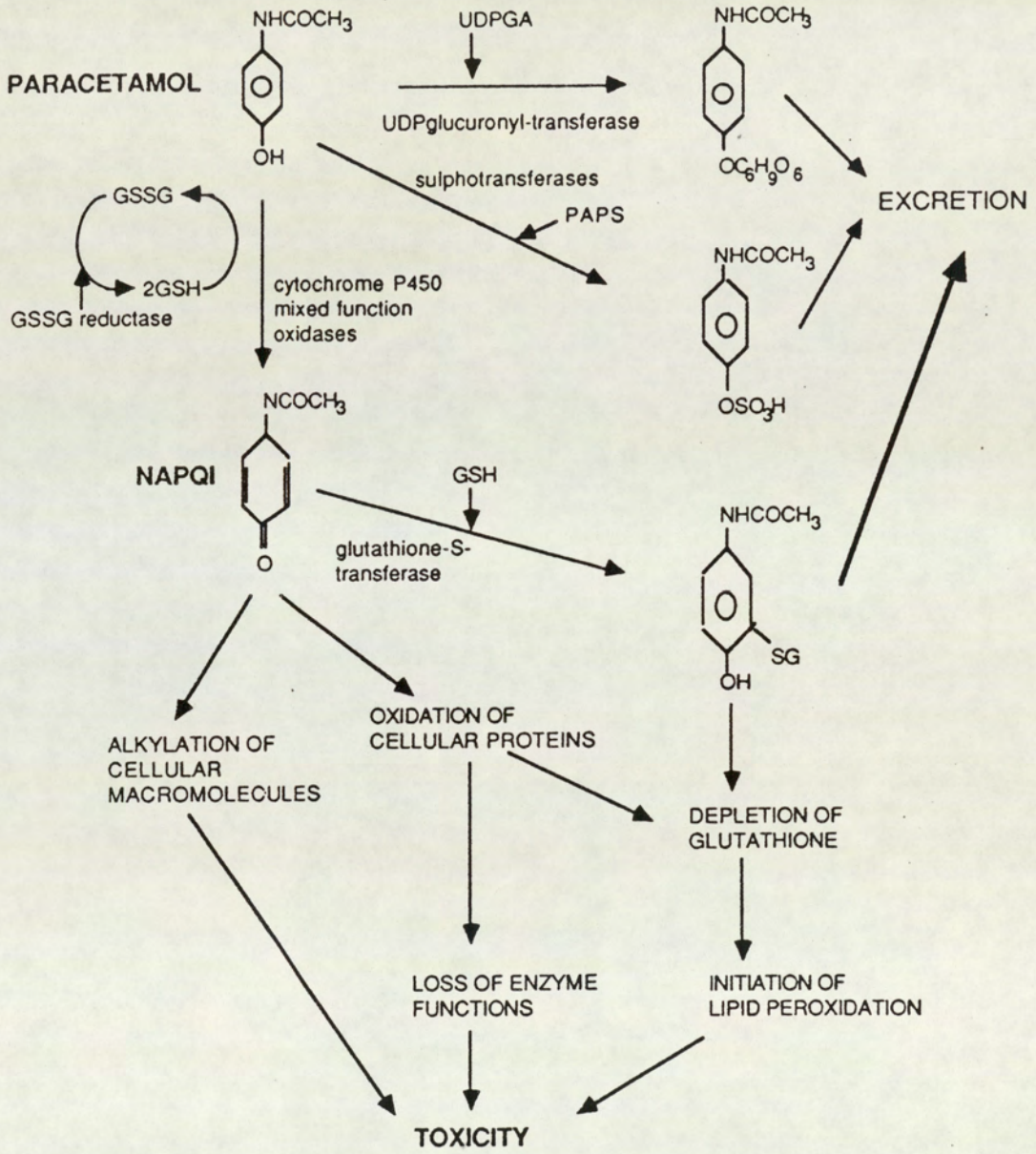
The metabolism and some possible hepatotoxic mechanisms

of APAP are summarised in figure 43. APAP is primarily metabolised by glucuronidation and sulphation to nontoxic conjugates. In addition the parent compound is metabolised to an unstable N-hyroxy derivative by the cytochrome P450 mixed function oxidase system. The N-hydroxy metabolite rapidly undergoes spontaneous degradation to N-acetyl-p-benzoquinone imine (NAPQI) which is believed to be the ultimate hepatotoxic species (Dahlin et al, 1984). At moderate doses of APAP the cell is protected by reaction of NAPQI with the nucleophilic sulphydryl of GSH (Mitchell et al, 1973c; Hinson et al, 1982). At high doses however the levels of hepatic glutathione are depleted by > 75% and covalent binding of the reactive species to cellular macromolecules occurs (Jollow et al, 1973; Mitchell et al, 1973c). The administration of compounds that are converted to the glutathione precursor L-cysteine in vivo, such as N-acetyl-L-cysteine, protects against APAP toxicity and the depletion of hepatic glutathione (Hazelton et al, 1986).

The role of covalent binding in the toxicity of APAP has been extensively investigated. Jollow et al (1973) demonstrated that covalent binding of $^{3}$H-APAP metabolites to hepatic macromolecules in vivo was negligible below the hepatotoxic dose threshold (300mg/kg) in mice. Pretreatment of mice with inducers of cytochrome P450 monooxygenases increased both covalent binding and the severity of liver necrosis. Conversely, pretreatment of mice with inhibitors of cytochrome P450 reduced both the covalent binding and the severity of hepatotoxicity. However covalent binding of APAP metabolites to cellular macromolecules has been dissociated from cell death (Devalia et al, 1982). Furthermore, radioactivity derived from 3-hydroxy-acetanilide, a nontoxic analogue of APAP, has been found to associate more with cellular macromolecules than radioactivity derived from APAP (Streeter et al, 1984a). Albano et al (1985) have shown that NAPQI covalently binds to proteins in isolated hepatocyte preparations at cytotoxic concentrations. However, the extent of binding and cytotoxicity of NAPQI are apparently dissociable: Cells pretreated with 1,3-bis-(2-chloroethyl)-1-nitrosourea, an inhibitor of glutathione reductase, were more susceptible to the cytotoxic effects of NAPQI whereas binding was unaffected. NAPQI-induced cytotoxicity did however parallel the oxidation of protein thiols suggesting that this may be an important step in the mechanism of toxicity and that the oxidation of GSH to its disulphide form may be an important protective mechanism.

Figure 43

## Possible mechanisms of paracetamol-induced hepatic injury.




Reference: Albano et al, 1985.

Appendix 2

## Plasma activity of hepatic enzymes in mice administered NMF and related amides.

### (a) CBA/CA mice

ALT

| Amide | Dose (mmol/kg) | n | Mean | S.D. | log mean | log (mean+S.D.) |
|---|---|---|---|---|---|---|
| Control | - | 13 | 36 | 20.9 | 1.56 | 1.76 |
| NMF | 1.7 | 6 | 21.7 | 3.0 | 1.34 | 1.39 |
| | 3.4 | 6 | 1550 | 1891 | 3.19 | 3.54 |
| | 6.8 | 6 | 5843 | 5852 | 3.76 | 4.07 |
| | 10.2 | 8 | 6162 | 6945 | 3.79 | 4.11 |
| NEF | 2.7 | 6 | 23.3 | 9.7 | 1.37 | 1.52 |
| | 5.5 | 6 | 124 | 213 | 2.09 | 2.53 |
| | 8.2 | 6 | 2245 | 3254 | 3.35 | 3.74 |
| F | 66.7 | 5 | 58.2 | 27.6 | 1.76 | 1.93 |
| NMA | 41.1 | 6 | 53.5 | 23.8 | 1.73 | 1.89 |
| DMF | 41.1 | 6 | 25.2 | 13.2 | 1.40 | 1.58 |

AST

| | | | | | | |
|---|---|---|---|---|---|---|
| Control | - | 13 | 105 | 63.7 | 2.02 | 2.23 |
| NMF | 1.7 | 6 | 87.7 | 31.9 | 1.94 | 2.08 |
| | 3.4 | 6 | 776 | 908 | 2.89 | 3.23 |
| | 6.8 | 6 | 4857 | 5012 | 3.69 | 3.99 |
| | 10.2 | 8 | 5813 | 6085 | 3.76 | 4.08 |
| NEF | 2.7 | 6 | 87.8 | 41.0 | 1.94 | 2.11 |
| | 5.5 | 6 | 223 | 93.5 | 2.35 | 2.50 |
| | 8.2 | 6 | 2328 | 2870 | 3.37 | 3.72 |
| F | 66.7 | 5 | 231 | 56.4 | 2.36 | 2.46 |
| NMA | 41.1 | 6 | 140 | 34.2 | 2.14 | 2.24 |
| DMF | 41.1 | 6 | 151 | 109 | 2.15 | 2.42 |

SDH

| | | | | | | |
|---|---|---|---|---|---|---|
| Control | - | 13 | 53.5 | 28.2 | 1.73 | 1.91 |
| NMF | 1.7 | 6 | 33.5 | 7.5 | 1.53 | 1.61 |
| | 3.4 | 6 | 1693 | 1773 | 3.23 | 3.54 |
| | 6.8 | 6 | 5984 | 5685 | 3.78 | 4.07 |
| | 10.2 | 8 | 5079 | 4188 | 3.71 | 3.97 |
| NEF | 2.7 | 6 | 40.5 | 15.6 | 1.61 | 1.75 |
| | 5.5 | 6 | 247 | 405 | 2.39 | 2.81 |
| | 8.2 | 6 | 2970 | 3787 | 3.47 | 3.83 |
| F | 66.7 | 5 | 144 | 47.0 | 2.16 | 2.28 |
| NMA | 41.1 | 6 | 71.5 | 27.1 | 1.85 | 1.99 |
| DMF | 41.1 | 6 | 57.3 | 30.2 | 1.76 | 1.94 |

## (b) Balb/C mice

ALT

| Amide | Dose (mg/kg) | n | Mean | S.D. | log mean | log (mean+S.D.) |
|---|---|---|---|---|---|---|
| Control | - | 6 | 21.2 | 8.0 | 1.33 | 1.47 |
| NMF | 100 | 3 | 29.0 | 6.6 | 1.46 | 1.55 |
| NMF | 200 | 6 | 5289 | 5385 | 3.72 | 4.03 |
| $^2H_3$-NMF | 100 | 3 | 20.0 | 1.7 | 1.30 | 1.34 |
| $^2H_3$-NMF | 200 | 6 | 2039 | 2143 | 3.31 | 3.62 |
| $^2H_1$-NMF | 100 | 3 | 25.7 | 7.2 | 1.41 | 1.52 |
| $^2H_1$-NMF | 200 | 8 | 94.6 | 84.3 | 1.98 | 2.25 |
| $^2H_1$-NMF | 300 | 6 | 1489 | 916 | 3.17 | 3.38 |
| SKF525A | | 3 | 26.7 | 1.2 | 1.43 | 1.45 |
| SKF525A+ NMF | 100 | 3 | 57.0 | 25 | 1.76 | 1.91 |
| SKF525A+ NMF | 200 | 4 | 1670 | 1753 | 3.22 | 3.53 |

AST

| | | | | | | |
|---|---|---|---|---|---|---|
| Control | - | 6 | 68.8 | 9.7 | 1.84 | 1.89 |
| NMF | 100 | 3 | 81.7 | 15.6 | 1.91 | 1.99 |
| NMF | 200 | 6 | 6895 | 8925 | 3.84 | 4.20 |
| $^2H_3$-NMF | 100 | 3 | 70.7 | 25.0 | 1.85 | 1.98 |
| $^2H_3$-NMF | 200 | 6 | 2163 | 2794 | 3.34 | 3.70 |
| $^2H_1$-NMF | 100 | 3 | 76.0 | 27.9 | 1.88 | 2.02 |
| $^2H_1$-NMF | 200 | 8 | 91.5 | 25.9 | 1.96 | 2.07 |
| $^2H_1$-NMF | 300 | 6 | 926 | 1220 | 2.97 | 3.33 |
| SKF525A | | 3 | 65 | 33.5 | 1.81 | 1.99 |
| SKF525A+ NMF | 100 | 3 | 100 | 53 | 2.00 | 2.18 |
| SKF525A+ NMF | 200 | 4 | 1784 | 1846 | 3.25 | 3.56 |

SDH

| Amide | Dose (mg/kg) | n | Mean | S.D. | log mean | log (mean+S.D.) |
|---|---|---|---|---|---|---|
| Control | - | 6 | 31.5 | 6.1 | 1.50 | 1.58 |
| NMF | 100 | 3 | 40.0 | 11.1 | 1.60 | 1.71 |
| NMF | 200 | 6 | 5156 | 4455 | 3.71 | 3.98 |
| $^2H_3$-NMF | 100 | 3 | 42.3 | 18.9 | 1.63 | 1.79 |
| $^2H_3$-NMF | 200 | 6 | 2810 | 2609 | 3.45 | 3.73 |
| $^2H_1$-NMF | 100 | 3 | 39.0 | 14.0 | 1.59 | 1.72 |
| $^2H_1$-NMF | 200 | 8 | 143 | 133 | 2.16 | 2.44 |
| $^2H_1$-NMF | 300 | 6 | 2452 | 1700 | 3.39 | 3.62 |
| SKF525A | | 3 | 65.0 | 12.3 | 1.81 | 1.89 |
| SKF525A+ NMF | 100 | 3 | 106 | 55 | 2.03 | 2.21 |
| SKF525A+ NMF | 200 | 3 | 1747 | 1219 | 3.24 | 3.47 |

Appendix 3

# HISTOPATHOLOGICAL FINDINGS.

Examination of livers extracted from CBA/CA mice 24h after administration of NMF and four structural analogues.

| | | N-METHYLFORMAMIDE | | | | |
|---|---|---|---|---|---|---|
| | DOSE (mg/kg) | 0 | 100 | 200 | 400 | 600 |
| | n | 4 | 3 | 4 | 1 | 2 |
| | NORMAL | 4 | 0 | 0 | 0 | 0 |
| **LIVER PATHOLOGY** | | | | | | |
| Periportal glycogen accumulation | grade 1 | 0 | 1 | 2 | 0 | 1 |
| | grade 2 | 0 | 2 | 1 | 0 | 0 |
| | grade 3 | 0 | 0 | 0 | 1 | 1 |
| Increased eosinophilia of centrilobular cells | grade 1 | 0 | 3 | 3 | 0 | 0 |
| | grade 2 | 0 | 0 | 0 | 0 | 1 |
| | grade 3 | 0 | 0 | 0 | 0 | 0 |
| Centrilobular haemorrhagic necrosis | grade 1 | 0 | 0 | 1 | 0 | 1 |
| | grade 2 | 0 | 0 | 0 | 0 | 0 |
| | grade 3 | 0 | 0 | 1 | 0 | 0 |
| | grade 4 | 0 | 0 | 0 | 1 | 1 |
| | grade 5 | 0 | 0 | 0 | 0 | 0 |
| Centrilobular degenerative change without necrosis | grade 1 | 0 | 0 | 0 | 0 | 0 |
| | grade 2 | 0 | 0 | 0 | 0 | 0 |
| | grade 3 | 0 | 0 | 0 | 0 | 0 |
| Pooling of blood in sinusoids without necrosis | grade 1 | 0 | 0 | 0 | 0 | 0 |
| | grade 2 | 0 | 0 | 0 | 0 | 0 |
| | grade 3 | 0 | 0 | 0 | 0 | 0 |
| Haemosiderin deposits | | 0 | 0 | 1 | 0 | 1 |

| | | N-METHYLFORMAMIDE | | | | |
|---|---|---|---|---|---|---|
| | DOSE (mg/kg) | 0 | 100 | 200 | 400 | 600 |
| | n | 4 | 3 | 4 | 1 | 2 |
| | NORMAL | 4 | 0 | 0 | 0 | 0 |
| **GALL BLADDER** | | | | | | |
| Mucosal erosion and necrosis | grade 1 | 0 | 0 | 0 | 1 | 1 |
| | grade 2 | 0 | 0 | 0 | 0 | 0 |
| Submucosal haemorrhage | grade 1 | 0 | 0 | 0 | 0 | 0 |
| | grade 2 | 0 | 0 | 0 | 0 | 0 |
| | grade 3 | 0 | 0 | 0 | 0 | 0 |
| Submucosal oedema | grade 1 | 0 | 0 | 0 | 0 | 1 |
| | grade 2 | 0 | 0 | 0 | 0 | 0 |

| | | N-ETHYLFORMAMIDE | | | |
|---|---|---|---|---|---|
| | DOSE (mg/kg) | 0 | 200 | 400 | 600 |
| | n | 4 | 3 | 4 | 2 |
| | NORMAL | 4 | 2 | 2 | 0 |
| **LIVER PATHOLOGY** | | | | | |
| Periportal glycogen accumulation | grade 1 | 0 | 0 | 1 | 0 |
| | grade 2 | 0 | 1 | 1 | 0 |
| | grade 3 | 0 | 0 | 0 | 0 |
| Increased eosinophilia of centrilobular cells | grade 1 | 0 | 0 | 2 | 0 |
| | grade 2 | 0 | 0 | 0 | 0 |
| | grade 3 | 0 | 0 | 0 | 0 |
| Centrilobular haemorrhagic necrosis | grade 1 | 0 | 0 | 0 | 0 |
| | grade 2 | 0 | 0 | 0 | 0 |
| | grade 3 | 0 | 0 | 0 | 1 |
| | grade 4 | 0 | 0 | 0 | 0 |
| | grade 5 | 0 | 0 | 0 | 0 |
| Centrilobular degenerative change without necrosis | grade 1 | 0 | 0 | 0 | 1 |
| | grade 2 | 0 | 0 | 1 | 0 |
| | grade 3 | 0 | 0 | 0 | 0 |
| Pooling of blood in sinusoids without necrosis | grade 1 | 0 | 1 | 1 | 1 |
| | grade 2 | 0 | 0 | 0 | 0 |
| | grade 3 | 0 | 0 | 0 | 0 |
| Haemosiderin deposits | | 0 | 0 | 0 | 0 |

| | | N-ETHYLFORMAMIDE | | | |
|---|---|---|---|---|---|
| | DOSE (mg/kg) | 0 | 200 | 400 | 600 |
| | n | 4 | 3 | 4 | 2 |
| | NORMAL | 4 | 2 | 2 | 0 |
| **GALL BLADDER** | | | | | |
| Mucosal erosion and necrosis | grade 1 | 0 | 0 | 0 | 1 |
| | grade 2 | 0 | 0 | 0 | 0 |
| | grade 3 | 0 | 1 | 0 | 1 |
| Submucosal haemorrhage | grade 1 | 0 | 0 | 0 | 0 |
| | grade 2 | 0 | 0 | 1 | 0 |
| | grade 3 | 0 | 0 | 0 | 2 |
| Submucosal oedema | grade 1 | 0 | 1 | 0 | 0 |
| | grade 2 | 0 | 1 | 0 | 0 |

| | | | F | NMA | DMF |
|---|---|---|---|---|---|
| | DOSE (mg/kg) | 0 | 3000 | 3000 | 800+1600 |
| | n | 4 | 3 | 3 | 2 |
| | NORMAL | 4 | 0 | 0 | 0 |
| **LIVER PATHOLOGY** | | | | | |
| Periportal glycogen accumulation | grade 1 | 0 | 0 | 0 | 0 |
| | grade 2 | 0 | 2 | 0 | 1 |
| | grade 3 | 0 | 0 | 3 | 1 |
| Increased eosinsphilia of centrilobular cells | grade 1 | 0 | 0 | 0 | 0 |
| | grade 2 | 0 | 0 | 3 | 2 |
| | grade 3 | 0 | 0 | 0 | 0 |
| Centrilobular haemorrhagic necrosis | grade 1 | 0 | 0 | 0 | 0 |
| | grade 2 | 0 | 0 | 0 | 0 |
| | grade 3 | 0 | 0 | 0 | 0 |
| | grade 4 | 0 | 0 | 0 | 0 |
| | grade 5 | 0 | 0 | 0 | 0 |
| Centrilobular degenerative change without necrosis | grade 1 | 0 | 1 | 0 | 0 |
| | grade 2 | 0 | 0 | 0 | 0 |
| | grade 3 | 0 | 0 | 0 | 0 |
| Pooling of blood in sinusoids without necrosis | grade 1 | 0 | 3 | 2 | 0 |
| | grade 2 | 0 | 0 | 0 | 0 |
| | grade 3 | 0 | 0 | 0 | 0 |
| Haemosiderin deposits | | 0 | 0 | 0 | 0 |

| | | | F | NMA | DMF |
|---|---|---|---|---|---|
| | DOSE (mg/kg) | 0 | 3000 | 3000 | 800+1600 |
| | n | 4 | 3 | 3 | 2 |
| | NORMAL | 4 | 0 | 0 | 0 |
| **GALL BLADDER** | | | | | |
| Mucosal erosion and necrosis | grade 1 | 0 | 0 | 0 | 0 |
| | grade 2 | 0 | 0 | 0 | 0 |
| | grade 3 | 0 | 0 | 0 | 0 |
| Submucosal haemorrhage | grade 1 | 0 | 1 | 0 | 0 |
| | grade 2 | 0 | 0 | 0 | 0 |
| | grade 3 | 0 | 0 | 0 | 0 |
| Submucosal oedema | grade 1 | 0 | 0 | 0 | 0 |
| | grade 2 | 0 | 0 | 0 | 0 |

# Appendix 4

## Publications

### Abstracts

A1. Threadgill, M.D., Gescher, A., Gledhill, A.P., Kestell, P., Shaw, A.J. and Farmer, P.B. (1986)
The metabolism and hepatotoxicity in the mouse of N-ethylformamide, a close analogue of N-methylformamide.
10th European Drug Metabolism Workshop, Guildford, U.K.

A2. Shaw, A.J. and Gescher, A. (1987)
Studies of the hepatotoxicity of N-methylformamide (NMF) in mouse hepatocytes.
3rd Stowe School Symposium on Drug Metabolism, U.K.

A3. Gescher, A., Shaw, A.J. and Mraz, J. (1987)
Studies of the hepatotoxicity and metabolism of formamides in mouse hepatocytes.
The Royal Society of Chemistry Toxicology Group Meeting, Birmingham, U.K.

A4. Shaw, A.J., Gescher, A. and Chipman, J.K. (1988)
Metabolism of N-methylformamide (NMF) to S-(N-methylcarbamoyl)glutathione in murine and human liver preparations.
11th European Drug Metabolism Workshop, Konstanz, F.R.G.

### Papers

P1. Kestell, P., Threadgill, M.D., Gescher, A., Gledhill, A.P., Shaw, A.J. and Farmer, P.B. (1987)
An investigation of the relationship between the hepatotoxicity and the metabolism of N-alkylformamides.
Journal Pharmacol. Exp. Ther., 240, 265-270.

P2. Threadgill, M.D., Axworthy, D.B., Baillie, T.A., Farmer, P.B., Farrow, K.C., Gescher, A., Kestell, P., Pearson, P.G. and Shaw, A.J. (1987)
The metabolism of N-methylformamide in mice. Primary kinetic deuterium isotope effect and identification of S-(N-methylcarbamoyl)glutathione as a metabolite.
Journal Pharmacol. Exp. Ther., 242, 312-319.

P3. Shaw, A.J., Gescher, A. and Mraz, J. (1988)
Cytotoxicity and metabolism of the hepatotoxin N-methylformamide and related formamides in mouse hepatocytes.
Toxicol. Appl. Pharmacol., in press.

P4. Baillie, T.A., Pearson, P.G., Threadgill, M.D., Howald, W.N., Han, D.-H., MacKenzie, N.E., Shaw, A.J. and Gescher, A. (1988)
Stable isotopes: Unique probes for mechanistic studies of xenobiotic metabolism. Application to studies on the bioactivation of N-methylformamide in vitro.
Proceedings of the ISSX conference, Kobe, Japan. Taylor and Francis, in press.

## A1 THE METABOLISM AND HEPATOTOXICITY IN THE MOUSE OF N-ETHYLFORMAMIDE, A CLOSE ANALOGUE OF N-METHYLFORMAMIDE.

M D Threadgill, A Gescher, A P Gledhill, P Kestell, [+]A Shaw and [*]P B Farmer. CRC Experimental Chemotherapy Group and [*]MRC Mechanisms of Drug Toxicity Group, Pharmaceutical Sci.Inst., Aston University, Birmingham and [+]MRC Toxicology Unit, Carshalton, Surrey.

N-Methylformamide (NMF) is an investigational antitumour drug with hepatotoxic properties. However, N-ethylformamide (NEF), a close analogue, exhibits only marginal activity against murine tumours (Gate et al, J. Med. Chem., in press), although it causes very similar histological and biochemical hepatotoxicity. NMF is metabolised to a reactive intermediate which may explain its toxicity (Pearson et al, submitted). S-(N-Methylcarbamoyl)-N-acetylcysteine and methylamine have been identified as major urinary metabolites of NMF (Kestell et al, submitted). The formation of the former metabolite involves the reaction of glutathione with a compound derived from NMF by oxidation at the formyl group. In an attempt to explain the similarity of toxicity which contrasts with the differences in antineoplastic properties between NMF and NEF, the metabolism of NEF was studied in CBA/CA mice and compared with that of NMF. S-(N-Ethylcarbamoyl)-N-acetylcysteine and ethylamine were identified by co-chromatography, 400 MHz $^1$H NMR and mass spectroscopy in the urine of mice which had received NEF. These data would suggest that the S-(N-alkylcarbamoyl)metabolites (or their carbamoylating agent precursors) may be responsible for the hepatotoxicity of these formamides but tend to discount their involvement in antineoplastic activity.

## A2 STUDIES OF THE HEPATOTOXICITY OF N-METHYLFORMAMIDE (NMF) IN MOUSE HEPATOCYTES

A.J.Shaw and A. Gescher MRC Mechanisms of Drug Toxicity Research Group, Pharmaceutical Sciences Institute, Aston University, Birmingham.

Evidence accrued in these laboratories suggests that the genesis of the hepatotoxic lesion caused by the experimental antitumour drug NMF is associated with its hepatic metabolism. But to investigate in vivo which metabolic intermediate of NMF might be responsible for its toxicity is virtually impossible. On incubation with liver fractions in vitro NMF is metabolised only to a minute extent. Therefore the hypothesis has been tested that suspensions of hepatocytes are a more suitable system to demonstrate a link between the toxicity and the metabolism of this drug. Mouse hepatocytes were isolated by retro perfusion of the liver with calcium- and magnesium-free Hank's salt solution followed by a medium with collagenase. In incubations containing Krebs buffer with 10% horse serum and 0.2% bovine serum albumin hepatocytes maintained their functional viability for more than 6 hours as assessed by their abilities to exclude trypan blue and to retain lactate dehydrogenase. On incubation with NMF their viability was affected drastically. Cell viability was decreased to the following percentages compared to controls, after incubation for 6 hours: 52.4 ± 12.0% (1 mM NMF), 14.6 ± 20.2% (5 mM NMF) and 28.7 ± 10.6% (50 mM NMF). These results contrast with the lack of cytotoxicity of NMF when incubated with TLX5 lymphoma cells as they have to be exposed for 48 hours to 106 mM in order to achieve 20% cell death compared to controls. Further studies will show whether the toxicity observed in hepatocytes is indeed due to metabolism.

A3 STUDIES OF THE HEPATOTOXICITY AND METABOLISM OF FORMAMIDES IN MOUSE HEPATOCYTES.

Andreas Gescher, Andrew J Shaw and Jaroslav Mraz. MRC Mechanism of Drug Toxicity Research Group and CRC Experimental Chemotherapy Research Group, Pharmaceutical Sciences Institute, Aston University, Birmingham, U.K.

Evidence accrued in these laboratories (Kestell et al., J. Pharm. Exp. Ther., 240, 265, 1987; Pearson et al. Biochem. Pharmacol., 36, 381 and 385, 1987) suggests that the genesis of the hepatotoxic lesion caused by the experimental antitumor drug N-methylformamide (NMF) is associated with its hepatic metabolism. The investigation in vivo of metabolic intermediates of NMF which might be responsible for its toxicity is virtually impossible. On incubation with liver fractions in vitro the extent of metabolism of NMF is minute and can be measured only by determination of the amount of drug-derived species which are bound covalently to microsomal proteins (Pearson et al., Biochem. Pharmacol., 36, 385, 1987). Here the hypothesis has been tested that suspensions of hepatocytes are a suitable in vitro system to demonstrate a link between the toxicity and the metabolism of this drug. Hepatocytes were isolated from male BALB/c mice by retro perfusion of the liver with calcium- and magnesium-free Hanks salt solution followed by a medium with collagenase. In incubations containing Krebs buffer with 10% horse serum and 0.2% bovine serum albumin hepatocytes maintained their viability for more than 6 hours as assessed by their ability to exclude trypan blue and to retain lactate dehydrogenase. On incubation with NMF their viability was affected drastically. Cell viability was decreased to the following percentages compared with controls after incubation for 6 hours: 52.4 ± 12.0% (1mM NMF), 14.6 ± 20.2% (5mM NMF) and 28.7 ± 10.6% (50mM NMF). These results contrast with the lack of cytotoxicity of NMF when incubated with TLX5 lymphoma cells which have to be exposed for 48 hours to 106mM NMF in order to achieve 20% cell death compared to controls. Cytotoxicity has also been observed on exposure of hepatocytes to N-ethylformamide (5mM) but not with dimethylformamide, formamide or N-methylacetamide (5mM). This observation is commensurate with the finding that in this series of NMF analogues only the monoalkylformamides are powerful hepatotoxins in vivo (Kestell et al., J. Pharm. Exp. Ther. 240, 265, 1987).

Extraction of the hepatocyte suspensions with propanol in the presence of $K_2CO_3$ afforded a product which was identified as propyl N-methylcarbamate on gas chromatography. On the basis of the observation that N-acetyl-S-(N-methylcarbamoyl)cysteine is a major urinary metabolite of NMF we tender the suggestion that the metabolite which reacted with propanol is S-(N-methylcarbamoyl) glutathione. It appears probable that the as yet unknown precursor of this conjugate is responsible for the hepatotoxicity of NMF.

This study was supported by travel grants from the British Council, the Nuffield Foundation and a NATO grant.

## A4 METABOLISM OF N-METHYLFORMAMIDE (NMF) TO S-(N-METHYLCARBAMOYL)GLUTATHIONE IN MURINE AND HUMAN LIVER PREPARATIONS

A. J. Shaw, A. Gescher and J. K. Chipman

MRC Mechanisms of Drug Toxicity Group, Pharmaceutical Sciences Institute, Aston University, Birmingham, U.K. (A.J.S., A.G.) and Department of Biochemistry, University of Birmingham, U.K. (J.K.C.)

NMF ($CH_3NHCHO$), an industrial solvent, is capable of inducing differentiation in certain human leukaemia cell lines and has undergone clinical evaluation as potential anticancer drug. Liver damage is one of the major unwanted effects associated with the administration of NMF to animals or humans. The mechanism by which NMF causes hepatotoxicity involves the metabolism of the molecule to a species which is excreted with the urine as N-acetyl-S-(N-methylcarbamoyl)cysteine (Threadgill et al., J. Pharm. Exp. Ther. 242, 312, 1987). S-(N-Methylcarbamoyl)-glutathione (SMG, $CH_3NHCO$-SG), the metabolic precursor of the mercapturate, has been identified unambiguously as the only metabolite of NMF found to date in vitro (Pearson et al., Biochem. Environm. Mass Spec., in press). As part of an investigation to elucidate the role of metabolism in the toxicity of NMF we studied details of the metabolism of NMF to SMG in suspensions of either hepatocytes or of liver 9000 g supernatant obtained from male BALB/c mice or humans. The formation of SMG was measured indirectly by derivatisation of N-methylcarbamoylating species with propanol under alkaline conditions to generate propyl N-methylcarbamate ($CH_3NHCOOC_3H_7$) (Shaw et al., Tox. Appl. Pharmac., in press). The rate of formation of SMG was dependent on NMF concentration at NMF concentrations < 5 mM. Sonication of cells which caused >96% cell death abolished the production of SMG. Depletion of hepatocytic GSH by pretreatment of mice with BSO (1600 mg/Kg i.p.) or by incubating cells with diethyl maleate (0.02%) exacerbated NMF-induced cytotoxicity and reduced the generation of the N-methylcarbamoylating metabolite from 5 mM NMF by 59 and 90 %, respectively. On incubation with 5 mM NMF hepatocytes isolated from a 4 year old male accident victim also generated SMG. Whereas mouse hepatocytes produced 47 ± 11 nmol SMG / $10^6$ cells, the human hepatocytes afforded only 3 nmol / $10^6$ cells within 4 hr. In postmitochondrial supernatant the amount of SMG which was found after incubation with NMF (10 mM) for 1 hr was 204 ± 40 nmol / g liver in the case of mice and 50 ± 12 nmol / g liver in the hepatic preparation obtained from a 20 month old boy. The results show that (i) suspensions of hepatocytes are suitable for for the study of the metabolism of NMF via the pathway which generates the hepatotoxic species, and (ii) preparations of human liver are possibly less capable of metabolising NMF in vitro than those of mice.

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# An Investigation of the Relationship between the Hepatotoxicity and the Metabolism of N-Alkylformamides[1]

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## ABSTRACT

The hepatotoxicity and metabolism of the following close analogs of the hepatotoxic antitumor agent N-methylformamide (NMF) were investigated in CBA/CA mice: N-ethylformamide (NEF), dimethylformamide (DMF), formamide and N-methylacetamide (NMA). Apart from NMF only NEF was potently hepatotoxic as measured by the elevation of plasma activities of the enzymes sorbitol dehydrogenase and alanine and aspartate aminotransferases 24 hr after drug administration. In freeze-dried urine samples of mice which had received NEF or NMF, but not in the case of DMF, formamide or NMA, thioesters were detected by thin-layer chromatography. Evidence based on high-pressure liquid chromatography analysis and 400 MHz $^1$H-NMR and mass spectrometry suggests that the thioester metabolite of NEF is *S*-(N-ethylcarbamoyl)-N-acetylcysteine. It has been shown previously that NMF is metabolized to *S*-(N-methylcarbamoyl)-N-acetylcysteine. NEF also underwent extensive metabolism to ethylamine; similarly NMF was biotransformed to methylamine. In contrast, the urine of mice which had received DMF contained only very small amounts of dimethylamine and methylamine could not be detected as a metabolite of NMA. Instead, the major metabolite of NMA was identified by 400 MHz $^1$H-NMR spectrometry as N-(hydroxymethyl)acetamide. DMF is known to undergo extensive metabolism to its N-hydroxymethyl derivative. The results suggest that two metabolic pathways of N-alkylformamides can be distinguished: Hydroxylation at the $\alpha$-carbon of the N-alkyl group and oxidation of the formyl moiety. The former pathway presumably constitutes a detoxification route, and the latter may well be associated with hepatotoxicity, and affords a glutathione conjugate, excreted in the urine as a mercapturate.

N-Alkylformamides are important industrial chemicals as starting materials for chemical syntheses and as solvents. Their solvent properties are related to their high dipole moment and to their extensive miscibility with both aqueous and organic media. Among the N-alkylformamides, DMF is the compound used most frequently as a solvent. It has been found to be hepatotoxic in rodents (Dexter *et al.*, 1982; Lundberg *et al.*, 1981; Massmann, 1956; Mathew *et al.*, 1980) and in workers after occupational exposure to high concentrations of the vapor (Chivers, 1978; Finzel, 1972; Potter, 1973; Reinl and Urban, 1965; Tolot *et al.*, 1969; von Klavis, 1970). NMF shows antineoplastic activity in mice (Clarke *et al.*, 1953; Furst *et al.*, 1955; Gescher *et al.*, 1982; Gate *et al.*, 1986) and its preliminary clinical investigation as an antitumor drug (Laird Myers *et al.*, 1956; McVie *et al.*, 1984; Ettinger *et al.*, 1985) as well as experiments in mice (Langdon *et al.*, 1985; Whitby *et al.*, 1984) have shown it to be a hepatoxin. The mechanisms by which DMF and NMF cause toxicity are poorly understood. NMF has been proposed as proximate or ultimate toxic metabolite through which DMF exerts its hepatotoxicity (Lundberg *et al.*, 1981). However, this is unlikely, as it has been demonstrated recently that NMF is not a major urinary metabolite of DMF in rodents (Brindley *et al.*, 1983; Scailteur and Lauwerys, 1984; Kestell *et al.*, 1986a). As far as the mechanism of toxicity of NMF is concerned, evidence has emanated from our laboratories which suggests that NMF is metabolized to a chemically reactive, potentially toxic, compound which is able to bind covalently to hepatic macromolecules (Pearson *et al.*, 1986a) and causes the depletion of hepatic glutathione stores (Gescher *et al.*, 1982). The major urinary metabolites of NMF are methylamine (Kestell *et al.*, 1985) and *S*-(N-methylcarbamoyl)-N-acetylcysteine (Kestell *et al.*, 1986b). The identification of the latter metabolite has prompted the postulate that its formation, which involves oxidation at the formyl carbon, is a biotransfor-

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ABBREVIATIONS: DMF, N,N-dimethylformamide; NMF, N-methylformamide; NEF, N-ethylformamide; F, formamide; NMA, N-methylacetamide; SDH, sorbitol dehydrogenase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; HPLC, high-pressure liquid chromatography; TLC, thin-layer chromatography; GLC, gas-liquid chromatography.

mation route which leads via the hepatotoxic intermediate. In the present paper, this hypothesis is being tested further. The hepatotoxic potential in the mouse of four close analogs of NMF has been investigated, namely of DMF, NEF, F and NMA (for structures see table 1). The hepatotoxic potencies of these compounds are compared with their proclivity to undergo oxidative and conjugative metabolism giving *S*-(*N*-carbamoyl)mercapturates via a pathway analogous to that which affords *S*-(*N*-methylcarbamoyl)-*N*-acetylcysteine in case of NMF (Kestell *et al.*, 1986b). The results presented in this paper demonstrate that, among the amides examined, only NEF and NMF are hepatotoxins in the mouse and also only NEF and NMF are metabolized to thiocarbamates.

## Materials and Methods

The amides NMF, NEF, DMF, F and NMA were purchased from Aldrich Chemical Co. (Poole, U.K.). *S*-(*N*-methylcarbamoyl)-*N*-acetylcysteine was prepared as described by Kestell *et al.* (1986b) and the synthesis of the following thiocarbamates will be reported elsewhere: *S*-(*N*-ethylcarbamoyl)-*N*-acetylcysteine, *S*-(*N*,*N*-dimethylcarbamoyl)-*N*-acetylcysteine and *S*-(carbamoyl)-*N*-acetylcysteine. *N*-(Hydroxymethyl)acetamide was made as described by Chwala (1948) and [$^{14}$C]methylene-labeled NEF (OHCNH$^{14}$CH$_2$CH$_3$) was synthesized according to a method published previously for [$^{14}$C]methyl-labeled NMF (OHCNH$^{14}$CH$_3$) (Threadgill and Gate, 1983).

Amides were dissolved in saline solution and administered via the i.p. route to male CBA/CA mice (18–25 g). The injection volume was 0.2 ml and, in the metabolism experiments, the dose was 400 mg/kg.

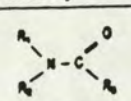
In order to measure the extent of hepatotoxicity, the activities in plasma of the following three enzymes were assayed 24 hr after drug administration. SDH, as described by Rose and Henderson (1975), and AST and ALT as described by Kachmar and Moss (1976).

For the detection of *S*-(*N*-alkylcarbamoyl)-*N*-acetylcysteines as metabolites of formamides, urine samples were freeze-dried after acidification with 9 M HCl to achieve pH 1. In acidic media these *S*-(*N*-carbamoyl)-mercapturates are of sufficient stability to allow detection. The residues were suspended in methanol and analyzed both by HPLC and TLC. For the HPLC analysis, a Waters trimodular system (Waters Associates, Northwich, U.K.) fitted with a Waters RCM-100 radial compression unit and a $C_{18}$ 5 μm reverse phase column was used, UV detection was achieved with a Waters ⎓480 LC spectrophotometer set at 205 nm. The eluant (0.01 M octylamine hydrochloride in water, pH 6.0: methanol, 3:1 v/v) was pumped through the column at a flow rate of 1 ml/min. TLC analysis was conducted on silica gel 60-coated plates (0.2 mm thickness, Merck, A.G., Darmstadt, W. Germany). Plates were developed in butan-1-ol-water-methanol (8:2:1 v/v). The mercapturates were detected by spraying the plates with either 1 M aqueous NaOH followed by Ellman's reagent (Glaser *et al.*, 1970) or with chloroplatinate reagent (Barnsley *et al.*, 1964). *S*-(*N*-Ethylcarbamoyl)-*N*-acetylcysteine was identified as an NEF metabolite after isolation by preparative TLC and esterification with methanolic hydrogen chloride as described previously for *S*-(*N*-methylcarbamoyl)-*N*-acetylcysteine (Kestell *et al.*, 1986b).

$^1$H-NMR and mass spectra of the methyl esters of metabolite and authentic thiocarbamate were obtained, respectively, at 400 MHz using a Bruker WH 400 spectrometer with $D_2O$ as solvent, and using a VG 7070 mass spectrometer (with a VG 2035 data system) in the chemical ionization mode, using 2-methylpropane as reagent gas, run at a scan rate of 1 sec/decade. 400 MHz $^1$H-NMR spectra of urine samples involved suppression of the $H_2O$ signal by selective presaturation (1.5 sec) followed by collection of free induction decay, with 4 preliminary dummy scans. Unchanged amides in the urine were quantitated by GLC as described previously by Gescher *et al.* (1982). *N*-(hydroxymethyl)-acetamide was identified as a metabolite of NMA by 400 MHz $^1$H-NMR analysis after separation of the constituents of freeze-dried urine samples by preparative TLC using benzene-acetone-acetic acid (3:2:2 v/v) as solvent and phenylhydrazine-ferric chloride reagent [prepared according to Nair and Francis (1980)] as detecting spray. After elution of the material from the TLC plate residual acetic acid and water were removed by freeze drying. *N*-(hydroxymethyl)acetamide was quantitated by GLC analysis under conditions used for the measurement of NMA. On the GLC column, this carbinolamide decomposes quantitatively to acetamide. Quantitative analysis was feasible as $^1$H-NMR spectra of urine obtained after NMA administration showed that there was no appreciable excretion of acetamide generated metabolically from NMA.

Aliphatic amines in the urine were detected by HPLC after derivatization with dinitrobenzenesulfonic acid and extraction into ether as described previously (Kestell *et al.*, 1985). Methylamine and ethylamine were quantitated by counting of radioactivity after TLC separation (Kestell *et al.*, 1985) of urinary constituents obtained from mice which had received OHCNH$^{14}$CH$_3$ or OHCNH$^{14}$CH$_2$CH$_3$.

TABLE 1
Structures of amides used in this study



| Amide | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|
| F | H | H | H |
| NMF | $CH_3$ | H | H |
| NEF | $C_2H_5$ | H | H |
| DMF | $CH_3$ | $CH_3$ | H |
| NMA | $CH_3$ | H | $CH_3$ |

## Results

In order to investigate the hepatotoxic potential of four close analogs of NMF, different doses of these amides were administered to mice and, after 24 hr, the plasma activities of three marker enzymes of hepatic damage, SDH, AST and ALT, were measured. In accordance with observations published previously (Langdon *et al.*, 1985), NMF was hepatotoxic at doses exceeding 200 mg/kg (3.4 mmol/kg) (fig. 1). Likewise, NEF exerted marked liver toxicity at 600 mg/kg (8.2 mmol/kg). However, none of the other three amides at doses of up to 3 g/kg caused plasma activities of the marker enzymes to increase as dramatically as seen after NMF. Nevertheless, even though F, DMF and NMA exhibited low hepatotoxic potential, at this dose they caused the mice to lose weight (table 2).

To study the formation of *S*-(*N*-alkylcarbamoyl)-mercapturates as urinary metabolites of the amides under test, freeze-dried urine samples from mice which had received either NMF, NEF, F or DMF were analyzed by TLC. The appropriate authentic mercapturates were prepared as reference compounds. Only the two monoalkylformamides NMF and NEF, but not DMF or F, gave rise to the appearance of thiocarbamates in the urine, as shown by TLC and HPLC analysis. The mercapturates arising from metabolism of NMF and NEF had $R_f$s identical to those of the respective synthetic materials, *S*-(*N*-methylcarbamoyl)-*N*-acetylcysteine (0.30) and *S*-(*N*-ethylcarbamoyl)-*N*-acetylcysteine (0.37). Both metabolites gave a positive reaction with Ellman's reagent after treatment with aqueous base as described under "Materials and Methods," indicating the presence of thioesters/thiocarbamates. The limit of detection by HPLC for unlabeled *S*-(*N*-alkylcarbamoyl)-mercapturates in the urine was near 10 μmol/ml. Therefore we cannot exclude the possibility that small amounts of DMF or

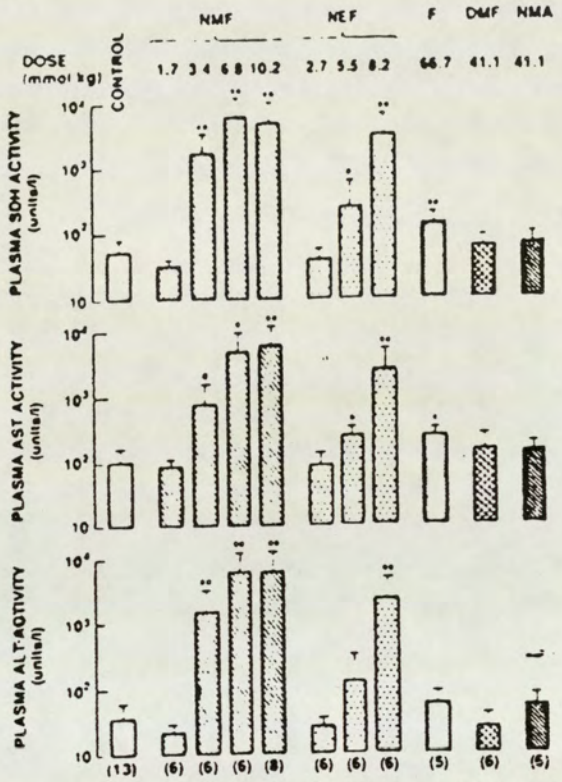



Fig. 1. Plasma levels of SDH (top) AST (middle) and ALT (bottom) in mice 24 hr after administration of amides. Numbers in parentheses, number of animals, bars denote S.D.; stars indicate significant difference from control (Mann-Whitney U-test), *P < .005, **P < .001.

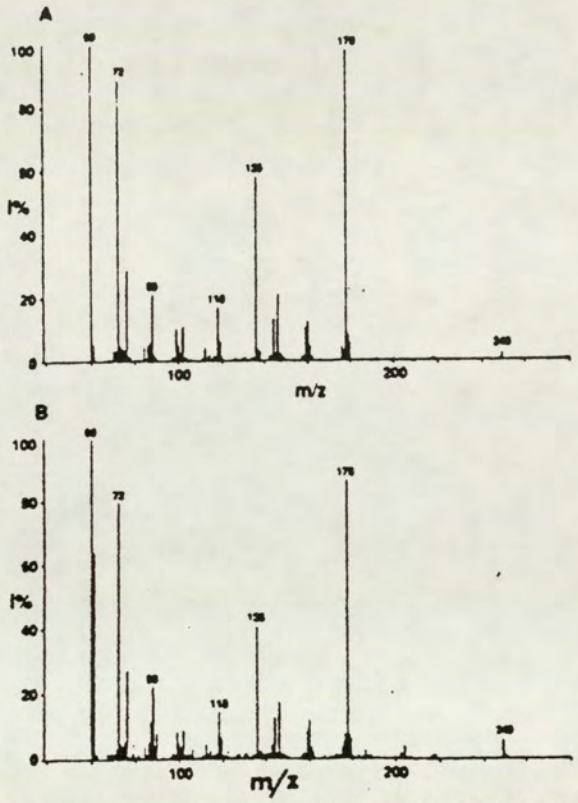



Fig. 2. Mass spectra of *S*-(N-ethylcarbamoyl)-N-acetylcysteine methylester (A) and of a urinary metabolite of NEF after reaction with methanolic hydrogen chloride (B).

TABLE 2
Weight change in mice 24 hr after administration of amides

| Amide | Dose | No. of Animals | Wt. Change, (% b.w. ± S.D.) |
|---|---|---|---|
| | *mmol/kg* | | |
| Control | | 14 | +1.5 ± 2.2 |
| NMF | 1.7 | 7 | +2.4 ± 1.4 |
| | 3.4 | 7 | +0.7 ± 2.3 |
| | 6.8 | 9 | −6.4 ± 2.7 |
| | 10.2 | 10 | −6.2 ± 2.6 |
| NEF | 2.7 | 6 | −2.0 ± 1.6 |
| | 5.5 | 6 | −8.0 ± 1.2 |
| | 8.2 | 7 | −9.2 ± 2.9 |
| F | 66.7 | 7 | −6.2 ± 1.0 |
| DMF | 41.1 | 6 | −4.0 ± 2.2 |
| NMA | 41.1 | 6 | −1.7 ± 4.0 |

F, less than 2% of the dose, were metabolized *via* this pathway but escaped detection. The mercapturate obtained in the urine of NMF-treated mice gave an HPLC peak with a retention time of 12 min and has been identified previously as *S*-(N-methylcarbamoyl)-N-acetylcysteine (Kestell *et al.*, 1986b). The mercapturate found in the urine of mice which had received NEF (retention time 15 min on HPLC analysis) was converted into its methyl ester by treatment with methanolic hydrogen chloride, isolated by HPLC and characterized by mass spectroscopy and 400 MHz $^1$H-NMR spectrometry. Figure 2 shows that the mass spectra of the methyl esters of the metabolite and of authentic *S*-(N-ethylcarbamoyl)-N-acetylcysteine are virtually identical. The $^1$H-NMR spectra of both methylated metabolite and authentic *S*-(N-ethylcarbamoyl)-N-acetylcysteine methyl ester (fig. 3) show the acetyl protons resonating as a 3H singlet at $\delta$2.01 ppm. The N-ethyl group gives rise to a 7 Hz triplet at $\delta$1.16 ppm and a 7 Hz quartet at $\delta$3.34 ppm which partially overlaps with the signal of the cysteine $\beta$-$CH_2$. The cysteine $\alpha$-CH resonates at $\delta$4.73 ppm (ddd, J 5.1, 6.0, 7.1 Hz). Quantitation of the metabolite excreted in the urine was achieved both by HPLC measurement and by radioactivity counting after TLC separation of constituents of urine samples obtained from mice which had received $OHCNH^{14}CH_2CH_3$. Of the dose of NEF administered, 10.3 ± 0.9% (mean ± S.D., $n$ = 4) was excreted as the mercapturate within 24 hr, which contrasts with the finding that 16% of a similar dose of NMF was excreted as *S*-(N-methylcarbamoyl)-N-acetylcysteine (Kestell *et al.*, 1985).

The $^1$H-NMR spectrum of a urine sample of mice which had received NEF shows clearly that ethylamine (in its protonated form) is a major metabolite of NEF [$\delta$1.16 ppm (3H, *t*, J 7 Hz) and 3.05 ppm (2H, q, J 7 Hz)]. Ethylamine as a metabolite of NEF comprised 42% of the dose (table 3). This amount exceeds the amount of NMF which was metabolized to methylamine, and contrasts markedly with the low or negligible biotransformation of DMF to dimethylamine and of NMA to methylamine respectively (table 3). The high field part of the $^1$H-NMR

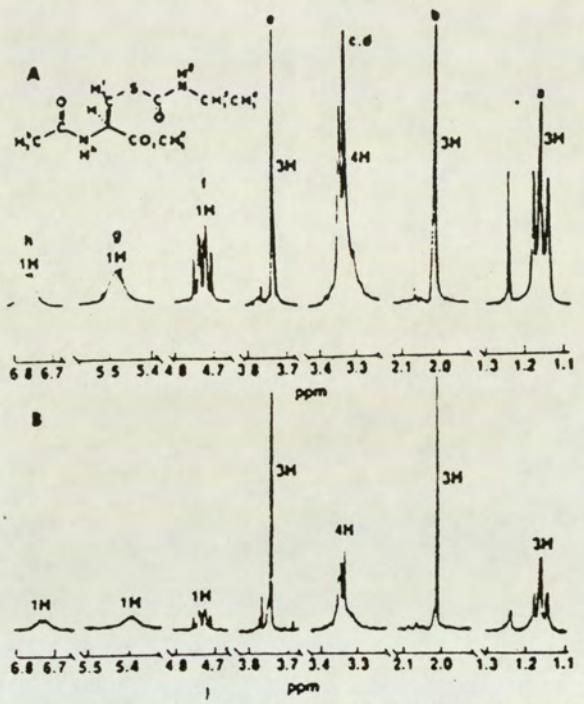



Fig. 3. Prominent signals in the 400 MHz $^1$H-NMR spectra of *S*-(*N*-ethylcarbamoyl)-*N*-acetylcysteine methyl ester (A) and of a urinary metabolite of NEF after reaction with methanolic hydrogen chloride (B). Details of the metabolite isolation are described under "Materials and Methods."

TABLE 3

Amounts of unchanged amides and of metabolically generated alkylamines excreted in the urine of mice which received NMF, NEF, DMF or NMA (400 mg/kg)

| Amide Administered | Amount Excreted Unchanged (% of dose ± S.D.) | Amine Detected[a] | Amount Excreted as Amine (% of dose ± S.D.) |
|---|---|---|---|
| NMF | 26.4 ± 1.9 (5)[b] | $CH_3NH_2$ | 15.0 ± 1.9 (3) |
| NEF | 4.8 ± 1.9 (5) | $C_2H_5NH_2$ | 42.1 ± 2.0 (4) |
| DMF | 4.9 ± 1.4 (5)[c] | $HN(CH_3)_2$ | 3.9 ± 1.9[d] |
| | | $CH_3NH_2$ | 4.1 ± 0.8[d] |
| NMA | 2.0 ± 2.0 (6) | N.D.[e] | |

[a] Urine was collected for 24 hr and amides and amines were detected and quantitated as described under "Materials and Methods."
[b] Numbers in parentheses, number of animals.
[c] As reported by Brindley *et al.* (1983).
[d] As reported by Kestell *et al.* (1986a).
[e] N.D., methylamine not detected.

spectrum of urine samples of mice which had received NMA is dominated by the acetyl proton resonance frequency of its major urinary metabolite (2.01 ppm). This metabolite was separated from other urine constituents by TLC. Figure 4B shows its $^1$H-NMR spectrum which is a virtually identical with that of synthetic N-hydroxymethylacetamide (fig. 4A). Whereas 54 ± 10% (mean ± S.D., $n$ = 5) of the dose of NMA was metabolized to N-hydroxymethylacetamide, only 2% was excreted as unchanged NMA (table 3).

## Discussion

Among the formamides, NMF is the most effective antineoplastic agent in mice (Gate *et al.*, 1986). According to the results

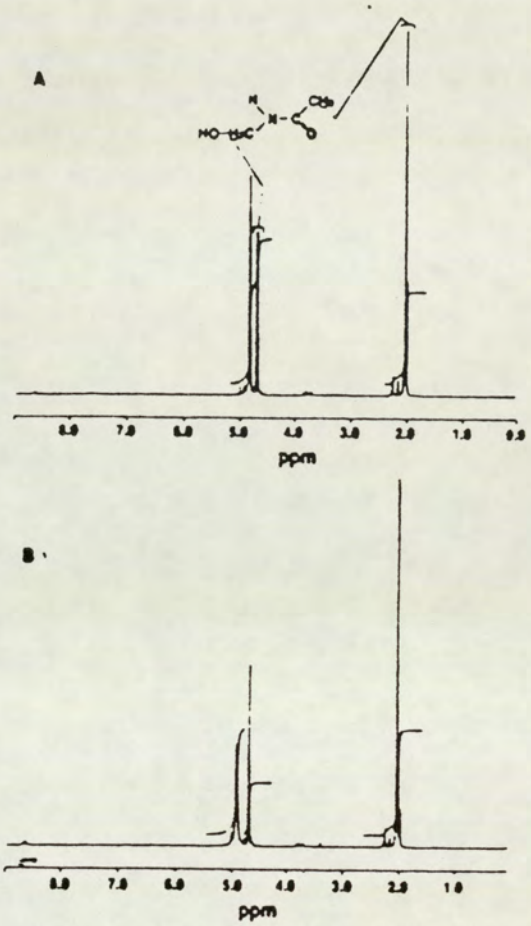



Fig. 4. $^1$H-NMR spectra of N-hydroxymethylacetamide (A) and the major urinary metabolite of NMA (B) in $D_2O$. Details of the synthesis of N-(hydroxymethyl)acetamide and of the metabolite isolation are described under "Materials and Methods."

presented in this paper, the monoalkylformamides NMF and NEF appear also to be more potently hepatotoxic than other formamides. The amides investigated here seem to exert toxicities also in targets other than the liver, as the derivatives which lacked marked hepatotoxic potential caused marked weight loss at high doses (3 g/kg).

Little is known about the mechanism by which NMF causes toxicity. Studies of the effect of NMF on glutathione status and of the effects of manipulation of glutathione status on hepatotoxicity and covalent binding of metabolic products of radiolabeled NMF suggest that NMF undergoes metabolic activation to a reactive, potentially toxic, metabolite (Whitby *et al.*, 1984; Pearson *et al.*, 1987a,b). The analytical chemical work described here demonstrates that the two hepatotoxins among the five amides tested in this study are also the ones which underwent metabolism to *S*-(N-alkylcarbamoyl)-mercapturates. This result is good evidence for the contention that the biotransformation route (fig. 5A) which yields thiocarbamates is linked closely with the formamide-induced generation of the hepatotoxic lesion. The formation of the N-alkylthiocarba-

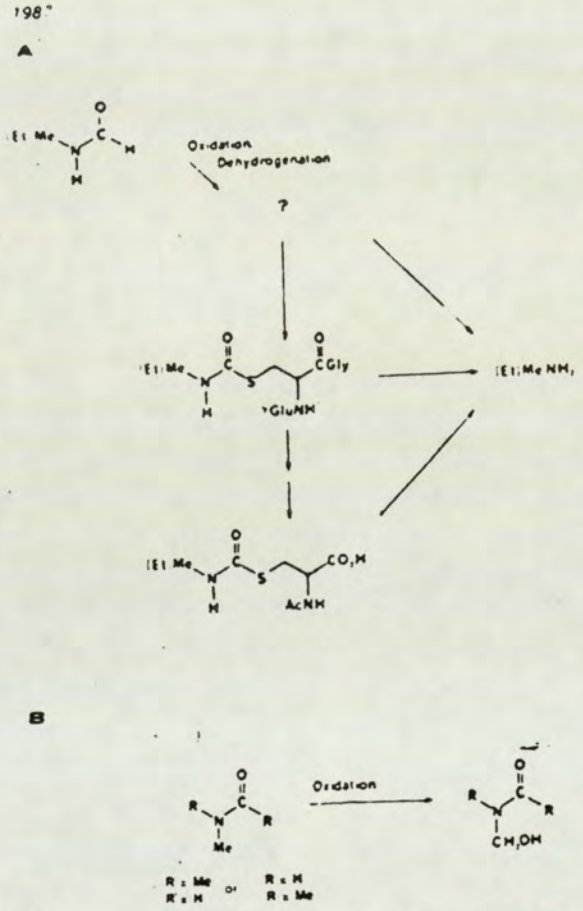



Fig. 5. Major metabolic toxification (A) and detoxification (B) pathways of N-alkylformamides and NMA.

mates from N-alkylformamides involves the oxidation of the formyl moiety and one could postulate a number of conceivable metabolic precursors, among them alkylisocyanates (alkyl-N=C=O). The formation of such a powerful electrophile would explain the fulminant hepatic necrosis observed in mice which had received NMF (Whitby *et al.*, 1984). It would appear (fig. 1) that NMF is slightly more toxic, on a molar basis, than is NEF and that this difference might correlate with the greater amount of mercapturic acid found in the urine, although this association probably does not indicate causality. It is worth noting that NMF and NEF are also the derivatives among the amides which gave rise to large amounts of metabolically produced alkylamines in the urine. The way in which the alkylamines are formed from the formamides is unclear. It is unlikely that direct hydrolysis of the formamide molecule occurs, as NMF and DMF are both resistant to hydrolysis catalyzed by liver or plasma enzymes (C. Brindley and D. Ross, unpublished observation). Indeed, the observation of a large primary H/D kinetic isotope effect on the metabolism of NMF to methylamine when $ODCNHCH_3$ and $OHCNHCD_3$ are coadministered [$k$(formyl$-H$)/$k$(formyl$-D$) = 5.5. ± 0.2] shows that NMF is not hydrolyzed to methylamine *in vivo* but rather that cleavage of the formyl C-H bond is involved (M. D. Threadgill, P. Kestell and A. Gescher, unpublished observation). It is therefore likely that the product of alkylformamide oxidation or, indeed, its conjugation product *S*-(*N*-alkylcarbamoyl)glutathione or its cysteinylglycine, cysteine or mercapturic acid derivatives are the immediate precursors of the alkylamines found in the urine.

Among the amides studied here, NMA is the only one incapable, in principle, of undergoing oxidation or dehydrogenation in its carbonyl moiety. It was metabolized predominantly to its hydroxymethyl amide. The major metabolic pathway of DMF in rodents is also N-methyl C-hydroxylation (Kestell *et al.*, 1986a; Scailteur and Lauwerys, 1984). This is remarkable in view of the fact that DMF possesses a formyl group which could be oxidized. However, oxidation at the formyl carbon does not appear to occur in DMF. On the basis of the demonstration of the very low toxicity *in vivo* of a related carbinolamide, *N*-(hydroxymethyl)formamide (Cooksey *et al.*, 1983), which has been found to be a minor urinary metabolite of NMF (Kestell *et al.*, 1985), we suggest that metabolic N-methyl C-hydroxylation (fig. 5B) is a detoxification pathway in this series. Therefore, the results of the study described here lead to the proposition that two metabolic pathways of N-alkylformamides can be distinguished (fig. 5): either hydroxylation at the $\alpha$-carbon of the N-alkyl group, which probably constitutes a detoxification reaction, or metabolic oxidation of the formyl moiety, which is a prelude to the generation of the hepatotoxic lesion.

In antitumor tests in mice of a large number of amides, only NMF possessed highly significant activity in several murine tumors (Gate *et al.*, 1986). Both NEF and DMF were only marginally inhibitory of tumor growth, each in one mouse tumor model. F and NMA lacked completely antitumor activity. Because, as shown in this paper, NMF and NEF (amongst the compounds examined) both exhibit hepatotoxicity and are metabolized significantly to *S*-(*N*-alkylcarbamoyl)-mercapturic acid but only NMF is an effective antitumor agent in mice, it would appear that the biochemical mechanisms of hepatotoxicity and antitumor activity are different.

**Acknowledgments**

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# Metabolism of N-Methylformamide in Mice: Primary Kinetic Deuterium Isotope Effect and Identification of *S*-(N-Methylcarbamoyl)Glutathione as a Metabolite[1]

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## ABSTRACT

*S*-(N-Methylcarbamoyl)glutathione has been identified by cesium ion liquid secondary ion mass spectrometry as a biliary metabolite in mice of the experimental antitumor agent and hepatotoxin N-methylformamide. Metabolism of N-methylformamide to urinary methylamine, urinary N-acetyl-*S*-(N-methylcarbamoyl)-cysteine and biliary *S*-(N-methylcarbamoyl)glutathione was found to be subject to large intermolecular primary kinetic isotope effects when hydrogen was replaced by deuterium in the formyl group ($k_H/k_D$ = 5.5 ± 0.2, 4.5 ± 1.0 and 7 ± 2, respectively), as shown by mass spectrometry of derivatives of these metabolites. These values indicate the existence of a common metabolic precursor for each of these metabolites. In particular, methylamine is shown not to arise from simple enzymatic hydrolysis of N-methylformamide but is associated with an oxidative process. Therefore, it is highly likely that N-methylformamide is oxidized and conjugated to form *S*-(N-methylcarbamoyl)glutathione which is metabolized further to N-acetyl-*S*-(*N*-methylcarbamoyl) cysteine. Either of these thiocarbamates could be hydrolyzed to give the parent thiol and the observed metabolic end products, methylamine and carbon dioxide. The presence of deuterium in the formyl moiety of N-methylformamide reduced markedly the hepatotoxicity of the compound, as shown by measurements of the activities of appropriate hepatic enzymes in plasma.

NMF (OHCNHCH$_3$; NSC 3051; fig. 1) is a powerful solvent with antineoplastic activity against implanted rodent tumors (Clarke *et al.*, 1953; Gescher *et al.*, 1982). Of a large series of analogs tested in these murine systems, only NMF showed consistent activity (Gate *et al.*, 1986). NMF has been the subject of clinical evaluation (Laird Myers *et al.*, 1956; McVie *et al.*, 1984; Eisenhauer *et al.*, 1986) and has shown hepatotoxicity both in humans (Laird Myers *et al.*, 1956) and in rodents (Whitby *et al.*, 1984; Langdon *et al.*, 1985). It is likely that this toxicity is mediated *via* one or more metabolites of NMF (Pearson *et al.*, 1987; Whitby *et al.*, 1984). It has also been suggested that the hepatotoxicity of the important industrial solvent (DMF) may be associated with its metabolism to NMF (Kimmerle and Eben, 1975a,b; Lundberg *et al.*, 1981), although a recent study has shown that the latter secondary amide is at most a very minor metabolic product when high doses of DMF are administered to mice (Kestell *et al.*, 1986a). NMF is metabolized extensively in mammals and the following urinary metabolites have been identified: N-(hydroxymethyl)formamide (mouse) (Kestell *et al.*, 1985b), methylamine (mouse and rat) (Kestell *et al.*, 1985a,b) and N-acetyl-*S*-(N-methylcarbamoyl)cysteine (mouse, rat and humans) (Kestell *et al.*, 1986b; Tulip *et al.*, 1986). Additionally, $^{14}CO_2$ was found in the breath of mice which had received [$^{14}$C]NMF (Kestell *et al.*, 1985b) and some 26% of the parent amide is excreted unchanged in this species (Brindley *et al.*, 1982).

In a limited structure-toxicity study, we have reported recently (Kestell *et al.*, 1987) the observation of a qualitative relationship between the hepatotoxic potential of formamides and the presence of an *S*-(N-alkylcarbamoyl)mercapturic acid as a urinary metabolite. Therefore, the metabolic pathway of

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ABBREVIATIONS: NMF, N-methylformamide; DMF, N,N-dimethylformamide; EI, electron impact; BPt, boiling point; HPLC, high-pressure liquid chromatography; LSIMS, liquid secondary ion mass spectrometry; TLC, thin-layer chromatography; CI, chemical ionization; SDH, sorbitol dehydrogenase; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

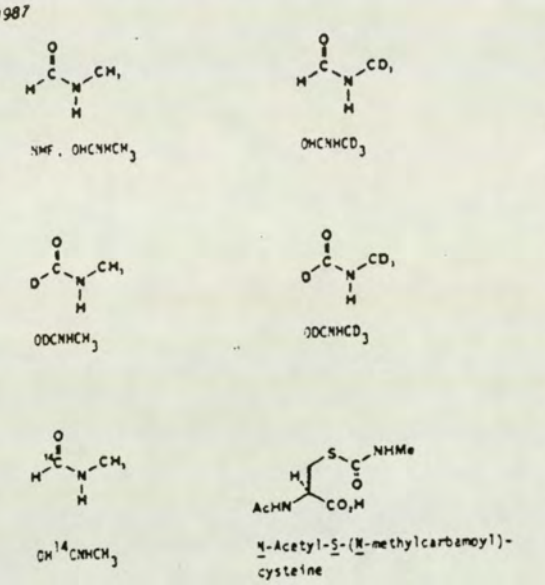


Fig. 1. Structures of synthetic compounds and isotopomers used in this study.

NMF which leads to the mercapturic acid appears to be the prime candidate for the route which generates the hepatotoxic intermediate. The chemical identity of this material and other details of the sequence of metabolic events leading from NMF to the mercapturic acid are unknown and difficult to establish directly as the reactive intermediate is likely to be a very short-lived species. Experiments are reported here which are designed to elucidate these details with the use of deuterated analogs of NMF. In particular, the following three hypotheses have been tested: 1) *S*-(*N*-methylcarbamoyl)glutathione is a precursor of the urinary mercapturic acid and is itself excreted in the bile; 2) the metabolic cleavage of the C-H bond in the formyl group of NMF is the rate-determining step in the metabolism of NMF to the thiocarbamates and to methylamine; and 3) the metabolic removal of the formyl hydrogen is involved in the genesis of the hepatotoxic lesions caused by NMF. Overall, it was hoped that this work may contribute to an understanding of the mechanisms of toxicity and of antitumor activity of NMF and perhaps to the design of a therapeutic analog in which these activities are separated.

## Methods

**Materials.** The radiochemical starting material ($H^{14}CO_2^-$, $Na^+$) was purchased from Amersham International P.L.C. (Amersham, U.K.). Deuterated starting materials and other chemicals were purchased from Aldrich Chemical Company Ltd. (Gillingham, Dorset, U.K.) or from Aldrich Chemical Company (Milwaukee, WI). N-[methyl-$^{14}$C]formamide ($OH^{14}CNHCH_3$) and N-(trideuteromethyl)formamide ($OCHNHCD_3$) were prepared as described previously (Threadgill and Gate, 1983) and the latter was found by EI mass spectrometry to be > 98% labeled in the methyl group. N-(trideuteromethyl)-deuteroformamide ($ODCNHCD_3$) was prepared as follows: sodium (161 mg, 7 mmol) was dissolved in anhydrous methanol (15 ml; freshly distilled from magnesium methoxide) and the solution of sodium methoxide was cooled to 0°C. Trideuteromethylamine hydrochloride ($CD_3NH_3^+$ $Cl^-$; 492 mg, 7 mmol) was added, followed after 20 min by methyl deuteroformate (1 ml). The reaction mixture was stirred for 16 hr before being filtered. Distillation (Kugelrohr) gave the product as a colorless liquid (70 mg, 22%) with BPt-$_{\infty}$ 183-187°C [literature BPt-$_{\infty}$ 180-185°C for protio compound (Weast, 1977)]. This material was found by EI mass spectrometry to consist of a mixture of 55% $C_3H_3D_3NO$ and 45% $C_3HD_4NO$. High field proton NMR analysis of this material indicated that the trideutero species corresponded to $OHCNHCD_3$ whereas the tetradeutero molecules were labeled at both the methyl and the formyl positions. N-Methyldeuteroformamide ($ODCNHCH_3$) was synthesized thus: methyl deuteroformate ($ODCOCH_3$) was added to anhydrous methanol (20 ml) which had been saturated with gaseous methylamine ($CH_3NH_2$) at 0°C. This mixture was stirred for 16 hr. Distillation (Kugelrohr) furnished N-methyldeuteroformamide (1.02 g, 75%) as a colorless liquid BPt-$_{\infty}$ 191-194°. EI mass spectrometry showed the material to be > 98% $ODCNHCH_3$. Infrared spectrum (liquid film) 3300, 2950, 2150 and 1650 $cm^{-1}$.

**HPLC.** HPLC purification of the N-ethoxycarbonylglutathione conjugate of NMF isolated from bile was performed using a Beckman model 342 instrument, equipped with a reverse phase 5 μM Ultrasphere ODS column (15 cm × 4.6 mm internal diameter, Rainin Instruments, Berkeley, CA). The mobile phase consisted of a linear 20 min gradient from 20 to 70% methanol in water, with 1% acetic acid throughout. The mobile phase was maintained at 70% methanol for a further 10 min. The flow rate was held at 1.0 ml $min^{-1}$ and 1-min fractions were collected. Under these conditions the ethoxycarbonyl-, ethoxycarbonyl methyl ester- and ethoxycarbonyl dimethyl ester derivatives of the NMF conjugate eluted in fractions 5 to 6, 8 to 9 and 10 to 11, respectively. The recovery of radioactivity in the HPLC eluate was > 95% of that applied to the column. Detection was carried out by liquid scintillation counting using a Beckman LS-7500 counter. HPLC equipment and conditions for the analysis of the urinary metabolites were as described previously by us (Kestell *et al.* 1986b) except that the mobile phase for the purification of N-methyl-2,4-dinitro-aniline was methanol-water (2:1 v/v) and UV absorption detection was carried out at 346 nm.

**Mass spectrometry.** LSIMS of the derivatized glutathione conjugates was performed on a Kratos MS-50S mass spectrometer, equipped with a 23-kG magnet and a postacceleration detector which was operated at −10 kV. Samples were dissolved in a glycerol matrix containing HCl and NaCl (to increase the intensity of $[M + H]^+$ and $[M + Na]^+$ species) and ionization was achieved by bombardment with a 1.0-μA primary beam of $Cs^+$ ions (Falick *et al.*, 1986). Chemical ionization mass spectrometry was carried out on a VG70-70 instrument as described previously (Kestell *et al.*, 1986b). EI mass spectrometry of deuterated analogs of N-methyl-2,4-dinitroaniline was carried out using a VG Micromass 12B instrument operating at an electron energy of 70 eV with a probe temperature of 250°C. The exact isotopic composition of the mixtures of the deuterated analogs which were administered to mice was determined by EI mass spectrometry using either a VG Micromass 12B or a VG 70-70H instrument.

**Animals and treatment procedures.** NMF or its deuterated or $^{14}$C-analogs (6.8 mmol $kg^{-1}$ in 200 μl of saline) were administered to male BALB/c mice (20-25 g) i.p. The total dose corresponds to the optimum antitumor dose (Gate *et al.*, 1986). Animals were sacrificed by cervical dislocation.

**Identification of the biliary glutathione conjugate and determination of the primary kinetic isotope effect on its formation.** Mice received NMF or its deuterated analogs together with $OH^{14}CNHCH_3$ as tracer (final specific activity, 30 mCi $mmol^{-1}$). In experiments designed to elucidate the position of the link of the NMF-derived moiety to glutathione, separate groups of four mice received either $OHCNHCH_3$ or $ODCNHCH_3$. In order to determine the magnitude of the primary kinetic isotope effect on the formation of this conjugate, a further group of mice received a mixture comprising $OHCNHCH_3$ (59%), $OHCNHCD_3$ (22.8%) (a byproduct of the synthesis of $ODCNHCD_3$) and $ODCNHCD_3$ (18.2%). Mice were sacrificed 4 hr after administration of NMF and bile was then collected *via* puncture of the gall bladder with a syringe.

Aliquots of bile (50 μl) were treated with a solution of ethyl chloro-

formate (100 μl) in 0.1 M phosphate buffer (pH 9.0, 3.0 ml). Excess ethyl chloroformate was removed by extraction with dichloromethane (5 ml) and the residual aqueous portion was acidified (to pH < 2) and applied to a prewashed $C_{18}$ Sep-Pak cartridge (Waters Associates, Milford, MA). The cartridge was rinsed with water and the ethoxycarbonyl derivatives were eluted with methanol and evaporated to dryness under a stream of nitrogen. The residues were subjected to analysis by HPLC. Fractions 5 to 7 (which contained the majority of the radioactivity) were pooled and processed, as described above, on a second Sep-Pak cartridge. The purified conjugates thus obtained were then treated with anhydrous methanolic hydrogen chloride at ambient temperature for 2 hr. The resulting methyl esters were purified further by HPLC, as described above, and fraction 10 was collected for mass spectrometric analysis. The precise isotopic composition of the fully derivatized NMF-glutathione conjugates, isolated from each of the separate studies, was determined under LSIMS conditions by sweeping the accelerating potential of the spectrometer repetitively to bring into focus the mass range 485 to 495. The duration of each limited mass scan was 10 sec and measurements of deuterium content were based on the $[M + Na]^+$ cluster (at $m/z$ 487 in the spectrum of the unlabeled conjugate) using the mean of 10 scans across this peak.

**Study on the metabolism of NMF to N-acetyl-*S*-(N-methylcarbamoyl)cysteine.** Collections of urine (24 hr) were made from three mice which had been given a mixture of $OHCNHCD_3$ (50%) and $ODCNHCH_3$ (50%). Each sample of urine was freeze-dried and the residues were subjected to preparative TLC as described previously (Kestell *et al.*, 1986b). The isolated mercapturic acid from each sample was esterified using methanolic hydrogen chloride (Kestell *et al.*, 1986b) and the resulting mixtures of isotopomers of N-acetyl-*S*-(N-methylcarbamoyl)cysteine methyl ester were subjected to CI mass spectrometry in order to determine the isotopic composition of the N-methyl groups. Averages of five spectra were taken for each sample.

**Study on the metabolism of NMF to methylamine.** Urine was collected from each of three male mice for 24-hr periods both before and after administration of a mixture of $OHCNHCD_3$ (47%) and $ODCNHCH_3$ (53%). To each urine sample (ca. 1 ml) was added 2 M ethanolic 2,4-dinitrofluorobenzene (2 ml) and the mixtures were allowed to stand for 1 hr at ambient temperature before addition of saturated aqueous sodium chloride (1 ml). Each sample was then extracted with diethyl ether (2 × 5 ml). The organic extracts were washed with 2.5 M aqueous sodium hydroxide (2 × 2 ml) and with saturated aqueous sodium chloride (2 ml) before being dried with anhydrous sodium sulfate and filtered. The solvent was then evaporated from each filtrate under reduced pressure. An aliquot of the residue from each sample was then assayed for total N-methyl-2,4-dinitroaniline by HPLC to determine the contribution of endogenous $CH_3NH_2$ to the total methylamine in the urine samples from animals after administration of NMF. The N-methyl-2,4-dinitroaniline from the urine of each treated animal was purified by preparative TLC [2 mm thick silica gel 60 plates, Merck, Darmstadt, FRG, dichloromethane-methanol, 9:1 (v/v)], followed by HPLC. Evaporation of the eluent from appropriate fractions gave the pure mixtures of isotopic analogs as yellow solids, the compositions of which were then determined by EI mass spectrometry.

**Studies on the effects of deuteration on hepatotoxicity of NMF.** Single doses of $OHCNHCH_3$ and $OHCNHCD_3$ (100 and 200 mg $kg^{-1}$) and of $ODCNHCH_3$ (100, 200 and 300 mg $kg^{-1}$) were administered i.v. in saline (200 μl) and control mice received saline (200 μl) alone. Twenty four hours after administration of the dose, the animals were anesthetized with diethyl ether and exsanguinated by cardiac puncture into syringes containing aqueous sodium heparin (2500 U $ml^{-1}$; 50 μl). Plasma was obtained from each sample by centrifugation. The activity of SDH was determined by the method of Rose and Henderson (1975) and those of AST and ALT were determined by the method of Kachmar and Moss (1976). Statistical comparison was effected using the Mann-Whitney *U* test.

## Results

The bile obtained from mice which had received $OH^{14}CNHCH_3$ contained a radioactive metabolite which was derivatized by treatment with ethyl chloroformate and the products were analyzed by HPLC. The exemplary chromatogram (fig. 2A) shows that a single radioactive component was present (fraction 5). This material was collected, esterified and analyzed in the same HPLC system (fig. 2B). One major, less polar, radioactive product (fraction 10) was observed, together with two minor components arising from incomplete esterification of the sample. The major product was isolated and subjected to structural analysis by $Cs^+$ ion LSIMS. The resulting mass spectrum (fig. 3) contained three prominent ions which correspond to the $[M + H]^+$ ($m/z$ 465), $[M + Na]^+$ ($m/z$ 487) and $[M + K]^+$ ($m/z$ 503) species of a compound of MW 464. This molecular weight corresponds to the sum of those of NMF (59) and N-(ethoxycarbonyl)glutathione dimethyl ester (407) minus two hydrogens.

In order to determine to which position in the NMF moiety the glutathione was attached, the conjugate excreted in the bile of mice which had received $ODCNHCH_3$ was derivatized, purified and analyzed, similarly. The partial mass spectra reproduced in figure 4 are representative of those arising from the fully derivatized glutathione conjugates from $OHCNHCH_3$ and $ODCNHCH_3$ (fig. 4, A and B, respectively). The strong simi-

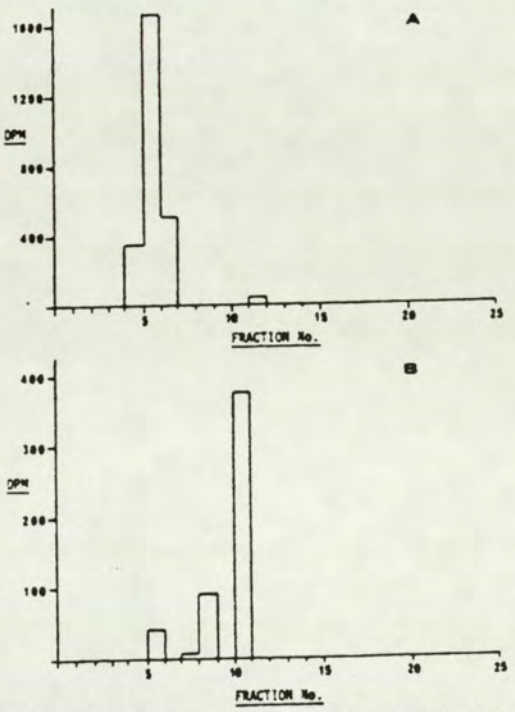


Fig. 2. A, reverse-phase high-pressure liquid chromatogram of bile of mice given $OH^{14}CNHCH_3$. After treatment of a sample of bile with ethyl chloroformate, metabolites were extracted and analyzed by HPLC as described under "Methods." Fractions (1 ml) were collected for radioactivity measurements. B, high-pressure liquid chromatograms of pooled fractions 4–6 from A, after treatment with methanolic hydrogen chloride.

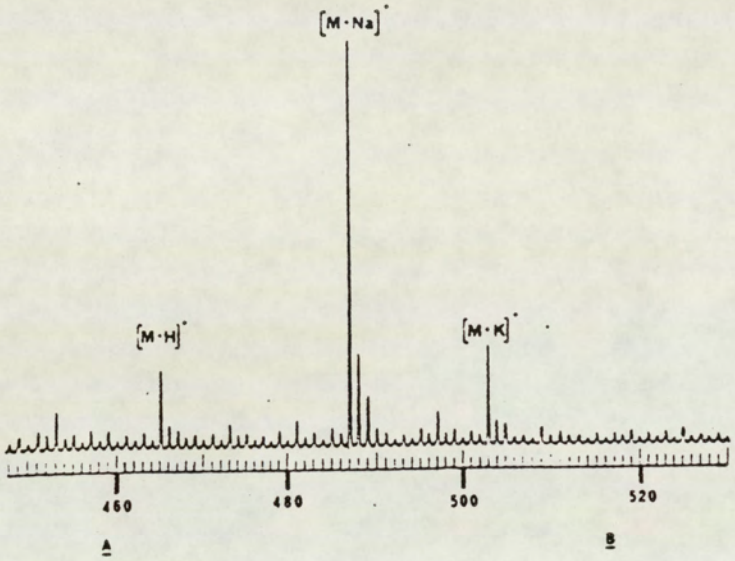


Fig. 3. Molecular ion region of the LSIMS spectrum of the fully derivatized NMF-glutathione conjugate as isolated by HPLC (fraction 10, fig 2B).

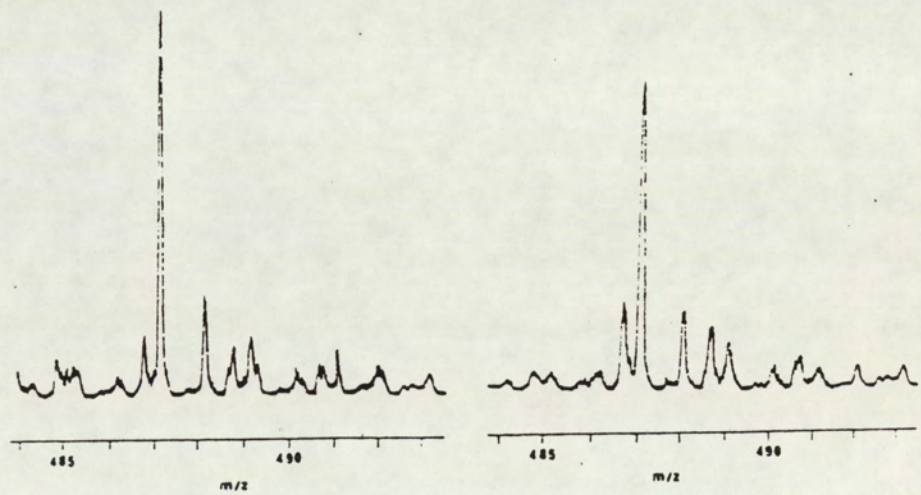


Fig. 4. A, the [M + Na]⁺ region of the LSIMS spectra of the fully derivatized NMF-glutathione conjugates isolated from the bile of mice which had received $OHCNHCH_3$ (A) or $ODCNHCH_3$ (B).

larity in relative intensities of the [M + Na]⁺ ions at *m/z* 487 and *m/z* 488 in the spectra of the two conjugates is indicative of the total loss of deuterium from the $ODCNHCH_3$ during the metabolic process to give the glutathione conjugate. Thus, the NMF moiety must be linked exclusively through the formyl group to glutathione. The structure of the fully derivatized conjugate, as determined in this study, is shown in figure 5.

The magnitude of the apparent primary kinetic deuterium isotope effect associated with formation of *S*-(N-methylcarbamoyl)glutathione from NMF labeled with deuterium at the formyl position was estimated by administering a known mixture of $OHCNHCH_3$, $OCHNHCD_3$ and $ODCNHCD_3$ to mice. Bile was collected from the animals, as before, and the conjugate was converted to its ethoxycarbonyl dimethyl ester derivative. Based on the relative intensities of the ions at *m/z* 487 and *m/z* 490 in the LSIMS spectrum of this derivatized conjugate, corresponding to [M + Na]⁺ ions for the —SCONHNCH₃ and —SCONHCD₃ species, respectively (fig. 6), and taking into account the GSCONHCD₃ generated from the impurity

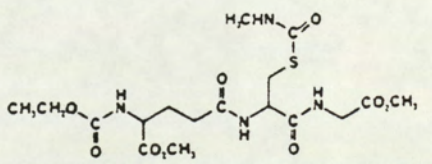

Fig. 5. Structure of the N-(ethoxycarbonyl) dimethyl ester derivative of the glutathione conjugate derived from NMF.

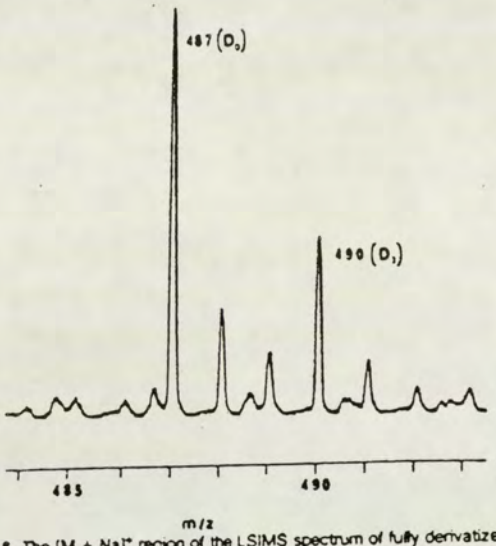


Fig. 6. The $[M + Na]^+$ region of the LSIMS spectrum of fully derivatized *S*-(*N*-methylcarbamoyl)glutathione isolated from the bile of mice which had received a mixture of $OHCNHCH_3$ and $ODCNHCD_3$.

$OHCNHCD_3$, it could be calculated that the apparent kinetic isotope effect associated with this metabolic process *in vivo* ($k_H/k_D$) is 7 ± 2 ($n$ = 3) as referred to the formyl carbon-hydrogen bond. In this experiment, the presence of a $—SCONHCH_3$ group in the conjugate reports on the metabolism of $OHCNHCH_3$ and the $—SCONHCD_3$ reports on the metabolism of the $ODCNHCD_3$ and $OHCNHCD_3$.

The primary kinetic deuterium isotope effect on the metabolism of NMF to *N*-acetyl-*S*-(*N*-methylcarbamoyl)cysteine was evaluated by administration of a known mixture of $ODCNHCH_3$ and $OHCNHCD_3$ to mice. The mixtures of deuterated analogs of the mercapturic acid were isolated from urine from each mouse and were esterified by treatment with methanolic hydrogen chloride before CI mass spectrometric analysis (fig. 7). Comparison of the intensities of the ions at $m/z$ 235 and $m/z$ 238, corresponding to the molecular ions of *N*-acetyl-*S*-(*N*-methylcarbamoyl)cysteine methyl ester and *N*-acetyl-*S*-[*N*-(trideuteromethyl)carbamoyl]cysteine methyl ester, respectively, indicated the magnitude of the apparent primary kinetic isotope effect ($k_H/k_D$) for this metabolic process to be 4.5 ± 1.0 ($n$ = 3). In this experiment, the mercapturic acid bearing the $SCONHCD_3$ reports on the metabolism of $OHCNHCD_3$ and that bearing the $SCONHCH_3$ group reports on the metabolism of $ODCNHCH_3$.

To evaluate the corresponding apparent primary kinetic isotope effect for the metabolism of NMF to methylamine, a similar mixture to that used for the *N*-acetylcysteine conjugate experiment was administered. Twenty four-hour urine collections were made both before and after the dose. Derivatization of the urinary methylamine was effected by treatment of the urine samples with ethanolic 2,4-dinitrofluorobenzene under neutral conditions. HPLC assay showed that endogenous methylamine ($CH_3NH_2$) in the control samples comprised < 5% of the total methylamine ($CH_3NH_2$ + $CD_3NH_2$) found in the urine after treatment of the animals; the contribution of endogenous methylamine to the total was therefore regarded as negligible. The *N*-methyl-2,4-dinitroaniline from the samples of urine after administration of NMF was purified by extraction into diethyl ether, followed by preparative TLC and HPLC. Based on the intensities of the ions in the EI mass spectra at $m/z$ 197 and $m/z$ 200, being the molecular ions for *N*-methyl-2,4-dinitroaniline and *N*-(trideuteromethyl)-2,4-dinitroaniline, respectively, an apparent kinetic isotope effect ($k_H/k_D$) of 5.5 ± 0.2 ($n$ = 3) was observed for the metabolism of NMF to methylamine, depending on the presence of a C—H or a C—D bond in the formyl group of the substrate. Again, the $—NCD_3$ group reports on the metabolism of $OHCNHCD_3$ and the $—NCH_3$ group reports the production of methylamine from $ODCNHCH_3$.

The elevations of the activities of the hepatocellular enzymes SDH, AST and ALT in the plasma of mice which had received NMF or its isotopic analogs (fig. 8) indicate that the threshold dose of $OHCNHCH_3$ and $OHCNHCD_3$ for hepatotoxicity lies between 100 and 200 mg $kg^{-1}$, whereas that of $ODCNHCH_3$ is markedly higher and falls between 200 and 300 mg $kg^{-1}$.

## Discussion

There is little doubt that the hepatotoxicity of NMF is associated with its metabolism (Whitby *et al.*, 1984; Pearson *et al.*, 1987). However, the structure of the hepatotoxic metabolite has hitherto eluded characterization. We have reported previously that NMF is metabolized (*inter alia*) to methylamine and *N*-acetyl-*S*-(*N*-methylcarbamoyl)cysteine (Kestell *et al.*, 1985b, 1986b). In this paper, the excretion of *S*-(*N*-methylcarbamoyl)glutathione as a biliary metabolite of NMF is demonstrated unambiguously. That the "NMF" moiety is linked through the formyl carbon to glutathione was shown by the complete loss of deuterium from $ODCNHCH_3$ during metabolic conjugation. Thus, the *S*-substitution is the same as that in the mercapturic acid derived from NMF and consequently it would appear that the glutathione conjugate characterized here is a metabolic precursor of the latter urinary conjugate *in vivo* (fig. 9).

In order to assess the importance of the metabolic cleavage of the formyl carbon-hydrogen bond on the metabolism and toxicity of NMF, the effect of replacement of C—H by C—D was studied. Carbon-deuterium bonds have a higher activation energy for cleavage than do carbon-hydrogen bonds, owing to the lower zero-point energy of the former (Alder *et al.*, 1971). If a carbon-hydrogen bond is cleaved during the rate-determining step of a multistep process, then substitution of deuterium for hydrogen will have the effect of slowing the rate of formation of all products (metabolites) downstream of this step. The formation of the glutathione conjugate of NMF was found to be subject to such an apparent intermolecular primary kinetic deuterium isotope effect in that it was slowed by a factor of 7 ± 2 when hydrogen in the formyl group of NMF was replaced by deuterium. A similarly significant kinetic isotope effect was observed for the formation of the mercapturic acid. These results confirm that both the *S*-substituted glutathione and the mercapturic acid lie downstream of the rate-limiting step in this metabolic pathway. Also implied is the conclusion that the formyl C—H bond is broken in this step. Interestingly, the formation of urinary methylamine is subject to an apparent kinetic isotope effect of the same magnitude when formyl-H is replaced by formyl-D in the substrate NMF. Hence, this amine is not the product of any biological hydrolysis of NMF as such

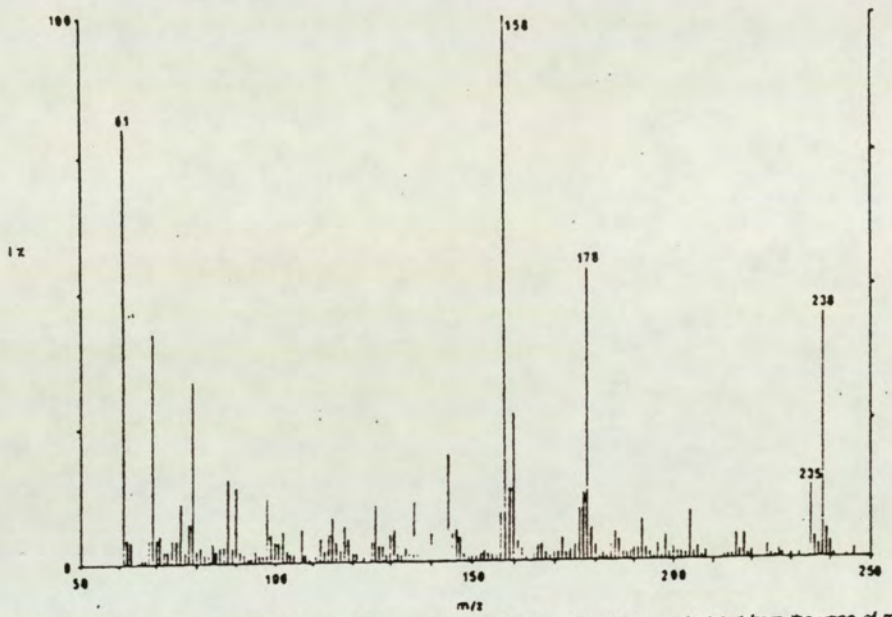


Fig. 7. Example of a CI mass spectrum of the methyl esters of N-acetyl-S-(N-methylcarbamoyl)cysteine isolated from the urine of mice which had received a 50 50 mixture of $ODCNHCH_3$ and $OHCNHCD_3$.

reactions necessarily would not involve cleavage of this formyl C—H bond and thus an hepatic formamidase similar to that reported by Shinohara and Ishiguro (1979) or to the bacterial N,N-dimethylformamidase described by Schär *et al.* (1986) is not involved in the metabolism of NMF. Therefore we propose that, in the metabolic sequence, NMF is first oxidized at the formyl group to give a short-lived reactive intermediate in which the formyl-H bond has already been broken (represented as ? in fig. 9) and that this compound is conjugated with glutathione either chemically or catalytically by a glutathione *S*-transferase. It is, of course, possible that the oxidation and conjugation steps are concerted, *i.e.*, that glutathione is a co-factor of the oxidizing enzyme and that there is no discrete intermediate (?). This glutathione conjugate would then be converted to the mercapturic acid in the usual way, presumably through the intermediacy of the cysteinylglycine and cysteine conjugates which are, as yet, undetected. The facile base-catalyzed chemical hydrolysis *in vivo* of any or all of these metabolites, including the postulated initial reactive intermediate, may well be responsible for the generation of the methylamine, as shown in figure 9. The $^{14}CO_2$ which is a metabolite of $OH^{14}CNHCH_3$ (Kestell *et al.*, 1985b) would be, in part, the by-product of this hydrolysis.

The observation and mechanistic significance of primary kinetic deuterium isotope effects occurring in biological systems has been recently reviewed briefly by Van Langenhove (1986) and, more extensively, by Pohl and Gillette (1985) and by Foster (1985). It has been shown that the intermolecular kinetic isotope effects on the oxidative demethylation catalyzed by cytochrome P-450, in rodent microsomes, of several substrates is low, with $k_H/k_D$ frequently equal to unity (Miwa *et al.*, 1980). The corresponding intramolecular kinetic isotope effects (representing more closely the intrinsic effect) have been reported to lie in the range $k_H/k_D$ = 1.45 to 3.9 (Miwa *et al.*, 1980; Hjelmeland *et al.*, 1977; Heimbrook *et al.*, 1984) and the intramolecular effect using a model porphyrin system is similar (Nee and Bruice, 1982). Whether cytochrome P-450 enzymes can catalyze the oxidation of N-formyl groups remains to be established. The mechanism of yeast formate dehydrogenase has been shown to involve hydride transfer from formate ion to $NAD^+$ in a process which is subject to a (necessarily intermolecular) kinetic deuterium isotope effect ($k_H/k_D$ = 2.3) on replacement of substrate $HCO_2^-$ by $DCO_2^-$ (Blanchard and Cleland, 1980; Hermes *et al.*, 1984). 10-Methylacridinium ion has been claimed to be a useful model for this enzymic reaction in that it also oxidizes formate to carbon dioxide through a hydride transfer mechanism with a similar retardation when the substrate is deuterated at carbon ($k_H/k_D$ = 2.7) (Hutch *et al.*, 1986). However, NMF was not oxidized by this chemical hydride acceptor (M. D. Threadgill, unpublished observations), despite the apparent structural similarity of the formamide to formate. In view of the large intermolecular deuterium kinetic isotope effects demonstrated for the metabolism of NMF, it is unlikely that cytochrome P-450-containing enzymes catalyze the oxidation of the N-formyl group, although an isoenzyme not examined in previous kinetic isotope effect studies may be involved. Conversely, it may be that NMF is metabolized by another enzyme system related to formate dehydrogenase, such as monoamine oxidase or xanthine oxidase. The results of studies *in vitro* to determine the enzyme responsible for this oxidation will be presented later.

Primary kinetic isotope effects on toxicity also have been described. Deuterochloroform ($CDCl_3$) was reported to be some 50 to 70% less cytotoxic in rodents than protiochloroform ($CHCl_3$) (Branchflower *et al.*, 1984), indicating that metabolic cleavage of the carbon-hydrogen bond is involved in the for-

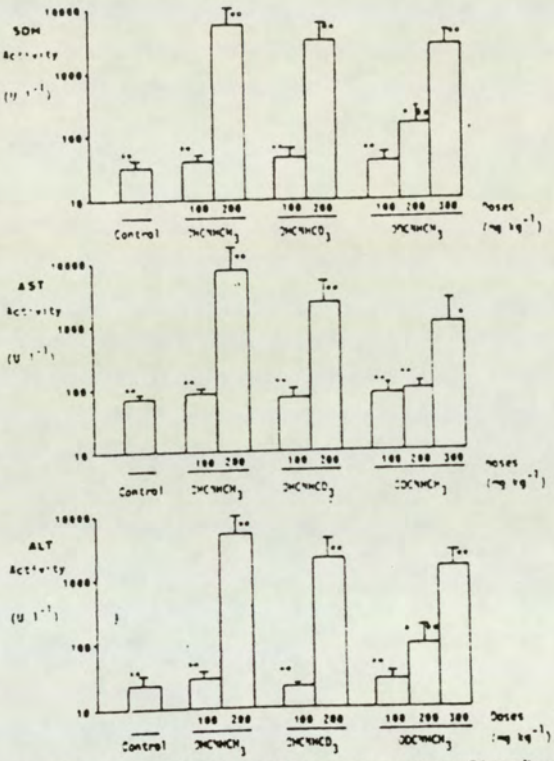


Fig. 8. Activities of SDH, AST and ALT in the plasma of mice 24 hr after doses of $OHCNHCH_3$, $OHCNHCD_3$ or $ODCHNCH_3$. Values are the mean ± SD. Significantly different from control at $P < .005$ (*) and $P < .002$ (**) (Mann-Whitney *U* Test). Significantly different from results from mice receiving 200 mg $kg^{-1}$ $OHCNHCH_3$ at $P < .005$ (†) and $P < .002$ (††).

mation of the ultimate toxic derivative. Conversely, tetradeutero-1,2-dibromoethane ($BrCD_2CD_2Br$) has been shown to be more genotoxic than the protio analog ($BrCH_2CH_2Br$) (White *et al.*, 1983), showing retardation of a competing detoxification pathway. In the present study, we have shown that the hepatotoxicity of NMF is reduced upon deuteration of the NMF in the formyl group. No such effect was in evidence for the trideuteromethyl analog. There appears to be a relationship between the hepatotoxicity of formamides and the metabolic generation of the corresponding N-acetyl-*S*-(N-alkylcarbamoyl)cysteine (Kestell *et al.*, 1987). These two pieces of evidence suggest strongly that either a *S*-(N-methylcarbamoyl)peptide or amino-acid metabolite or the (as yet) unknown reactive intermediate arising from oxidation of the formyl group of NMF (possibly methyl *iso*cyanate) may be the hepatotoxic entity. It is unlikely that methylamine is responsible for the damage to the liver, inasmuch as this amine is present in untreated animals, albeit in small amounts. Further pharmacological studies will show whether replacement of the formyl hydrogen by deuterium will lead to loss of antineoplastic potency similar to the diminution of hepatotoxicity caused by $ODCNHCH_3$.

Acknowledgments

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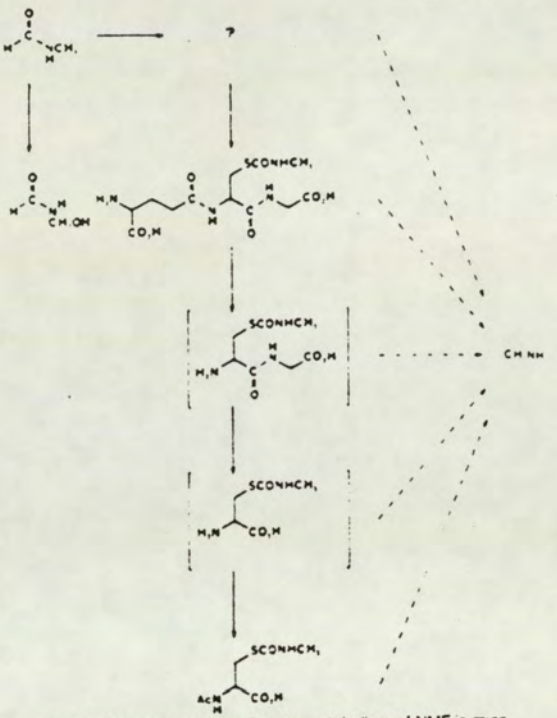


Fig. 9. Proposed pathway for the metabolism of NMF in mice

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P3 TOXICOLOGY AND APPLIED PHARMACOLOGY 95, 000–000 (1988)

# Cytotoxicity and Metabolism of the Hepatotoxin *N*-Methylformamide and Related Formamides in Mouse Hepatocytes

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Cytotoxicity and Metabolism of the Hepatotoxin *N*-Methylformamide and Related Formamides in Mouse Hepatocytes. SHAW, A. J., GESCHER, A., AND MRÁZ, J. (1988). *Toxicol. Appl. Pharmacol.* 95, [redacted]. Some *N*-alkylformamides such as *N*-methylformamide (NMF) possess hepatotoxic properties *in vivo*. To study the mechanism of this toxicity, suspensions of mouse hepatocytes were tested as an *in vitro* model system suitable for the study of the relationship between (i) the toxic potential of formamides, (ii) their metabolism to *N*-alkylcarbamoylating species, and (iii) their ability to deplete hepatic glutathione pools. The effects of NMF were compared with those of its analogs *N*-ethylformamide (NEF), *N,N*-dimethylformamide (DMF), formamide (F), *N*-methylacetamide (NMA), and *N*-methyldeuteroformamide ([$^2$H]NMF). Only NEF and [$^2$H]NMF share with NMF the ability to cause liver damage *in vivo* in mice. Hepatocellular toxicity was determined by measuring LDH leakage into the extracellular medium; metabolism to *N*-alkylcarbamoylating species was measured by GLC after derivatization with propanol to form propyl *N*-alkylcarbamate; glutathione concentrations were determined spectrophotometrically. Of the formamide analogs studied, only NMF and NEF caused cytotoxicity, being apparently equipotent. NMF, NEF, and [$^2$H]NMF gave rise to the formation of detectable levels of *N*-alkylcarbamoylating metabolites and depleted glutathione pools. Toxicity, metabolism, and glutathione depletion were dependent on NMF concentration. [$^2$H]NMF was markedly less cytotoxic than NMF, yielding only 35% of the amount of *N*-methylcarbamoylating metabolite compared to NMF and caused less depletion of glutathione than did NMF. These results parallel closely the *in vivo* hepatotoxic potential of NMF and its analogs, their metabolism to urinary *S*-(*N*-alkylcarbamoyl)mercapturates and their ability to deplete hepatic glutathione in mice. The results provide support for the contention that metabolism is involved with formamide-induced hepatotoxicity and suggest that suspensions of isolated mouse hepatocytes are an appropriate *in vitro* model for the further study of the mechanism by which formamides cause toxicity. 

*N*-Methylformamide (NMF[2]; $OHCNHCH_3$) is an excellent polar solvent of use in the chemical laboratory and in industrial processes. NMF possesses antineoplastic properties in mouse tumor models (Clarke *et al.*, 1953; Furst *et al.*, 1955; Gescher *et al.*, 1982) and induces terminal differentiation in certain leukemia cell lines *in vitro* (Langdon and Hickman, 1987). The potential application of NMF as anticancer agent in the clinic has been evaluated in recent years and the major toxicities of NMF in patients are liver damage and nonspecific but severe malaise (Laird Myers *et al.*, 1956; McVie *et al.*, 1984; Et-

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[2] Abbreviations used: DMF, *N,N*-dimethylformamide; F, formamide; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-ethane-2-sulfonic acid; LDH, lactate dehydrogenase; NEF, *N*-ethylformamide; NMA, *N*-methylacetamide; NMF, *N*-methylformamide; [$^2$H]NMF, *N*-methyldeuteroformamide.



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tinger *et al.*, 1985; Eisenhauer *et al.*, 1986). Further investigation of the therapeutic usefulness of NMF is improbable. The hepatotoxic potential of NMF has also been demonstrated in animals (Whitby *et al.*, 1984; Langdon *et al.*, 1985). Studies conducted mainly *in vivo* of the mechanism by which NMF causes hepatotoxicity suggest that it is metabolized to a chemically reactive, potentially toxic species, which is bound covalently to hepatic macromolecules (Pearson *et al.*, 1987b) and causes the depletion of hepatic glutathione stores (Pearson *et al.*, 1987a). The major urinary metabolites of NMF are methylamine (Kestell *et al.*, 1985) and *N*-acetyl-*S*-(*N*-methylcarbamoyl)cysteine [$H_3CNHCOSCH_2CH(NHCOCH_3)$-COOH] (Kestell *et al.*, 1986; Tulip *et al.*, 1986). The generation of these metabolites involves oxidation at the formyl carbon which presumably leads to a hepatotoxic intermediate (Threadgill *et al.*, 1987). A *S*-(*N*-alkylcarbamoyl)-mercapturate has also been found as a metabolite of *N*-ethylformamide (NEF) but such thiocarbamates have not hitherto been observed at detectable levels in the urine of mice which received *N,N*-dimethylformamide [DMF, $OHCN(CH_3)_2$], formamide (F, $OHCNH_2$), or *N*-methylacetamide (NMA, $CH_3OCNHCH_3$) (Kestell *et al.*, 1987). The hepatotoxic potential of these compounds in mice paralleled their ability to undergo metabolism to thiocarbamates so that, in addition to NMF, only NEF, but not DMF, F, or NMA were hepatotoxic (Kestell *et al.*, 1987). *N*-Acetyl-*S*-(*N*-methylcarbamoyl)cysteine has also been identified as a urinary metabolite of DMF in a human volunteer (Mráz and Tureček, 1987). These authors describe the facile reaction of the mercapturate, a *N*-methylcarbamoylating agent, with ethanol to form ethyl *N*-methylcarbamate, a product which, unlike the parent mercapturate, is well suited to chemical analysis by GLC.

The link between the metabolism of *N*-alkylformamides and their toxicity is the focus of the work presented here. A major obstacle to a detailed elucidation of this link has been the fact that it has hitherto been impossible to detect any metabolites of formamides in incubations *in vitro* (Brindley *et al.*, 1982). In the study described here the hypothesis has been tested that mouse hepatocytes in suspension are a model system suitable for the study of formamide cytotoxicity and metabolism. Using this *in vitro* system the following properties have been investigated of NMF, NEF, DMF, F, NMA, and of *N*-methyldeuteroformamide ($[^2H]NMF$, $ODCNHCH_3$), a deuterated form of NMF with reduced hepatotoxic potential *in vivo* (Threadgill *et al.*, 1987): their cytotoxic potential, their metabolism to *N*-alkylcarbamoylating species, and their ability to interfere with glutathione homeostasis.

## METHODS

*Chemicals.* Formamides and NMA were purchased from Aldrich Chemical Co. (UK) and purified by distillation. $[^2H]NMF$ was synthesized by Dr M. D. Threadgill as described previously (Threadgill *et al.*, 1987). Collagenase was purchased from Boehringer Mannheim (West Germany). Bovine serum albumin and biochemicals for the LDH and glutathione assays were supplied by Sigma Chemical Co. (UK). Horse serum and a concentrate of Hanks' buffered salt solution were obtained from GIBCO (UK).

*Preparation and incubation of isolated hepatocytes.* Male BALB/c mice (18–23 g) obtained from Bantin and Kingman Ltd (UK) were anesthetized with an overdose of pentobarbital (250 mg/kg) given via the ip route. Parenchymal hepatic cells were prepared by the methods of Berry and Friend (1969) and Seglen (1973) adjusted by Klaunig *et al.* (1981) to the specific problems posed by mice. The method involves retroperfusion of the liver with $Ca^{2+}$-free Hanks' buffered salt solution containing Hepes (10 mM) and then with a solution of collagenase (typically 1.5 mg/ml) in Krebs–Henseleit buffer including $Ca^{2+}$. Cells were washed three times by sedimentation on ice and suspended in the incubation medium consisting of Krebs–Henseleit buffer containing Hepes (10 mM), bovine serum albumin (0.2% w/v), and horse serum (10% w/v). Initial viability measured by trypan blue exclusion was better than 80%. Incubations were carried out at 37°C in silanized conical flasks with rubber stoppers under gentle shaking. The final incubation volume was 3.5 ml and included typically $1.4 \times 10^6$ cells/ml and amides at concentrations between 0.1 and 100 mM. Incubates were gassed with a $O_2$:$CO_2$ (95:5) mixture.

*Assays of cytotoxicity and glutathione content.* Viability of cells was routinely assessed by the determination of the release from cells of LDH according to Leathwood and Plummer (1969) using a Cecil CE594 double beam spectrophotometer. In some cases samples of the cell suspension were mixed with a solution of trypan blue in saline (to give 0.3% final concentration) and the percentage of cells which excluded the dye was determined using a hemocytometer. Glutathione (GSH + GSSG) was measured spectrophotometrically by the recycling method originally developed by Tietze (1969) as described by Akerboom and Sies (1981) in samples (160 μl) with approximately $2 \times 10^5$ cells. In control experiments the influence of hepatocytic protein on the spectrophotometric quantitation of glutathione was assessed. The calibration curve for glutathione was constructed using solutions with and without hepatocytes. The slopes of the absorbance vs. glutathione concentration plots were not affected by the presence of hepatocytes. In selected experiments, GSSG was measured and was never >10% of total glutathione.

*Determination of N-alkylcarbamoylating metabolites.* The method used here is a modification of the procedure described by Mráz and Tureček (1987) for the determination of *N*-acetyl-*S*-(*N*-methylcarbamoyl)cysteine in urine samples. A sample of the incubate (320 μl) was diluted with 80 μl water and mixed in a glass stoppered tube with propanol (0.8 ml) containing quinoline (as internal standard). Powdered anhydrous potassium carbonate (0.6 g) was added and the mixture was shaken for 2 min. The principle of the method is encapsulated in the following equation:

$$H_3CNHCOR + C_3H\text{-}OH \overset{base}{\rightleftharpoons} H_3CNHCOOC_3H_7.$$

Any *N*-alkylcarbamoylating species ($H_3CNHCOR$ in the equation above, R = glutathionyl, for example) thus reacted to give propyl *N*-alkylcarbamate. In the original method (Mráz and Tureček, 1987), ethanol was used as derivatizing agent but control cells treated in this way displayed a peak on GLC analysis which cochromatographed with ethyl *N*-methylcarbamate. After centrifugation at 500 g for 2 min, the organic layer (1 μl) was analyzed by GLC, using a glass column (1.5 m × 2 mm) packed with 5% potassium hydroxide and 10% Carbowax 20M on silanized Chromosorb W, 80–100 mesh, in a Pye Unicam series 204 chromatograph. Nitrogen was used as the carrier gas (50 ml/min) and detection was achieved by a nitrogen-sensitive detector. The temperature of injection port, column, and detector were 200, 170, and 250°C, respectively. The calibration curve was constructed using *N*-acetyl-*S*-(*N*-methylcarbamoyl)cysteine as an *N*-alkylcarbamoylating agent in the absence of authentic *S*-(*N*-methylcarbamoyl)glutathione. *N*-Acetyl-*S*-(methylcarbamoyl)cysteine was synthesized as described previously (Kestell *et al.*, 1986). The limit of detection for the thiocarbamate was 2 nmol/$10^6$ cells. The method was characterized by the following coefficient of variation values calculated for six samples prepared with two concentrations of *N*-acetyl-*S*-(*N*-methylcarbamoyl)cysteine (20 and 100 μM): 6.70% for 20 μM and 4.52% for 100 μM obtained within 1 day and 7.79% for 20 μM and 3.94% for 100 μM obtained between 6 days. It has to be emphasised that this indirect method measured total *N*-alkylcarbamoylating compounds. Moreover, the assumption was made that all *N*-alkylcarbamoylating species present reacted with propanol under alkaline conditions as did the thiocarbamate used for the calibration curve.

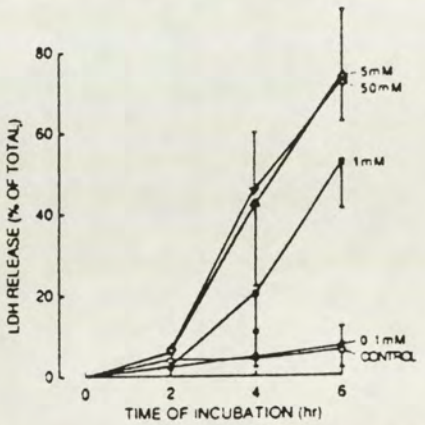


FIG. 1. Effect of concentration of NMF on viability of hepatocytes. Toxicity was quantified by measuring the LDH content of the medium (see Methods). Values represent the means ± SD of 4 to 15 separate preparations of hepatocytes. For the sake of clarity, the error bars have been omitted for the values obtained at 2 hr where they did not exceed 52% of the mean.

## RESULTS

The cytotoxic potential of the formamides was assessed by measurement of both release of LDH from and uptake of trypan blue by the cells. In preliminary experiments, both methods gave almost identical cytotoxicity values. Therefore, in subsequent experiments, only release of enzymes was determined. NMF-induced cytotoxicity was dependent on cell density with cells suspended at low density being more susceptible to lethal injury than those in incubations with high cell numbers (result not shown). Consequently, a constant medium hepatocyte density of $1.4 \pm 0.1 \times 10^6$ cells/ml was used. Cytotoxicity was only apparent beyond 2 hr of exposure to NMF and depended on drug concentration (Fig. 1). At a concentration of 0.1

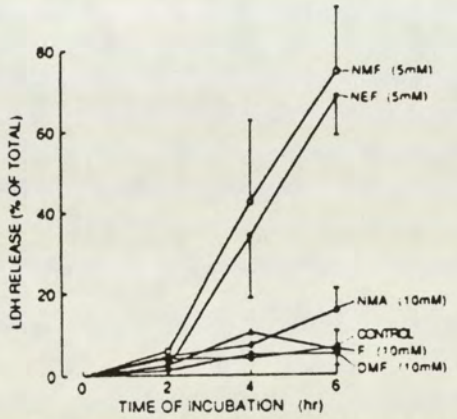


FIG. 2. Effect of amides related to NMF on viability of hepatocytes. Toxicity was quantified by measuring the LDH content of the medium (see Methods). Values represent the means ± SD of 4 to 15 separate preparations of hepatocytes. Error bars on some of the points have been excluded for the sake of clarity. In these cases, the SD did not exceed 65% (at 2 hr), 60% (4 hr), or 31% (6 hr) of the mean.

mM NMF was innocuous and maximum toxicity was observed at concentrations of 5 mM or greater. Concentrations of >50 mM of NMF and other formamides interfered with the LDH assay and for higher concentrations toxicity was assessed by trypan blue exclusion. Of the formamide analogs studied, only NMF and NEF were cytotoxic at concentrations <10 mM (Fig. 2). F, DMF, and NMA did not cause toxicity at this concentration. At 100 mM, these derivatives decreased cell viability (result not shown), however, not to the extent seen with 5 mM NMF or NEF (Fig. 2).

[$^2$H]NMF was markedly less cytotoxic toward hepatocytes than was NMF. After incubation of hepatocytes with 5 mM [$^2$H]NMF for 6 hr release of LDH was only 7.3 ± 1.6% ($n$ = 5) and thus was indistinguishable from controls.

Biotransformation of NMF to an *N*-methylcarbamoylating species was determined by GLC. The metabolite was derivatized with propanol under alkaline conditions and measured as propyl *N*-methylcarbamate. Formation of metabolites was observed during the whole of the 6-hr incubation period but decreased with time (Fig. 3). The rate of this biotransformation was dependent on the concentration of NMF at concentrations <5 mM and formed a plateau at higher concentrations. Sonication of cells for 20 sec, which caused >96% cell kill as established by trypan blue exclusion, abolished production of detectable levels of the metabolite. On incubation of hepatocytes with F, DMF, or NMA (10 or 50 mM), a carbamoylating metabolite could not be detected. However, a metabolite which reacted with propanol to yield a product with a retention time indistinguishable from that of propyl *N*-methylcarbamate was found in incubations of hepatocytes with NEF (5 mM). The amount of this species formed within 4 hr of incubation was 156 ± 16% ($n$ = 4) of the amount of metabolite found in incubations with NMF at the same concentration. This percentage value is based on the assumption that the NEF metabolite behaved exactly like the NMF metabolite in

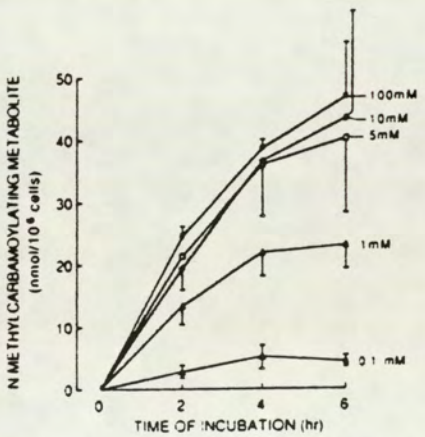


FIG. 3. Time course of the formation of the *N*-methylcarbamoylating metabolite from NMF on incubation with hepatocytes. Formation of metabolites was determined by GLC after derivatization with propanol as described under Methods. *N*-Acetyl-*S*-(*N*-methylcarbamoyl)cysteine was used for the calibration curve. Values represent the means ± SD of three to six separate preparations of hepatocytes. Error bars on the points representing formation of metabolites for 5 or 10 mM NMF have been omitted for the sake of clarity; here the SD was 18% (2 and 4 hr) and 26% of the mean (6 hr).

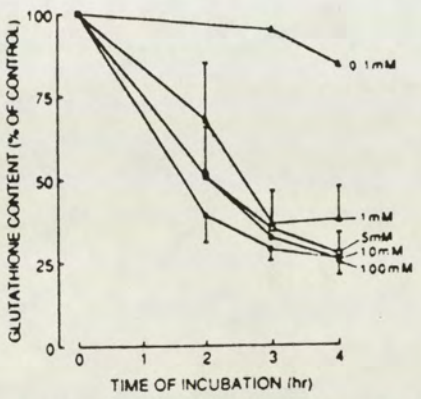


FIG. 4. Effect of concentration of NMF on the loss of intracellular glutathione from hepatocytes. Glutathione was measured spectrophotometrically as described under Methods. Values are the mean of 2 (in case of 0.1 mM NMF) or the means ± SD of 3 to 11 separate preparations of hepatocytes. For the sake of clarity, error bars on the points representing 5 or 10 mM NMF have been omitted. In these cases, the SD did not exceed 28% of the mean. Control concentrations of glutathione were 44 ± 5.3 nmol per $10^6$ cells ($n$ = 9) at the start of the incubations and decreased by <10% of original values throughout control incubations.

the analytical assay as the value was calculated using the calibration curve employed for the metabolite of NMF. [$^2$H]NMF was also metabolized to a *N*-methylcarbamoylating species. The metabolite yield obtained after 4 hr under these incubation conditions with 5 mM [$^2$H]NMF was only 35 ± 3% ($n$ = 5) of that measured in the presence of 5 mM NMF.

Cytotoxic concentrations of NMF caused a dramatic depletion of hepatocytic glutathione pools. Maximal depletion to 26% of control values occurred after 4 hr of exposure to 10 mM NMF (Fig. 4). NEF (5 mM) decreased intracellular glutathione down to 7% of control values after 3 hr incubation, a depletion even more dramatic than that seen with NMF (Fig. 5). [$^2$H]NMF was also able to affect concentrations of glutathione but to a lesser extent than NMF. After 4 hr incubation with 5 mM [$^2$H]NMF glutathione pools were depleted to 54% of controls whereas the concentration of glutathione measured after incubation with 5 mM NMF was 27% of controls. Neither F, DMF, or NMA at 10 mM concentration caused significant depletion of glutathione (Fig. 5).

## DISCUSSION

There is good evidence to suggest that the mechanism by which NMF causes hepatotoxicity involves a metabolite. Whereas tumor cells in culture had to be exposed to 0.5 M NMF to achieve cell killing (Gescher *et al.*, 1982), fulminant hepatic periacinar necrosis occurred in mice (Whitby *et al.*, 1984) after administration of doses which led to peak plasma concentrations below 10 mM (Brindley *et al.*, 1982). Similarly, the concentration of NMF measured in the plasma of patients who received the drug in a phase I clinical trial did not exceed 2 mM (Griffiths *et al.*, 1983), yet patients on NMF frequently exhibited liver damage (Laird Myers *et al.*, 1953; McVie *et al.*, 1984; Ettinger *et al.*, 1985; Eisenhauer *et al.*, 1986). These results together with those of mechanistic studies in mice, which showed that NMF depletes hepatic

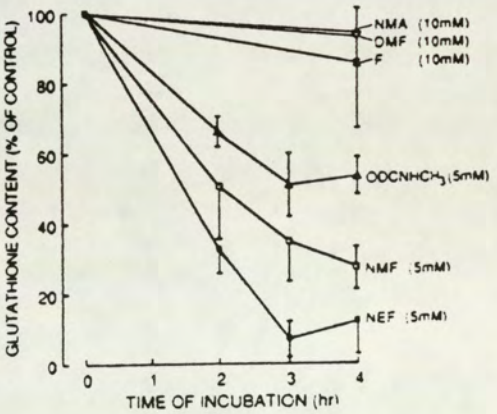


FIG. 5. Effect of amides related to NMF on the loss of intracellular glutathione from hepatocytes. Glutathione was measured spectrophotometrically as described under Methods. Values are the means ± SD of 3 to 11 separate preparations of hepatocytes. The depletion caused by NEF after 3 hr was significantly different from that observed in the case of NMF ($p < 0.05$).

glutathione stores (Pearson *et al.*, 1987a) and that $^{14}C$-labeled NMF is covalently bound to hepatic proteins (Pearson *et al.*, 1987b), are consistent with the involvement of a reactive metabolite in the generation of the NMF-induced toxic lesion. The major urinary metabolites of NMF have been characterized as methylamine (Kestell *et al.*, 1985) and *N*-acetyl-*S*-(*N*-methylcarbamoyl)cysteine (Kestell *et al.*, 1986; Tulip *et al.*, 1986) and they appear to be end products of the biotransformation route which proceeds via a hepatotoxic species (Threadgill *et al.*, 1987). The results presented here show for the first time that NMF is metabolized by liver cells *in vitro* to a *N*-methylcarbamoylating species, and that NMF is cytotoxic toward hepatocytes at concentrations of 1 mM or above, concentrations which have been achieved after administration of hepatotoxic doses of NMF *in vivo* (Brindley *et al.*, 1982; Griffiths *et al.*, 1983). Moreover, NEF, an analog of NMF which is also hepatotoxic *in vivo* (Kestell *et al.*, 1987), was also found to be cytotoxic to hepatocytes *in vitro* and, like NMF, capable of depleting hepatic glutathione and generating a *N*-alkylcarbamoylating metabolite. The NMF analogs F, DMF, and NMA were previously shown to be devoid of toxicity *in vivo* and not to undergo metabolism to detectable amounts of *S*-(*N*-alkylcarbamoyl)mercapturates (Kestell *et al.*, 1987). It is demonstrated here that these analogs also lack cytotoxic potential in hepatocytes and that they do not deplete hepatic glutathione or generate *N*-alkylcarbamoylating metabolites. The toxicity of NMF and its metabolism to *N*-acetyl-*S*-(*N*-methylcarbamoyl)cysteine and methylamine *in vivo* are subject to a primary kinetic isotope effect when the molecule is deuterated in the formyl moiety: [$^2H$]NMF was significantly less toxic *in vivo* than NMF and its metabolism occurred at a slower rate then that observed for NMF (Threadgill *et al.*, 1987). Likewise, in mouse hepatocytes, [$^2H$]NMF was less potent than NMF as cytotoxin and as a glutathione depleting agent and the rate of its metabolism to the *N*-methylcarbamoylating species was only a third of that observed with NMF.

In summary, there is excellent agreement between the observations recorded *in vivo* (Kestell *et al.*, 1987; Threadgill *et al.*, 1987) and reported here *in vitro* concerning the structure–activity relationship in toxicity, glutathione depletion, and metabolism of formamides. This conclusion characterizes mouse hepatocytes in suspension as a suitable model system *in vitro* for further investigations of the metabolism and the biochemical changes associated with the hepatotoxicity of this class of compound. The results presented here also provide further evidence for the contention that formamide-induced toxicity, glutathione depletion, and formation of the *N*-alkylcarbamoylating metabolite are mechanistically linked. NMF-induced hepatotoxicity *in vivo* in mice has been shown to develop after a lag period of at least 10 hr (Whitby *et al.*, 1984). In the isolated hepatocytes, NMF caused cytotoxicity only after a delay of between 2 and 4 hr during which the cells remained viable. The difference in the time lag preceeding the emergence of toxic manifestations between the liver *in vivo* and liver cells *in vitro* can be explained on the basis of the assumption that glutathione is important in protecting the cells against the necrogenic activity of the reactive NMF metabolite. The time lag correlates to the time required for a sufficient amount of the reactive NMF metabolite to be synthesized and cause glutathione depletion in the liver to concentrations below the threshold critical for protection of the cells. Hepatocytes lose some of their original complement of glutathione during isolation (Vina *et al.*, 1978), and may be less efficient than the whole liver in glutathione synthetic activity. Consequently, isolated hepatocytes may be vulnerable toward NMF-induced toxicity more rapidly than are cells contained in the intact organ.

It is puzzling that NEF depleted glutathione to a larger extent than did NMF. Glutathione depletion caused by NMF formed a

TABLE I

COMPARISON OF AMOUNTS OF N-METHYLCARBAMOYLATING METABOLITE FORMED IN HEPATOCYTES AND OF GLUTATHIONE REMOVED FROM HEPATOCYTES ON INCUBATION WITH NMF

| Incubation time (hr) | NMF concentration (mM) | Amount of metabolite formed[a] (nmol/$10^6$ cells) | Amount of glutathione depleted[a] (nmol/$10^6$ cells) |
|---|---|---|---|
| 2 | 1 | 13.4 ± 2.9[b] | 14.3 ± 7.7[b] |
| 4 | 1 | 22.0 ± 3.7 | 27.6 ± 4.4 |
| 2 | 5 | 21.4 ± 6.1 | 21.7 ± 6.5 |
| 4 | 5 | 36.3 ± 8.5 | 32.0 ± 2.7 |
| 2 | 10 | 19.4 ± 3.4 | 21.8 |
| 4 | 10 | 36.7 ± 6.4 | 32.7 ± 1.4 |
| 2 | 100 | 24.6 ± 1.5 | 27.0 ± 3.5 |
| 4 | 100 | 38.7 ± 1.8 | 32.7 ± 1.8 |

[a] Amounts of N-methylcarbamoylating metabolite and of residual glutathione were determined as described under Methods.

[b] Values are the mean of two or the mean ± SD of three to six experiments.

plateau at 26% of control values whereas NEF caused a depletion down to 7% of control values. Likewise, NEF gave rise to the production of more N-alkylcarbamoylating metabolite than did NMF, assuming that the intensity of the GLC detection signal was identical for both the methyl and the ethyl derivatives. This is a reasonable assumption since a nitrogen-sensitive detector was used. These results suggest that the necrogenic metabolite of NEF influences glutathione pools unaffected by the analogous metabolite of NMF. This apparent difference in reactivity seems to be of little consequence to cell survival as the toxic potentials of these two N-alkylformamides are very similar both *in vivo* (Kestell *et al.*, 1987) and *in vitro*.

DMF is an occupational hazard of moderate toxicity (Scailteur and Lauwerys, 1987). In man, DMF undergoes metabolism to N-acetyl-S-(N-methylcarbamoyl)cysteine (Mráz and Tureček, 1987) and the extent of this biotransformation may be important for the assessment of the occupational risk associated with exposure to DMF. Yet, in mouse hepatocytes, the amount of N-methylcarbamoylating metabolite generated from DMF was below the detection limit of the assay. We have recently found N-acetyl-S-(N-methylcarbamoyl)cysteine as a metabolite of DMF also in the urine of mice and rats which had received DMF; however, amounts of this metabolite were very low, at or below 2% of the dose (Cross, Mraz, Gescher, and Threadgill, [illegible]).

The method used here for metabolite detection measured, without discrimination, all species derived from NMF or NEF capable of N-alkylcarbamoylating propanol. Therefore, it cannot be excluded that hepatocytes produced a mixture of such species. However, a comparison of the amount of metabolite(s) generated from NMF with the amount of glutathione which was lost by the cells during exposure to NMF shows an astonishing congruity (Table 1). This comparison is tentative, as the amount of metabolite was calculated on the basis of a calibration curve constructed using N-acetyl-S-(N-methylcarbamoyl)cysteine rather than S-(N-methylcarbamoyl)glutathione. Nevertheless the high degree of congruity suggests strongly that the metabolic species detected after derivitization as propyl N-methylcarbamate was S-(N-methylcarbamoyl)glutathione. S-(N-Methylcarbamoyl)glutathione has previously been identified as a metabolite of NMF in mouse bile (Threadgill *et al.*, 1987), even though work

on the synthesis of authentic reference material is still in progress. Recent evidence obtained in rat hepatocytes using HPLC and MS indeed support the suggestion that *S*-(*N*-methylcarbamoyl)glutathione is a metabolite of NMF in isolated liver cells (Pearson, Threadgill, Howald, and Baillie, manuscript submitted).

As the hepatotoxicity of NMF in mice could be ameliorated by pretreatment with cysteine or *N*-acetylcysteine and exacerbated by pretreatment with the glutathione synthesis inhibitor buthionine sulfoximine (Pearson *et al.*, 1987a), it seems likely that *S*-(*N*-methylcarbamoyl)glutathione, taken to be the precursor of the propyl *N*-methylcarbamate measured here, is a detoxification product of the ultimate NMF-derived hepatotoxin. We offer the suggestion that the ultimate toxin may be methyl isocyanate ($OCNCH_3$) (Kestell *et al.*, 1987) or an ester of *N*-methylcarbamic acid ($HOOCNHCH_3$). Future studies using suspensions of hepatocytes should help to unravel questions concerning the formation of this metabolic intermediate and the enzymes which catalyze the oxidation of the formyl moiety of *N*-alkylformamides.

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# P4 STABLE ISOTOPES : UNIQUE PROBES FOR MECHANISTIC STUDIES OF XENOBIOTIC METABOLISM. APPLICATION TO STUDIES ON THE BIOACTIVATION OF N-METHYL-FORMAMIDE IN VITRO

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SUMMARY

Substrates labeled at specific positions with $^{2}H$, $^{13}C$ and $^{15}N$ were employed to study the metabolism of NMF to a GSH conjugate in isolated hepatocyte preparations from rats and mice. The conjugate was identified by a combination of FAB MS/MS and $^{13}C$-NMR techniques, and was shown to be formed from NMF in a process which involves cleavage of the formyl C-H bond in the rate-limiting step. These findings indicate that metabolism of NMF leads to the generation of a reactive intermediate, e.g. methyl carbamic acid and/or methyl isocyanate, which may be responsible for the hepatotoxic effects of NMF.

## INTRODUCTION

Following the first applications, in the early 1970's, of stable isotope techniques to studies in drug metabolism, stable isotopes became adopted with increasing frequency for a host of qualitative, quantitative and mechanistic investigations of foreign compound biotransformation in animals and man (Baillie, 1981). Studies on the molecular mechanism by which xenobiotics undergo metabolic activation to chemically reactive, potentially toxic intermediates have benefited greatly from stable isotope tracer experiments in that analysis of heavy isotope loss from labeled substrate during metabolism, or of incorporation of isotope from labeled environment into metabolites, can often provide a wealth of information on the nature of short-lived, electrophilic species formed *in vitro* or *in vivo*.

In order to illustrate the utility of stable isotope tracers in the field of biochemical toxicology, the following example is presented from some of our recent work on the metabolic activation of *N*-methylformamide (NMF, 1, Fig. 1), an industrial solvent and candidate antineoplastic agent. Although this compound has been shown to be active against implanted rodent tumors (Gescher *et al.*, 1982) and has

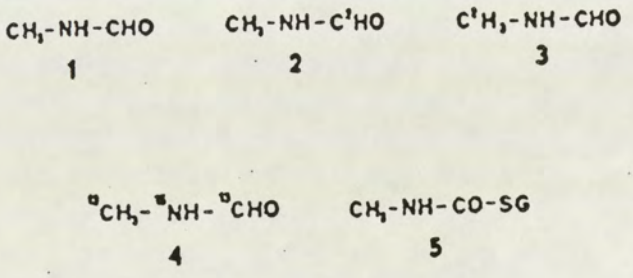


Fig. 1 Structures of compounds referred to in the text.

been the subject of clinical investigation (McVie et al., 1984; Eisenhauer et al., 1986), hepatotoxicity has proved to be a serious side-effect of NMF therapy in clinical trials (Laird Myers et al., 1956) and is also evident in animal models (Whitby et al., 1984; Pearson et al., 1987). Recent studies have suggested that this toxicity is mediated by a reactive metabolite of NMF, and the identification of a glutathione (GSH) conjugate of the drug (5, Fig. 1) in bile of mice dosed with NMF has added support to this view (Threadgill et al., 1987).

In order to investigate the mechanism by which NMF undergoes metabolic activation, appropriate in vitro systems were required to study the conversion of NMF to its GSH conjugate, and freshly-isolated hepatocytes from both mice (Shaw et al., 1988) and rat (Pearson et al., 1988) have been found recently to be suitable for this purpose. The specific objectives of the work described in this paper were: (i) to compare the utility of tandem mass spectrometry (MS/MS) and $^{13}C$-NMR techniques for the detection of conjugate 5 in incubates of NMF with hepatocytes, and (ii) to determine the influence of deuterium substitution at different sites on NMF on the rate of metabolism of the parent compound to 5. For the latter objective, compounds 2-4 (Fig. 1) served as substrates for metabolism.

## METHODS

Compounds 2 (96 atom % excess $^{2}H$) and 3 (97 atom % excess $^{2}H_3$) were synthesized by published methods (Threadgill and Gate, 1983; Threadgill et al., 1987), while 4 (90 atom % excess $^{13}C_2$, 95 atom % excess $^{15}N$) was purchased from MSD Isotopes (St. Louis, MO). An authentic sample of 5 was prepared by treating GSH in aqueous buffer with excess methylisocyanate; full details of the method will be reported elsewhere. Hepatocytes were isolated from either adult male Balb/c mice or adult male Sprague-Dawley rats by a collagenase perfusion technique (Moldéus et al., 1978), and incubations were performed in Krebs-albumin buffer ($2 \times 10^6$ cells $ml^{-1}$) with substrate (5 mM) for 4 hr at 37°C under an atmosphere of $O_2/CO_2$ (95/5).

Conjugate 5 was isolated from incubations with rat hepatocytes, converted to its N-benzoxycarbonyl dimethyl ester derivative, and analyzed by fast atom bombardment (FAB) MS/MS techniques as described previously (Pearson et al., 1988). Deuterium isotope effect experiments were conducted with approximately equimolar mixtures of 2 and 4, or of 3 and 4, as substrate. For $^{13}C$-NMR studies, mouse hepatocytes were incubated with 4 and acetone was added to precipitate proteins. The resultant supernatants were lyophylized, the residues treated with $^{2}H_2O$ and the proton-decoupled $^{13}C$-NMR spectra were recorded with a Varian VXR 300 spectrometer operating at 76 MHz. Chemical shifts were expressed relative to tetramethylsilane.

## RESULTS AND DISCUSSION

### Detection of 5 by MS/MS

When the derivatized GSH conjugate isolated from an incubation of 4 with rat hepatocytes was subjected to analysis by conventional FAB MS, the spectrum exhibited a weak $MH^+$ ion at m/z 530 which was barely distinguishable from the matrix background (data not shown). However, when this $MH^+$ species was selected for collisionally-activated dissociation (CAD) in a tandem mass spectrometer, the daughter ion spectrum shown in Fig. 2 was obtained. By virtue of the presence of the stable isotopes $^{13}C$ and $^{15}N$ in the NMF moiety, the $MH^+$ species and daughter ions retaining the elements of NMF were shifted (by 3 Daltons) from the m/z values observed in the spectrum of the corresponding unlabeled reference compound (Pearson et al., 1988). By this approach, the $^{13}C_2$, $^{15}N$-analog of 5 was identified unequivocally from a complex biological extract with approximately 5 µg of sample.

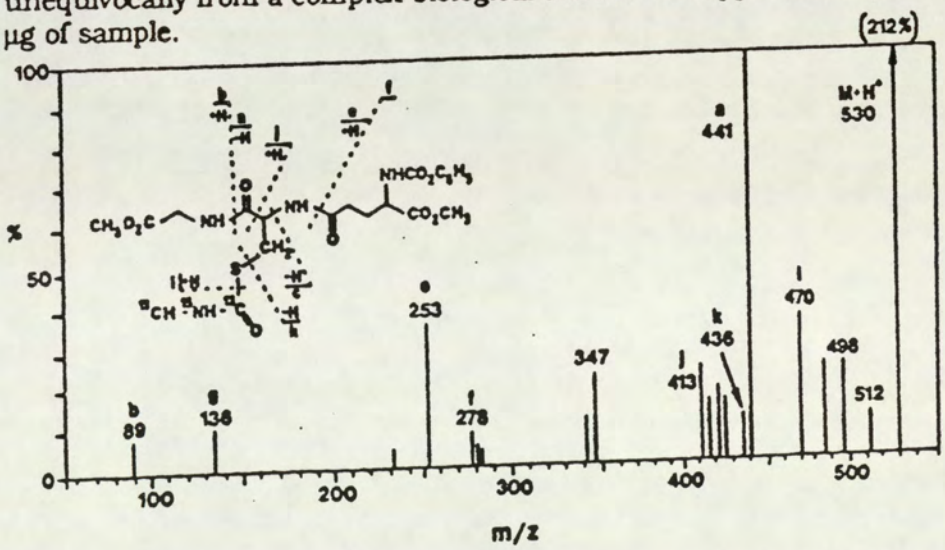


Fig. 2 Spectrum of daughter ions obtained by CAD of m/z 530, the $[M+H]^+$ ion of the devivatized GSH conjugate of 5.

Detection of 5 by $^{13}C$-NMR

Deproteinized samples of mouse hepatocytes which had been incubated with 4 were examined by $^{13}C$-NMR and afforded the partial spectrum reproduced in Fig. 3. The resonances centered at 167.9 and 171.0 ppm were attributed to the carbonyl carbons of the two rotamers of residual 4, the magnitude of the $^{13}C$-$^{15}N$ coupling constant being 15 Hz in each case. Some fine structure is visible in the signals centered at 171.0 ppm, resulting from two-bond $^{13}C$-$^{13}C$ coupling (J = 1.5 Hz). Vertical expansion of the region from 173-174 ppm revealed the doublet shown in the inset (centered at 173.5 ppm, J = 14 Hz), which exhibited the correct chemical shift and splitting pattern for the NMF carbonyl carbon of the GSH conjugate. Thus, despite a signal-to-noise ratio of only ~ 3:1, the target compound was clearly detectable in the incubation mixture by $^{13}C$-NMR and was distinguished from extraneous signals by virtue of the characteristic $^{13}C$-$^{15}N$ coupling.

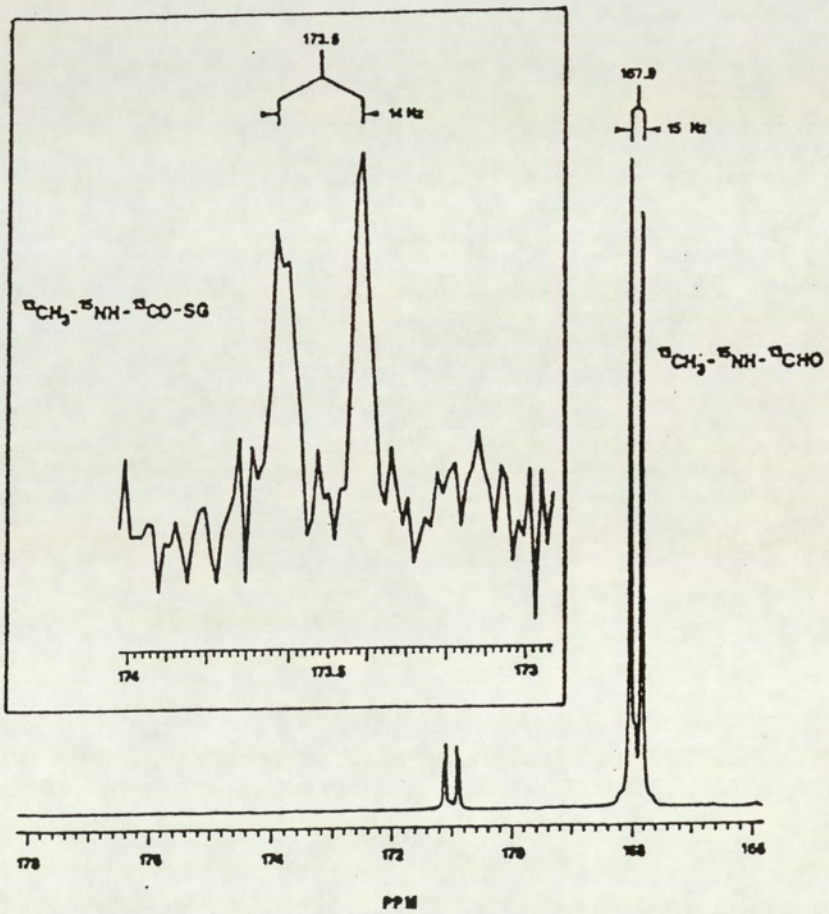


Fig. 3 $^{13}C$-NMR Spectrum (carbonyl region) of an extract of hepatocytes incubated with 4.

Deuterium Isotope Effects on GSH Conjugate Formation

Incubations of roughly equimolar quantities of 2 and 4, and of 2 and 3, with rat hepatocytes generated the GSH conjugates whose partial FAB mass spectra are shown in Figs. 4A and 4B, respectively. Based on the relative abundances of ions (m/z 527, $MH^+$; m/z 549, $[M+Na]^+$) corresponding to unlabeled 5 and ions (m/z 530, 552) derived from isotopically-labeled 5, it was deduced that: (i) the primary kinetic deuterium isotope effect ($k_H/k_D$) associated with metabolism of NMF at the formyl carbon was ~ 6, a value in good agreement with that derived from corresponding in vivo studies (Threadgill et al., 1987), and (ii) $^2H$ substitution at the methyl carbon of NMF has little effect on metabolism of this substrate to 5. Thus, formyl C-H bond cleavage is rate-limiting in the overall metabolism of 1 to 5.

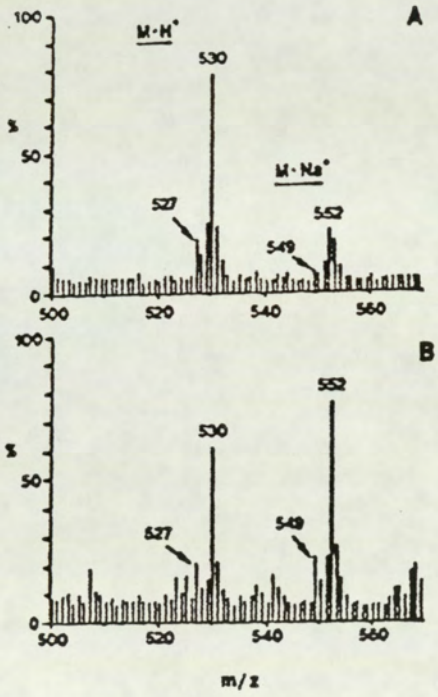


Fig. 4 Molecular ion regions of the FAB mass spectra of the derivatized NMF-GSH conjugate obtained from hepatocytes incubated with approximately equimolar mixtures of 2 and 4 (A) and of 2 and 3 (B). The low abundances of ions corresponding to the unlabeled conjugate relative to those derived from the stable-isotope-enriched conjugate are indicative of a large primary kinetic deuterium isotope effect associated with formyl C-H bond cleavage during the metabolism of 2 to 5.

CONCLUSIONS

The results of these studies further support the contention that freshly-isolated hepatocytes from rats and mice represent suitable in vitro model systems with which to examine the metabolic activation of NMF. Moreover, the results are consistent with the emerging view of NMF metabolism depicted in Fig. 5, in which oxidative attack occurs at the formyl carbon to generate an unstable intermediate (possibly methyl carbamic acid and/or methyl isocyanate) which, in turn, reacts with GSH to produce the observed conjugate, 5. In these investigations, stable isotopes served a dual purpose in (a) facilitating detection of the GSH adduct of NMF by two complementary analytical techniques, viz. tandem mass spectrometry and $^{13}C$-NMR spectrocopy, and (b) providing mechanistic information, through the expression of $^{2}H$ isotope effects, on the biotransformation of NMF to its GSH adduct. Such examples illustrate the power of stable isotope tracer techniques in studies of metabolic activation, and highlight the value of $^{2}H$, $^{13}C$ and $^{15}N$ for mechanistic applications in biochemical toxicology.

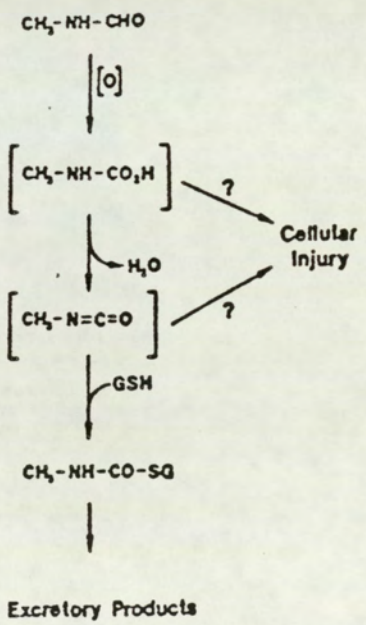


Fig. 5 Proposed metabolic activation pathway for 1 in mammalian liver. Intermediates shown in brackets have not been identified.

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