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INVESTIGATION OF THE METABOLISM OF N,N-DIMETHYLFORMAMIDE
AND ITS METABOLITES

HILARY JEAN CROSS
Doctor of Philosophy

THE UNIVERSITY OF ASTON IN BIRMINGHAM

October 1989

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INVESTIGATION OF THE METABOLISM OF N,N-DIMETHYLFORMAMIDE AND ITS METABOLITES
by
Hilary Jean Cross

Submitted for the degree of Doctor of Philosophy, 1989

The industrial solvent N,N-dimethylformamide (DMF) causes liver damage in humans. The hepatotoxicity of N-alkylformamides seems to be linked to their metabolism to N-alkylcarbamoyl acid thioesters. To clarify the role of metabolism in DMF hepatotoxicity, the metabolic fate of DMF was investigated in rodents. DMF was rapidly metabolised and excreted in the urine as N-hydroxy-methyl-N-methylformamide (HMMF), N-acetyl-S-(N-methylcarbamoyl)cysteine (AMCC) and a metabolite measured as formamide by GLC. At high doses (0.7 and 7.0mmol/kg) a small proportion of the dose was excreted unchanged. AMCC, measured by GLC after derivatisation to ethyl N-methylcarbamate, was a minor metabolite. Only 5.2% of the dose (0.1mmol/kg) in rats or 1.2% in mice was excreted as AMCC. The minor extent of this metabolic pathway in rodents might account for the marginal liver damage induced by DMF in these species. In a collaborative study, volunteers were shown to metabolise DMF to AMCC to a greater extent than rodents. Nearly 15% of the inhaled dose (0.049mmol/kg) was excreted as AMCC. This result suggests that the metabolic pathway leading to AMCC is more important in humans than in rodents. Consequently the risk associated with exposure to DMF might be higher in humans than in rodents.

The metabolism of formamides to S-(N-alkylcarbamoyl) glutathione, the metabolic precursor of the thioester mercapturates, was studied using mouse, rat and human hepatic microsomes. The metabolism of NMF (10mM) to S-(N-methylcarbamoyl)glutathione (SMG) required the presence of GSH, NADPH and air. Generation of SMG was inhibited when incubations were conducted in an atmosphere of CO:air (1:1) or when SKF 525-A (3.0mM) was included in the incubations. Pre-treatment of mice with phenobarbitone (PB, 80mg/kg for 4 days) or betanaphthoflavone (BNF, 50mg/kg for 4 days) failed to increase the microsomal formation of SMG from NMF. This result suggests that the oxidation of NMF is catalysed by a cytochrome P-450 isozyme which is unaffected by PB or BNF. Microsomal incubations with DMF (5 or 10mM) failed to generate measurable amounts of SMG although DMF was metabolised to HMMF. Incubations of microsomes with HMMF resulted in the generation of a small amount of SMG which was affected by inhibitors of microsomal enzymes in the same way as in the case of NMF. HMMF was metabolised to AMCC by rodents in vivo. This result suggests that HMMF is a major intermediate in the metabolic activation of DMF.

Keywords: dimethylformamide, N-alkylformamides, hepatotoxicity, metabolic activation, mercapturic acids.
ACKNOWLEDGEMENTS

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</table>
HM[\(^{14}\text{C}]\text{MF} \quad \text{HMMF labelled with } [^{14}\text{C}] \text{ in the methyl group}

HPLC \quad \text{high performance liquid chromatography}

IARC \quad \text{International Agency for the Research on Cancer}

ip \quad \text{intraperitoneal}

IS \quad \text{internal standard}

3-MC \quad 3\text{-methylcholanthrene}

MeOH \quad \text{methanol}

MIC \quad \text{methyl isocyanate}

NADP \quad \text{nicotinamide adenine dinucleotide phosphate}

NADPH \quad \text{NADP (reduced form)}

NaOH \quad \text{sodium hydroxide}

NEF \quad \text{N-ethylformamide}

NMA \quad \text{N-methylacetamide}

NMF \quad \text{N-methylformamide}

"NMF" \quad \text{metabolite measured as NMF by GLC}

NMR \quad \text{nuclear magnetic resonance}

\(^{18}\text{O} \quad \text{stable isotope of oxygen (atomic wt = 18)}

OEL \quad \text{occupational exposure limit}

PB \quad \text{phenobarbitone}

PLC \quad \text{preparative liquid chromatography}

PMC \quad \text{propyl N-methyl carbamate}

Pr-1-OH \quad \text{propan-1-ol}

Rf \quad \text{distance travelled by compound in TLC relative to solvent front}

SDH \quad \text{sorbitol dehydrogenase}

SKF 525-A \quad \text{diethylaminoethyl 2,2-diphenylvalerate HCl}

SMG \quad \text{S-(N-methylcarbamoyl)glutathione}

TLC \quad \text{thin layer chromatography}

UV \quad \text{ultra-violet}
SECTION 1

INTRODUCTION
1.1 USES OF N,N-DIMETHYLFORMAMIDE

N,N-Dimethylformamide (DMF) is an organic solvent possessing properties which have led to its widespread use in the manufacturing and chemical industry. The physical and chemical properties of DMF are summarised in Table 1.1. The industrial processes which take advantage of its solvent properties include the manufacture of synthetic leathers and acrylic fibres, the production of surface coatings, adhesives and inks, inclusion in some paint strippers, as a solvent for dyes and pigments, and in resin and rubber applications.

The excellent solvent properties of DMF have also led to its useage in non-industrial applications. DMF, for instance, is frequently used in the chemical laboratory and has been used as a vehicle for the administration of non-water-soluble chemicals in experimental biological and pharmaceutical studies. In the latter case, increasing awareness of its toxicity have in many cases caused it to be replaced by dimethylsulphoxide (Mathew et al, 1980).

In addition to its role as a solvent, DMF has been investigated as a potential anti-tumour agent due to its ability to induce differentiation in certain malignant cells (Cordeiro and Savarese, 1986; Dexter et al, 1982; Spremulli and Dexter, 1984; Langdon and Hickman, 1987; van Dongen et al, 1989).
Relative molecular mass 73.1
Melting point -60.5°C
Boiling point (760mm Hg) 152°C
Specific gravity (water = 1) 0.9445
Vapour density (air = 1) 2.51 (at boiling point of DMF)
Evaporation rate (butyl acetate = 1) 0.71
Vapour pressure
- 0.68mm Hg at 0°C
- 2.65mm Hg at 20°C
- 8.95mm Hg at 40°C
- 16mm Hg at 50°C
- 26mm Hg at 60°C
Flash point 58°C (closed cup)
67°C (open cup)
Autoignition temperature 445°C
Explosive limits (vol % in air) 2.2 - 16
Appearance - colourless liquid
Solubility in water - miscible in all proportions
Solubility in organic solvents - miscible in ether, ketones, aromatic hydrocarbons, and ethanol
Stability - stable under normal conditions
Odour threshold limit - 300mg/m³
Thermal decomposition products - dimethylamine, CO

Table 1.1

SOME PHYSICAL AND CHEMICAL PROPERTIES OF N,N-DIMETHYLFORMAMIDE

It can be seen that DMF is extensively used in a variety of environments. As a consequence, many people are potentially exposed to its toxic effects.
1.2 HEALTH EFFECTS OF DMF IN OCCUPATIONALLY-EXPOSED PERSONS

Current knowledge of the health effects of DMF in humans has been derived from investigations of occupationally-exposed persons. These studies, summarised in Tables 1.2 and 1.3, may be broadly divided into two groups according to whether the exposure was acute or chronic.

The most prominent effect of DMF in occupationally-exposed humans is its ability to cause liver damage. This is most clearly illustrated in cases of acute exposure and a typical example is described by Potter (1976). In this instance the worker was involved in an accidental spillage of DMF in which he experienced substantial skin contact with the liquid and was also exposed to high concentrations of vapour. Shortly after the incident skin irritation and anorexia developed, followed by vomiting and abdominal pain. Elevation of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) suggested liver damage and this was subsequently confirmed by biopsy. The symptoms subsided two weeks after the incident, indicating that the condition was reversible. Symptoms of abdominal pain accompanied by nausea and vomiting were similarly reported in a group of five workers exposed to DMF (Chary, 1974). In this study a diagnosis of acute pancreatitis was suggested.

Liver damage associated with chronic exposure to DMF has been reported in several studies (Cirla et al, 1984;
<table>
<thead>
<tr>
<th>AUTHOR</th>
<th>WORKPLACE</th>
<th>NO. IN STUDY</th>
<th>LEVELS/ROUTE OF EXPOSURE</th>
<th>SYMPTOMS</th>
<th>DIAGNOSIS/COMMENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>&quot;Aldyeva &amp; Gafurov, 1980&quot;</td>
<td>Synthetic fibre factory</td>
<td>0</td>
<td></td>
<td>Disorders of GIT, liver, nervous system and heart</td>
<td></td>
</tr>
<tr>
<td>Chivers, 1974</td>
<td>-</td>
<td>5 σ</td>
<td></td>
<td>Skin rash, GIT disorders, abdominal pain, alc.intol.</td>
<td>Acute pancreatitis</td>
</tr>
<tr>
<td>Chivers, 1978</td>
<td>-</td>
<td>1 σ</td>
<td>30ppm inhal'n &amp; skin</td>
<td>Chest tightness, reddening of skin</td>
<td>Alcohol intolerance</td>
</tr>
<tr>
<td>&quot;Kang-de &amp; Hui-lan, 1981&quot;</td>
<td>Synthetic rubber plant</td>
<td>3 σ</td>
<td>Skin</td>
<td>Anorexia, dizziness, raised serum enzymes, abdominal pain, skin reddening</td>
<td></td>
</tr>
<tr>
<td>Paoletti et al, 1982</td>
<td>-</td>
<td>1 σ</td>
<td>&quot;High&quot;</td>
<td>Abdominal pain</td>
<td>Reversible liver damage</td>
</tr>
<tr>
<td>Potter, 1973</td>
<td>Fabric coating plant</td>
<td>1 σ</td>
<td>&quot;High&quot; inhal'n &amp; skin</td>
<td>Abdominal pain, vomiting, skin irritation, raised ALT &amp; AST</td>
<td>Liver damage</td>
</tr>
<tr>
<td>&quot;Shligina &amp; Nemolchev, 1981&quot;</td>
<td>-</td>
<td>σ</td>
<td></td>
<td></td>
<td>Persistent liver &amp; kidney damage</td>
</tr>
<tr>
<td>Tolot et al, 1958</td>
<td>Synthetic fibre plant</td>
<td>11 σ</td>
<td></td>
<td>Nausea, vomiting, anorexia, GIT pain, skin irritation, alcohol intolerance</td>
<td></td>
</tr>
<tr>
<td>&quot;Weiss, 1971&quot;</td>
<td>-</td>
<td>1 σ</td>
<td>(poor vent'n)</td>
<td>Raised ALT &amp; AST, GIT disorders</td>
<td>Reversible liver damage</td>
</tr>
</tbody>
</table>

*Source: IPCS (1987)
<table>
<thead>
<tr>
<th>Author</th>
<th>Workplace</th>
<th>No. in study</th>
<th>Level/Route of exposure</th>
<th>Symptoms</th>
<th>Diagnosis/Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>*Aldyrev &amp; Gafurov, 1980</td>
<td>Synthetic leather plant</td>
<td>115</td>
<td>10 - 50ppm Inhal'n, skin</td>
<td>Abdominal pains, vomiting, dizziness, diarrhoea, heart disorders, headache</td>
<td>Liver damage, GIT disorders</td>
</tr>
<tr>
<td>*Aldyrev &amp; Gafurov, 1980</td>
<td>Synthetic leather plant</td>
<td>70</td>
<td>-</td>
<td>Menstrual disorders</td>
<td>Gynaecological disturbances</td>
</tr>
<tr>
<td>Catenacci et al, 1984</td>
<td>Acrylic fibre plant</td>
<td>54</td>
<td>3 - 6ppm</td>
<td></td>
<td>No evidence of liver damage</td>
</tr>
<tr>
<td>Chen et al, 1988</td>
<td>Du Pont employees</td>
<td>3859</td>
<td>[Cancer study]</td>
<td>Increased cancer of buccal cavity &amp; pharynx, &amp; malig't melanoma</td>
<td>No definite causal link with DMF exposure</td>
</tr>
<tr>
<td>Chen et al, 1988</td>
<td>Du Pont employees</td>
<td>393</td>
<td>[Mortality study]</td>
<td>Increased deaths from ischaemic heart disease</td>
<td>No definite causal link with DMF exposure</td>
</tr>
<tr>
<td>Cirila et al, 1984</td>
<td>Artificial leather plant</td>
<td>100</td>
<td>8ppm</td>
<td>Eye &amp; throat irritation, liver disorders, alcohol intolerance, headaches</td>
<td>10ppm is not &quot;no adverse-effect limit&quot;</td>
</tr>
<tr>
<td>Cirila et al (follow-up)</td>
<td>&quot;</td>
<td>38</td>
<td>10ppm</td>
<td>Insomnia, irritability, liver disorders, alcohol intolerance, skin irrit'n</td>
<td>Subsidence of some symptoms, persistance of liver &amp; skin disorders, &amp; alcohol intol.</td>
</tr>
<tr>
<td>*Dilorenzo &amp; Graziooli, 1972</td>
<td>Artificial fibre plant</td>
<td>28</td>
<td>-</td>
<td>GIT disorders, leukopenia, lymphocytosis</td>
<td>-</td>
</tr>
<tr>
<td>Ducatman et al, 1986</td>
<td>Aircraft repair workshop</td>
<td>7</td>
<td>[Case study]</td>
<td>Germ cell tumours of testicle</td>
<td>Raised possible assoc'n with DMF exposure</td>
</tr>
<tr>
<td>Imbriani et al, 1986</td>
<td>Chemical factory</td>
<td>43</td>
<td>9ppm</td>
<td>Decreased platelet no., increased coagulation time</td>
<td>Altered platelet activity</td>
</tr>
<tr>
<td>Lauwers et al, 1980</td>
<td>Acrylic fibre factory</td>
<td>22</td>
<td>&lt;4ppm Inhal'n, skin</td>
<td>Alcohol intolerance</td>
<td>No signs of liver damage, alcohol intolerance common</td>
</tr>
<tr>
<td>Levin et al, 1987</td>
<td>Leather tannery</td>
<td>3</td>
<td>[Case study] &quot;High&quot;</td>
<td>Germ cell tumours of testicle</td>
<td>Possible causal link with DMF raised</td>
</tr>
<tr>
<td>Lyle et al, 1979</td>
<td>Chemical plant</td>
<td>102</td>
<td>&lt;10ppm</td>
<td>Alc.intol. and dizziness in 19/102</td>
<td>Alcohol intolerance</td>
</tr>
<tr>
<td>Paololetti &amp; Iannoccene, 1981</td>
<td>Synthetic leather factory</td>
<td>27</td>
<td>3 - 26ppm (180ppm peaks)</td>
<td>GIT disorders, alcohol intolerance, some cases of liver damage</td>
<td>-</td>
</tr>
<tr>
<td>Redlich et al, 1988</td>
<td>Polyurethane fabric coating plant</td>
<td>58</td>
<td>Poor vent'n Inhal'n, skin</td>
<td>Raised AST &amp; ALT, nausea, alc. intol., nausea, dizziness, abdominal pain, headaches</td>
<td>Liver damage common, formerly undetected</td>
</tr>
<tr>
<td>AUTHOR</td>
<td>WORKPLACE</td>
<td>NO. IN STUDY</td>
<td>LEVEL/ROUTE OF EXPOSURE</td>
<td>SYMPTOMS</td>
<td>DIAGNOSIS/REMARKS</td>
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<td>-------------------------------------------------------</td>
</tr>
<tr>
<td>Reinh &amp; Urban, 1965</td>
<td>Acrylic fibre factory</td>
<td>12 $^a$</td>
<td>&lt;20ppm</td>
<td>Alcohol intolerance, raised AST &amp; ALT, jaundice, vomiting</td>
<td></td>
</tr>
<tr>
<td>*Soldtek, 1970</td>
<td>-</td>
<td>207 $^b$</td>
<td>33ppm</td>
<td>-</td>
<td>Increased incidence of miscarriages</td>
</tr>
<tr>
<td>Taccola et al, 1981</td>
<td>Artificial leather plant</td>
<td>39 $^b$</td>
<td>10 - 50ppm</td>
<td>-</td>
<td>No effect on cardio-circulatory system</td>
</tr>
<tr>
<td>Tomasini et al, 1983</td>
<td>Artificial leather factory</td>
<td>14 $^a$</td>
<td>5 - 20ppm</td>
<td>Alc. intol., nausea, raised AST &amp; ALT, liver tenderness, eye &amp; URT irritation</td>
<td>Symptoms subsided</td>
</tr>
<tr>
<td>*Wink (1970)</td>
<td>Synthetic rubber plant</td>
<td>30 $^a$</td>
<td>-</td>
<td>Headache, nausea, dizziness, alcohol intolerance</td>
<td>Decrease in steroid levels</td>
</tr>
</tbody>
</table>

*Source: IPCS (1987)
Redlich et al, 1988; Tomasin et al, 1983). An increased incidence of hepatic disorders was found amongst 100 employees at an Italian factory inspite of the levels of airborne DMF being within the recommended limits of 10 ppm (30mg/m³) (Cirla et al, 1984). In a five-year follow-up study of 38 of the exposed workers, hepatotoxicity was still evident (Cirla et al, unpublished report). Clinical signs of liver damage were detected in 62% of a group of 58 employees in a polyurethane-coated fabric manufacturing plant (Redlich et al, 1988). Elevated levels of ALT and AST were measured and in some individuals these were five times the normal value. The factory had poor safety practices with inadequate ventilation and little or no skin protection for exposed workers. Levels of DMF were not obtained in this study. An important feature of this particular study was that prior to the investigation, hepatotoxicity amongst the workforce had largely gone undetected.

In contrast to these reports, a number of other studies of workers exposed to DMF have suggested that airborne concentrations which are below 10 ppm (30mg/m³) present no increased risk of liver disorders (Catenacci et al, 1984; Lauwerys et al, 1980).

Other health effects associated with DMF exposure which have been commonly reported include gastrointestinal disorders, irritation of the eyes and upper respiratory tract, dizziness, headaches, cardiac troubles, chest
tightness and alcohol intolerance. Alcohol intolerance is a particularly common reaction in workers exposed to DMF (Tolot et al, 1958; Chivers, 1978; Lyle et al, 1979; Lauwerys et al, 1980; Paoletti and Iannaccone, 1982; Tomasini et al, 1983; Cirla et al, 1983; Redlich et al, 1988). Chivers (1978) describes a classic case of alcohol intolerance in a maintenance fitter who was exposed to DMF. On the consumption of alcohol at lunchtime the same day, the individual developed reddening of the face and tightness in the chest. These symptoms reoccurred after drinking more alcohol in the evening. Acting on medical advice, the individual's alcohol intake was decreased for a week and his symptoms subsequently subsided. In another study 19 out of a group of 102 employees reported a similar reaction after consuming alcohol (Lyle et al, 1979). Flushing generally affected the face, neck, hands, arms and chest, and was sometimes accompanied by dizziness.

In the last few years attention has focussed on the potential carcinogenicity of DMF in humans. Clusters of testicular germ cell tumours were found amongst employees in two aircraft repair workshops (Ducatman et al, 1986). Although workers were exposed to a whole range of chemicals, the unique process used at the two workshops led the authors to suggest DMF involvement in the aetiology of the cancers. Three cases of germ cell tumours of the testicle were also found amongst workers at a leather tannery (Levin et al, 1987). The relatively
high levels of DMF in the stage of the process where the individuals were working, with little ventilation and no skin protection, led the authors to raise the possibility of DMF involvement.

The association between DMF and testicular cancer has been disputed (Chen and Kennedy, 1988) on the basis of the results of an extensive epidemiological study. The study, conducted by the Medical Division of Du Pont, investigated cancer incidence amongst 3859 employees (Chen et al, 1988). No increased incidence of testicular cancer was observed. However, the study reported an increased incidence of buccal and pharyngeal cancer and malignant melanoma in workers exposed to DMF compared to a control population. An increased incidence of prostate cancer was found in workers exposed to both DMF and acrylonitrile. On the basis of a lack of association between cancer incidence and the duration and level of exposure to DMF, the authors avoided drawing any conclusions on the role of DMF in these cancers.

In the light of these reports the potential for DMF to cause cancer has recently been assessed by the International Agency for Research on Cancer (IARC). After reviewing all available evidence, the overall evaluation was that "DMF is possibly carcinogenic to humans" and was thus classified as a Group 2B carcinogen (IARC, 1989).
A wide range of health effects are associated with occupational exposure to DMF. Some of these effects are clearly recognised such as its ability to elicit liver damage and the association with alcohol intolerance. Others, primarily its potential to cause cancer, are the subject of some debate and continuing investigation. The studies presented in this thesis focus on the hepatotoxic properties of DMF.
1.3 EFFECTS OF DMF ON EXPERIMENTAL ANIMALS

Widespread use of DMF in industry and reports of adverse effects in exposed workers have prompted the need to investigate its toxicity in laboratory animals. A summary of some of the animal studies which have been conducted is shown in Table 1.4 (for review see Scailteur and Lauwerys, 1987).

In general the animal studies which have been conducted have verified the liver as the main target for DMF toxicity. The concentrations of DMF to which animals have been exposed have in many cases been high. Fischer rats and B6C3F1 mice, for instance, exposed to 1200ppm DMF for periods of up to 12 weeks incurred liver damage (Craig et al, 1984). The severity of the damage was found to be dose-related and it was concluded that the no-effect dose of DMF for mice and rats was below 150ppm. Hepatotoxic effects were demonstrated in cats and rabbits after acute exposure, and in rats and cats chronically exposed to 100, 230 or 450ppm DMF (Massman, 1956). Elevated plasma levels of sorbitol dehydrogenase (SDH), an indicator of damaged liver cells, were measured in Sprague Dawley rats which received either 240 or 479 mg/kg DMF ip (Lundberg et al., 1981). SDH increases were found to correlate well with liver damage observed at histopathological examination. It was noted in this study that the onset of hepatotoxicity was delayed in the case of the higher dose. In accordance with this finding, levels of plasma SDH were increased in rats 16hr after exposure to 565ppm.
<table>
<thead>
<tr>
<th>AUTHOR</th>
<th>SPECIES/STRAIN</th>
<th>SEX</th>
<th>ROUTE</th>
<th>DOSE</th>
<th>FINDINGS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antoine et al, 1983</td>
<td>BALB/c mice</td>
<td>♀</td>
<td>ip</td>
<td>0.2mg/kg, 20mg/kg or 2g/kg x 1</td>
<td>No detection of sperm abnormalities</td>
</tr>
<tr>
<td>Brondeau et al, 1983</td>
<td>S.D.rat</td>
<td>♀</td>
<td>Inhal'n</td>
<td>126ppm x 4hr, 126ppm x 6day</td>
<td>Liver damage - raised SDH and GDH</td>
</tr>
<tr>
<td>Clayton et al, 1963</td>
<td>Rat, mouse, g.pig, rabbit</td>
<td>♂ + ♀</td>
<td>Inhal'n</td>
<td>23ppm + peaks - chronic exposure</td>
<td>Hepatoxicity, cardiovascular changes (dogs), other organs damaged</td>
</tr>
<tr>
<td>Craig et al, 1984</td>
<td>Fischer rat, B6c3F1, mouse</td>
<td>♂ + ♀</td>
<td>Inhal'n</td>
<td>Up to 1200ppm - chronic exposure</td>
<td>Liver damage - rat at 1200ppm; mouse at all doses</td>
</tr>
<tr>
<td>Itch et al, 1987</td>
<td>F344 rat</td>
<td>♂ + ♀</td>
<td>Oral</td>
<td>0.75 or 1.0ml/kg weekly x 12weeks</td>
<td>Liver damage - histological and clinical signs, centrilob.hernosis</td>
</tr>
<tr>
<td>Kennedy et al, 1986</td>
<td>Crl:CD rat, CD-1 mouse, NZ white rabbit</td>
<td>♂ + ♀</td>
<td>Various</td>
<td>Acute and sub-chronic dosing</td>
<td>Eye irritant, weight loss, lung damage, liver damage (acute &amp; chronic dosing)</td>
</tr>
<tr>
<td>Kimmerle &amp; Machemer, 1975</td>
<td>FB rat</td>
<td>♂ + ♀</td>
<td>Inhal'n</td>
<td>18 to 172ppm x 10day during gestation</td>
<td>Some weight reduction in foetuses at high dose; concluded that DMF is not teratogenic in rats</td>
</tr>
<tr>
<td>Llewellyn, 1984</td>
<td>Gerbils</td>
<td>♂ + ♀</td>
<td>Dermal</td>
<td>1 or 2 applications per week x 35day</td>
<td>No observed organ damage</td>
</tr>
<tr>
<td>Lundberg et al, 1981</td>
<td>S.D.rat</td>
<td>♂</td>
<td>ip</td>
<td>DMF 240 or 479mg/kg, other formamides</td>
<td>Liver damage - raised SDH, onset delayed at higher dose; histol. damage; NMF more toxic than DMF or F</td>
</tr>
<tr>
<td>Lundberg et al, 1983</td>
<td>S.D.rat</td>
<td>-</td>
<td>Inhal'n</td>
<td>565 or 2250ppm</td>
<td>Increased SDH, onset delayed at higher dose</td>
</tr>
<tr>
<td>Massman, 1956</td>
<td>Mouse, cat, rat, rabbit</td>
<td>-</td>
<td>Various</td>
<td>Acute and chronic exposure</td>
<td>Liver damage with both exposures</td>
</tr>
<tr>
<td>Mathew et al, 1980</td>
<td>Albino rat</td>
<td>♂</td>
<td>ip</td>
<td>0.6 to 1.2ml/kg - single dose</td>
<td>Liver damage - raised enzymes, histotologial damage</td>
</tr>
<tr>
<td>*Scailletteur, 1984</td>
<td>Rat</td>
<td>-</td>
<td>ip</td>
<td>0.5 or 1.0ml/kg - x 1 or x 4</td>
<td>No increased SDH with single dose, increased SDH with repeated doses</td>
</tr>
<tr>
<td>Scailletteur et al, 1981</td>
<td>S.D.rat</td>
<td>♂ + ♀</td>
<td>ip</td>
<td>DMF up to 2ml/kg, other formamides</td>
<td>Toxicity - NMF &gt; DMF &gt; F; sex difference</td>
</tr>
<tr>
<td>Scailletteur &amp; Lauwerys, 1984a</td>
<td>S.D.rat</td>
<td>♂</td>
<td>ip</td>
<td>Up to 2.5ml/kg - x 1 or x 4</td>
<td>Toxicity - NMF &gt; HMNF &gt; DMF</td>
</tr>
<tr>
<td>AUTHOR</td>
<td>SPECIES/STRAIN</td>
<td>SEX</td>
<td>ROUTE</td>
<td>DOSE</td>
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<td>---------------------------------------------------</td>
</tr>
<tr>
<td>*Spinnazola et al, 1969</td>
<td>Rabbit</td>
<td>-</td>
<td>sc</td>
<td>2ml/kg - single dose</td>
<td>Liver damage - raised AST, ALT and SDH</td>
</tr>
<tr>
<td>Stula &amp; Kraus, 1977</td>
<td>S.D.rat, NZ white rabbit</td>
<td>♀</td>
<td>Dermal</td>
<td>During pregnancy</td>
<td>Embryotoxicity found but only at doses which killed dam</td>
</tr>
<tr>
<td>Tanaka, 1971</td>
<td>S.D.rat</td>
<td>♀</td>
<td>Inhal'n</td>
<td>200ppm daily x 4week</td>
<td>Liver damage - younger animals more susceptible</td>
</tr>
<tr>
<td>Ungar et al, 1976</td>
<td>Syrian hamster</td>
<td>♂</td>
<td>ip</td>
<td>0.5 or 1.0ml/kg - single dose</td>
<td>Liver damage and death</td>
</tr>
</tbody>
</table>

* Source: Scailleur and Lauwerys, 1987
DMF but not after exposure to 2250ppm (Lundberg et al, 1983). Raised plasma levels of SDH and glutamate dehydrogenase (GDH) were also demonstrated in this strain of rat after exposure to 126ppm DMF for short periods (Brondeau et al, 1983). However, lower concentrations of DMF (66ppm) did not cause hepatotoxicity.

One study was conducted which was aimed at reproducing realistic DMF workplace concentrations, consisting of continuous low levels plus occasional peak exposures (23ppm for 5.5hr plus 426ppm for 0.5hr each day for 58 days) (Clayton et al, 1963). Rats, mice, guinea pigs, rabbits and dogs were found to have damaged livers on histopathological examination, although clinical signs of toxicity were absent. It was concluded that workplace exposure should be limited to 20ppm (60mg/m³).

Histopathological examination of liver tissue damaged by DMF shows affected tissue to be mainly located in the centrilobular region (Tanaka, 1971; Ungar et al, 1976; Mathew et al, 1980; Itoh et al, 1987). The severity of damage depended on dose and ranged from mild degenerative changes with cloudy swelling and some fatty change (Tanaka, 1971) to extensive coagulative necrosis with pigment deposition and massive fibrosis (Itoh et al, 1987). Regression of hepatic lesions was evident during longer periods of exposure (Mathew et al, 1980; Tanaka, 1971).
Apart from the liver, only a few studies have reported other organs to be affected by DMF. Histological examination revealed some damage to the kidneys, spleen, pancreas, adrenals, thymus and heart in a number of animal species exposed to DMF (Clayton et al, 1963). Some lung and kidney damage was observed in animals exposed to DMF vapours (Massman, 1956). The reproductive toxicity of DMF has been examined in a number of studies (Kimmerle and Machemer, 1975; Merkle and Zeller, 1980; Stula and Kraus, 1977; Thiersch, 1962) and has been reviewed by Barlow and Sullivan (1982). The review article concludes that DMF is non-teratogenic and only embryolethal at doses which cause maternal toxicity.

Liver damage is clearly the main feature of DMF toxicity in animals. However, high concentrations are generally required for DMF to elicit its hepatotoxic effects and a comparison with some other N-alkylformamides (see Section 1.5) leads to the conclusion that in experimental animals DMF exerts marginal hepatotoxicity.
1.4 ABSORPTION, METABOLISM AND EXCRETION OF DMF AND OTHER N-ALKYLFORMAMIDES

An understanding of the metabolism of a drug or an industrial chemical in biological systems can satisfy several objectives. Elucidating the metabolic pathways may provide an insight into the biological activities of the compound. With drugs these may be unwanted toxic effects or desirable medicinal properties. The ultimate aim in understanding the metabolism of drugs is to minimise the former while enhancing, or at least not reducing, the latter (Nelson, 1982). In the case of industrial chemicals, knowledge of its metabolic fate can explain biological effects and enable the development of an appropriate biological monitoring system for assessing human exposure in the workplace (Bernard and Lauwerys, 1986).

Several early experiments using animal models concluded that N-methylformamide (NMF) was a major metabolite of DMF, appearing in the blood and then the urine shortly after the beginning of exposure (Barnes and Ranta, 1972; Kimmerle and Eben, 1975a; Scailteur et al, 1981). Barnes and Ranta (1972) suggested that measurement of urinary NMF in humans could provide a means of quantifying exposure to DMF. This method was indeed applied in several studies of human exposure to DMF which will be described later in this section.
In addition to NMF, another metabolite, formamide (F), was found at low concentrations in the urine of rats and dogs following inhalation of DMF (Kimmerle and Eben, 1975a).

Subsequent investigations suggested that the metabolites considered to be NMF and F were in fact their N-hydroxymethyl analogues, N-hydroxymethyl-N-methylformamide (HMMF) and N-hydroxymethylformamide (HMF) respectively (Brindley et al, 1983; Scailteur et al, 1984; Scailteur and Lauwerys, 1984a). The compound previously identified as NMF was in fact thought to be the decomposition product of the actual metabolite HMMF which, at the high temperatures required by GLC analysis, breaks down to liberate formaldehyde. The identity of HMMF, formed by C-hydroxylation of DMF, was confirmed by high field ¹H-NMR spectroscopy in the urine of CBA/CA mice injected with DMF (Kestell et al, 1986a) and by chemical ionisation mass spectrometry (Scailteur et al, 1984). On the basis of studies which measured formaldehyde-release after alkaline hydrolysis, F was likewise assumed to be the decomposition product of the metabolite HMF (Brindley et al, 1983; Scailteur et al, 1984; Scailteur and Lauwerys, 1984a).

The existence of NMF as a metabolite of DMF has been confirmed, although quantitatively it is a minor urinary metabolite compared to HMMF (Scailteur and Lauwerys, 1984a). By employing the simultaneous analytical methods
of GLC (for the detection of HMMF and NMF combined) and colorimetric assay of formaldehyde-release after alkaline hydrolysis (HMMF only), 4% of the dose administered to rats was found to be excreted in the urine as NMF, compared to 50% as HMMF. The metabolic pathways leading to the formation of NMF and HMMF were considered to be independent of each other.

For the remainder of this thesis, the urinary metabolites of DMF which are measured by GLC as NMF and F will be referred to as "NMF" and "F" respectively.

Methylamine and dimethylamine have been identified as minor metabolites of DMF in the urine of CBA/CA mice (Kestell et al, 1986a). Although these two alkylamines are known to be endogenous urinary constituents, their elevated concentration in urine samples enabled quantification by GLC and each was found to constitute about 4% of the administered DMF.

In agreement with animal studies, quantitatively the most important urinary metabolite of DMF in exposed humans was found to be "NMF" (Maxfield et al, 1975; Kimmerle and Eben, 1975b; Krivanek et al, 1978; Catenacci et al, 1980; Lauwerys et al, 1980; Yonemoto and Suzuki, 1980). Small amounts of "F" were also measured in the urine of volunteers exposed to 26 or 87 ppm DMF for 4hr, with unchanged DMF excreted only at the higher dose (Kimmerle and Eben, 1975b).
More recently studies of humans exposed to DMF have led to the discovery of another urinary metabolite. Humans exposed to DMF were found to have increased concentrations of urinary thioethers compared to a non-exposed group (Bardodej et al, 1985). The thioethers measured by a non-specific colorimetric method were considered to be mercapturic acids. The identity of this sulphur-containing metabolite was confirmed by NMR spectroscopy and mass spectrometry as N-acetyl-S-(N-methylcarbamoyl)cysteine (AMCC) following its isolation from the urine of a volunteer who had inhaled DMF (Mraz and Turecek, 1987). AMCC has been confirmed as a urinary metabolite of another N-alkylformamide, NMF, in humans and rodents (Kestell et al, 1986b; Tulip et al, 1986; see Section 1.5). Confirmation of AMCC as a metabolite of DMF (Mraz and Turecek, 1987) has important implications with regard to understanding the toxicity of DMF. The significance of N-alkylformamide metabolism to N-(alkylcarbamoyl)mercapturates is reported in Section 1.5.

Animal studies have shown no evidence that DMF is metabolised to AMCC, although the presence of unidentified metabolites in the urine of rodents has been reported (Brindley et al, 1983; Scailteur and Lauwerys, 1984a).

Following absorption, DMF is rapidly metabolised and eliminated in the urine of experimental animals. The liver has been confirmed as the main site for
biotransformation of DMF. Rats that had undergone partial hepatectomy excreted significantly more DMF in the unchanged form and less as "NMF" and "F" (Scailteur et al, 1984). Unchanged DMF was detectable in the blood of rats and dogs which had inhaled DMF vapours for only a few hours after the end of exposure and very little was excreted unchanged in the urine (Kimmerle and Eben, 1975a). In this study most of the administered dose was eliminated within 24hr of exposure. DMF administered to CBA/CA mice by ip injection was similarly rapidly excreted (Brindley et al, 1983). Unchanged DMF rapidly disappeared from the blood and over 80% of the radiolabelled dose was recovered from the urine within 24hr. Less than 5% of the dose was excreted unchanged. Sprague Dawley rats excreted more than 80% of the dose within 48hr and 90% within 72hr of treatment (Scailteur and Lauwerys, 1984a). Only 15% of the dose was excreted unchanged in this study.

Investigations of the time course of DMF metabolism in human volunteers have reported a similarly extensive biotransformation and rapid excretion (Kimmerle and Eben, 1975b; Krivanek et al, 1978; Maxfield et al, 1978; Yonemoto and Suzuki, 1980). "NMF" appeared in the urine of volunteers who had inhaled DMF vapours during a single 4hr period or repeatedly for 5 days within 4hr and most of the dose was excreted within 24hr (Kimmerle and Eben, 1975b). Unchanged DMF was detectable in the blood only at higher doses.
The extent of DMF metabolism in both humans and animals has been shown to be influenced by a number of factors. High concentrations of DMF, for example, appears to inhibit its own metabolism resulting in the excretion of more unchanged DMF and less "NMF" (Kimmerle and Eben, 1975b; Lundberg et al, 1983). These findings correlate with the delayed onset of hepatotoxicity that was observed in rats treated with 479mg/kg DMF compared to those treated with 240mg/kg (see Section 1.3; Lundberg et al, 1981). Another study reported a similar correlation between the inhibition of DMF metabolism at high doses and a decrease in toxicity (Scailteur et al, 1984).

The speed with which metabolites appeared in the urine of volunteers was found to be influenced by the route of exposure (Maxfield et al, 1975). Percutaneous absorption of DMF vapour caused metabolites to be excreted later than when skin absorption of liquid DMF occurred or when DMF was inhaled.

Co-administration of ethanol has been shown to inhibit the metabolism of DMF in volunteers and experimental animals (Eben and Kimmerle, 1976; Yonemoto and Suzuki, 1980). The administration of ethanol to rats and dogs prior to their exposure to DMF resulted in elevated blood levels of unchanged DMF and a delayed appearance of metabolites (Eben and Kimmerle, 1976). In the same study DMF similarly inhibited the metabolism of ethanol. The consumption of alcohol by workers exposed to DMF caused
the excretion of "NMF" to be delayed (Yonemoto and Suzuki, 1980). These studies reflect the interaction between DMF and alcohol which has frequently been reported in workers (see Section 1.2). This interaction is thought to involve altered activities of the enzymes which are required for the metabolism of ethanol and its metabolite acetaldehyde (Elovaara et al., 1983; Sharkawi, 1979). The characteristic alcoholic flush, reported in Section 1.2, is suggested as resulting from an accumulation of acetaldehyde in the blood (Hanasono et al., 1977).

Species difference in the excretion of DMF was reported by Kimmerle and Eben (1975a). Dogs exposed to DMF by inhalation retained "NMF" in the blood and urine for longer periods than rats exposed to the same concentrations. The same experiment also demonstrated sex differences in the rate of metabolism and excretion of DMF, and this has been supported by a number of other studies (Eben and Kimmerle, 1976; Scailteur et al., 1984). Different rates of metabolism in male and female rats has been reflected by differential toxicity of DMF. Male rats were more susceptible than females to DMF toxicity as demonstrated by raised GDH and SDH enzyme levels, and mortality data (Scailteur et al., 1981; Scailteur et al., 1984).

Inhalation of DMF vapours and percutaneous absorption are the main routes of exposure for humans in the occupational
setting. Skin absorption of DMF vapours or liquid has been shown to contribute significantly to the total body burden in humans (Lauwerys et al, 1980; Maxfield et al, 1975). Several studies of human exposure have been conducted with the aim of developing a biological monitoring assay which might reflect human absorption of DMF more accurately than measurement of airborne concentrations (Bardodej et al, 1985; Catenacci et al, 1980; Kimmerle and Eben, 1975b; Krivanek et al, 1978; Lauwerys et al, 1980; Maxfield et al, 1975; Mraz and Turecek, 1987; Yonemoto and Suzuki, 1980). Many of these studies have recommended the measurement of urinary levels of "NMF" as an indicator of exposure to DMF, since this has been found to be the major metabolite of DMF. The metabolism of DMF to "NMF" in humans has been shown to be affected by several factors, such as route of exposure and individual variability (Lauwerys et al, 1980; Maxfield et al, 1975), duration and level of exposure (Kimmerle and Eben, 1975b; Krivanek et al, 1978) and alcohol consumption (Yonemoto and Suzuki, 1980). The timing of urine collection has been observed as being particularly crucial in evaluating DMF exposure and it was noted in one study that "values obtained from single urine samples could produce a completely false picture (of exposure)" (Kimmerle and Eben, 1975b).

Investigations of DMF metabolism in animals and humans have shown that DMF is rapidly metabolised and excreted in the urine, mainly as "NMF", some "F" and at high doses
unchanged DMF. AMCC has been identified as a metabolite of DMF in human urine. Studies of human metabolism have led to the recommendation that measurement of urinary levels of "NMP" provides a useful biological monitoring method in the assessment of exposure to DMF. However, the limitations of this method have been noted.
1.5 THE RELATIONSHIP BETWEEN METABOLISM AND TOXICITY OF DMF AND OTHER N-ALKYLFORMAMIDES

The requirement for metabolic activation is a recognised feature of the toxicity of many chemicals. Examples of drugs and industrial chemicals which are now known to cause adverse effects in humans and animals as a result of their metabolism to reactive compounds include paracetamol, chloroform, halothane, carbon tetrachloride and bromobenzene (Nelson, 1982; Monks and Lau, 1988).

Metabolic studies, reported in Section 1.4, have provided evidence which indicates that the hepatotoxic properties of DMF may be associated with its biotransformation. DMF is rapidly metabolised; the liver is the main site of biotransformation and is also the main target for toxic effects; variation in the extent of metabolism, for instance as a result of sex differences, is matched by variation in the severity of toxicity.

Early reports which suggested that NMF was a major metabolite of DMF provided a convenient explanation for DMF's toxicity, as NMF was recognised as a hepatotoxin. However, it did not explain why NMF was more hepatotoxic than DMF, nor why DMF displayed poor anti-neoplastic activity compared to NMF. Confirmation of HMMF as the actual metabolite, not NMF, reopened the case for examining the relationship between metabolism and toxicity of DMF.
With NMF and HMMF being confirmed as minor and major metabolites of DMF respectively, their relative toxicities in rats were compared with DMF itself (Scailteur and Lauwerys, 1984a). The metabolism of HMMF was also investigated and compared with that of DMF. Results from acute toxicity studies (plasma enzyme levels, pentobarbital sleeping time and mortality) enabled them to be ranked in the following order of toxicity: \( \text{NMF} > \text{HMMF} > \text{DMF} \). In contrast to NMF, neither DMF or HMMF caused depletion of hepatic GSH levels. Although HMMF was more toxic than DMF, it was found to be stable enough to be excreted mainly unchanged. The involvement of HMMF in the hepatotoxicity of DMF was therefore considered unlikely.

The identification of AMCC as a urinary metabolite of DMF in humans has confirmed the existence of a metabolic pathway for DMF previously unsuspected (Mraz and Turecek, 1987). Mercapturic acids, such as AMCC, are end-products of a metabolic pathway which involves the conjugation of endogenous glutathione (GSH) with potentially toxic, electrophilic compounds (Chasseaud, 1976). Evidence of such metabolites in the urine is thus indicative of the generation of reactive species. The metabolism of DMF to AMCC in humans may well be a bioactivation pathway, particularly in the light of the mechanism which has been proposed for N-alkylformamide toxicity by Kestell et al (1987). This proposal is outlined later in this section.
Much of the available information on the mechanisms of toxicity of N-alkylformamides has originated from studies on NMF. The toxicity of NMF has received much attention in view of interest in this compound as an anti-neoplastic agent (Gescher et al, 1982; Langdon and Hickman, 1987; Spremulli and Dexter, 1984). In patients undergoing clinical trials it has been shown to cause liver damage (Ettinger et al, 1985; McVie et al, 1984; Murphy et al, 1987; Myers et al, 1956; Planting et al, 1987). Animal studies have endorsed these findings (Langdon et al, 1985; Pearson et al, 1987a; Tulip and Timbrell, 1988; Whitby et al, 1984). In addition, the animal studies have revealed strain and species variation in susceptibility to NMF-induced liver damage. Elevated plasma levels of ALT, AST and SDH demonstrated BALB/c mice to be more vulnerable to hepatotoxic effects than BDF₁ mice (Langdon et al, 1985) or CBA/CA mice (Pearson et al, 1987a). BALB/c mice were similarly more susceptible to NMF-induced liver injury than Sprague Dawley rats (Tulip and Timbrell, 1988).

Investigations of NMF toxicity clearly demonstrate the involvement of metabolic activation. The onset of NMF-induced hepatotoxicity in mice was delayed for about 10 hr after dosing (Whitby et al, 1984). Treatment of animals with NMF caused depletion of hepatic GSH levels, the extent of which was paralleled by the severity of toxicity in different strains of mice (Pearson et al, 1987a) and in different species (Tulip and Timbrell,
Furthermore, modulation of GSH status by butathionesulphoximine (Tulip and Timbrell, 1988) or N-acetylcysteine caused exacerbation or reduction of hepatotoxicity respectively (Pearson et al, 1987a). Radiolabelled metabolites of NMF were shown to covalently bind to hepatic macromolecules, and the extent of covalent binding in three strains of mice correlated with differences in their susceptibility to liver damage (Pearson et al, 1987b). Covalent binding is an event which is indicative of the formation of reactive metabolites (Nelson, 1982).

Similar to DMF, NMF is rapidly metabolised and excreted mainly in the urine, although some is exhaled as CO₂ (Brindley et al, 1982; Kestell et al, 1985b). The urinary metabolites of NMF are methylamine, HMF and AMCC. Neither methylamine (Kestell et al, 1985a) or HMF (Cooksey et al, 1983) appear to be involved in NMF toxicity.

AMCC, accounting for about 16% of the administered dose in mice (Kestell et al, 1985b), was identified in the urine of rodents and patients which had received NMF (Kestell et al, 1986b; Tulip et al, 1986). Several compounds related to NMF, including DMF, were investigated for their ability to be metabolised to their corresponding N-(alkylcarbamoyl) mercapturates in CBA/CA mice (Kestell et al, 1987). Only NMF and N-ethylformamide (NEF), which were the most hepatotoxic of the compounds studied, were biotransformed to N-(alkylcarbamoyl)
mercapturates. The authors proposed that two metabolic pathways existed for N-alkylformamides — one, involving hydroxylation of the N-alkyl group, acted as a detoxification pathway; the second route, proceeding via the oxidation of the formyl group and leading to the excretion of thioethers, was a toxification pathway. The ability of N-alkylformamides to be metabolised by the latter pathway was suggested as accounting for their differential hepatotoxicity.

Prior to the study of Mraz and Turecek (1987), reported in Section 1.4, there was no evidence that DMF was metabolised to AMCC to any detectable extent in any species. Accordingly, in contrast to NMF and NEF, DMF failed to deplete hepatic GSH levels and demonstrated little toxicity in isolated hepatocytes (Shaw et al, 1988). NMF and NEF caused hepatotoxicity in CBA/CA mice at doses of 200mg/kg and 600mg/kg respectively, whereas up to 3g/kg DMF was non-hepatotoxic (Kestell et al, 1987). The low toxicity of DMF compared to that of NMF and NEF was in accordance with the apparent lack of its metabolism to AMCC.

Metabolic studies using deuterium-labelled NMF have provided firm evidence that metabolic activation of NMF involves oxidation of the formyl moiety, and that AMCC is a metabolic end-product of this bioactivation pathway (Threadgill et al, 1987). In addition, S-(N-methylcarbamoyl)glutathione (SMG), the metabolic
precursor of AMCC was unequivocally identified in this study in the bile of treated BALB/c mice.

The hepatotoxicity of monoalkylformamides, particularly NMF and NEF, appears to be associated with their metabolism, and the biotransformation pathway leading to the excretion of N-(alkylcarbamoyl)mercapturates seems to be involved. The discovery of this metabolic pathway in humans exposed to DMF (Mraz and Turecek, 1987) indicates that DMF may be metabolically activated in the same manner. At the time of commencing this study, the biotransformation of DMF to AMCC had not been observed in experimental animals.
Chemical structures of some N-alkylformamides and their derivatives.
1.6 AIMS OF THIS STUDY

Investigations of the toxicity of DMF in occupationally-exposed humans and in experimental animals, described in Sections 1.2 and 1.3, have demonstrated a wide range of effects of this compound on health. An important feature of the toxicity of this industrial solvent is its ability to cause liver damage, which it shares with certain other N-alkylformamides. The hepatotoxic properties of DMF are the subject of the studies reported here.

Evidence that has been accumulated on DMF and other N-alkylformamides (see Section 1.4 and 1.5) suggests that the hepatotoxicity of these compounds is linked to their metabolism. The metabolic pathway of N-alkylformamides which involves formyl oxidation and glutathione conjugation and leads to the excretion of urinary mercapturates is now thought to be a bioactivation pathway. The existence of this metabolic route has been confirmed in rodents and humans treated with NMF and in mice dosed with NEF. Recently AMCC has also been detected in the urine of humans exposed to DMF. It is therefore possible that DMF and other N-monoalkylformamides undergo a similar metabolic activation which results in their hepatotoxic properties. So far, however, the metabolic pathway leading to the urinary excretion of mercapturates has not been identified in experimental animals exposed to DMF.
The first part of this investigation concentrates on the *in vivo* metabolism of DMF. Rodents are commonly used to study the metabolism of chemicals, and results from these studies may contribute significantly to the understanding of toxic effects in humans and in assessing the health risks for humans. Nevertheless, frequently there are differences between experimental animals and humans in the biological fate of a chemical which make extrapolation of animal data to humans difficult (Travis, 1987). It is therefore sensible to identify these differences so that they may be taken into consideration when results are extrapolated to assess the risk to humans. They may also help to select an animal model suitable in predicting toxic features.

The *in vivo* metabolic fate of DMF was investigated in a range of rodent models with the objective of identifying common and contrasting features compared to the qualitative and quantitative metabolism of DMF in humans. Attention was specifically focussed on whether or not and to what extent rodents are able to metabolise DMF to AMCC via the metabolic pathway which has been implicated in the hepatotoxicity of N-alkylformamides and which has been confirmed in humans.

A quantitatively major urinary metabolite of DMF in animals and in humans is now known to be the carbinolamine HMMF (see Section 1.4). Indeed, concentrations of this metabolite in the urine, measured
by GLC as NMF, are used as a biological monitor in the
determination of the extent of occupational exposure to
DMF. The properties of this metabolite have hitherto
received little attention and its involvement in the
toxic effects of DMF remain unknown. The in vivo
metabolic fate of HMMF was therefore examined in rodents
with the objective of establishing its role in the
hepatotoxicity of DMF.

The existence of the proposed bioactivation pathway
leading to the urinary excretion of S-(N-
alkylcarbamoyl)mercapturates has been confirmed in
several species exposed to N-alkylformamides (see Section
1.5). However, the precise mechanisms involved in this
metabolic route and the chemical nature of any
intermediate(s) formed remain to be elucidated.

The second part of this study was aimed at addressing
this issue. Using in vitro methods as a means of
focussing on a particular metabolic pathway, the
metabolism of N-alkylformamides was studied in hepatic
microsomes. Specifically, the proposed bioactivation
pathway was investigated, using the formation of S-(N-
methylcarbamoyl)glutathione (SMG) as an appropriate
endpoint. The objectives of these investigations was to
establish the metabolic steps involved in the
"bioactivation" pathway from the parent N-alkylformamide
to the formation of SMG, to clarify the enzymatic nature
of this pathway, and to provide more information which
information which might contribute to the identification of the reactive intermediate(s) considered to be responsible for the hepatotoxicity of these compounds.

It is hoped that information gained from these in vivo and in vitro studies will contribute not only to the general body of knowledge in the area of xenobiotic metabolism, but also to the understanding of the mechanisms of hepatotoxicity of N-alkylformamides, more particularly of the industrial solvent DMF. Extending the knowledge of the metabolism of industrial chemicals will, hopefully, lead to the development of improved methods of biological monitoring, increased accuracy in assessing health risks resulting from occupational exposure, and ultimately to minimising adverse health effects in humans.
SECTION 2

ANIMALS AND MATERIALS
2.1 ANIMALS
Two strains of mice were used in these studies, BALB/c (18 to 25g) and CBA/CA (20 to 25g). The two other rodent species studied were Sprague-Dawley rats (235 to 300g) and Syrian hamsters (100 to 130g). Only male animals were used. Animals were purchased from Bantin and Kingman, Hull and were supplied with food (Rat and Mouse Breeding Diet, Pilsbury's Ltd.) and water ad libitum.

2.2 N-ALKYLFORMAMIDES AND THEIR ANALOGUES
DMF and F (ACS grade) were purchased from Sigma Chemical Company Ltd., UK. NMF was purchased from Aldrich Chemical Company Ltd., Gillingham, UK.

HMMF had previously been synthesised by A.Gledhill, Aston University.

AMCC and SMG were synthesised and kindly provided by Dr D-H Han, University of Washington, Seattle. These compounds were synthesised according to the method described by Han et al (1989).

2.3 RADIOLABELLED CHEMICALS
Di[\(^{14}\)C]methylamine hydrochloride, which was used for the synthesis of D[\(^{14}\)C]MF, was purchased from Amersham International plc., UK.
[\textsuperscript{14}C]-Methylamine hydrochloride was used in the synthesis of HM[\textsuperscript{14}C]MF and was also purchased from Amersham International plc., UK.

Other chemicals required for the syntheses were supplied as follows:

Dimethylamine hydrochloride and methylamine hydrochloride (Aldrich Chemical Company Ltd., Gillingham, UK)

Ethyl formate and dichloromethane (BDH Chemicals Ltd., Poole, UK)

Sodium metal and sodium hydroxide (Fisons plc, Loughborough, UK)

Potassium carbonate (anhydrous; Sigma Chemical Company Ltd., UK)

Paraformaldehyde (Hopkin and Williams, UK).

2.4 LIQUID SCINTILLATION COUNTING

OptiPhase 'MP' scintillation fluid was purchased from Fisons plc, Loughborough, UK.

2.5 MATERIALS FOR CHROMATOGRAPHY AND AUTORADIOGRAPHY

Chromatography plates

Thin-layer chromatography (TLC) plates (DC-Plastikfolien Kieselgel, 60F_{254}, 0.2mm thickness) were purchased from E.Merck, Darmstadt.

For preparative chromatography, PLC plates (silica gel 60 coated, without fluorescent indicator, 2mm thickness, no.5745) were purchased from E.Merck, Darmstadt.
Autoradiography

Singul-XRP medical X-ray film was purchased from Ceaverken AB Strangnas, Sweden. Photographic processing chemicals, D-19 developer and Unifix fixer, were purchased from Eastman Kodak.

Solvent systems

All solvents used were supplied by BDH Chemicals Ltd., Poole, UK and were SLR grade. The following solvent systems were employed to separate metabolites:

- Chloroform/MeOH 4:1 v/v (DMF, HMMF, NMF, P)
- Toluene/ethyl acetate 2.5:1 v/v (methyl- and dimethyl-amine)
- Bu-1-OH/H$_2$O/MeOH 8:2:1 v/v (AMCC)

2.6 MATERIALS FOR SPRAY-DETECTION OF METABOLITES

Chemicals for detection of metabolites were purchased as follows:

Thioesters

5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB) and Tris Base (Sigma Chemical Company Ltd., UK); NaOH and concentrated HCl (Fisons plc, Loughborough, UK); EtOH (SLR grade; BDH Chemicals Ltd., Poole, UK).

HMMF

Concentrated sulphuric acid (Aldrich Chemical Company Ltd., Gillingham, UK); phenylhydrazine (Sigma Chemical Company Ltd., UK); ferric chloride (BDH Chemicals Ltd., Poole, UK).
2.7 CHEMICALS FOR DERIVATISATION OF SAMPLES
Materials used for derivatising metabolites prior to analysis were purchased as follows:

AMCC
Potassium carbonate (anhydrous; Sigma Chemical Company Ltd., UK); EtOH, Pr-1-OH and ethyl acetate (SLR grade; BDH Chemicals Ltd., Poole, UK).

Methyl- and dimethylanline
2,4-Dinitrofluorobenzene (DNFB; Sigma Chemical Company Ltd., UK); sodium hydrogen carbonate and sodium sulphate (Fisons plc., Loughborough, UK); diethyl ether and EtOH (SLR grade; BDH Chemicals Ltd., Poole, UK). Authentic dinitrophenylalkylamines had been previously synthesised by Dr M.D. Threadgill.

2.8 MATERIALS FOR MICROSOMAL INCUBATIONS
Chemicals used in the preparation of hepatic microsomes, in the microsomal incubations and in the assays were supplied as follows:
Phosphate buffer

Potassium dihydrogen orthophosphate (anhydrous) and di-sodium hydrogen orthophosphate (BDH Chemicals Ltd., Poole, UK)

Cofactors

The following chemicals were all purchased from Sigma Chemical Company Ltd., UK:
NADPH (reduced form; tetrasodium salt)
Glucose-6-phosphate (monosodium salt)
NADP (sodium salt)
Glucose-6-phosphate dehydrogenase (type VII; from Bakers yeast)
Glutathione (reduced form)
Magnesium chloride (MgCl₂·6H₂O; Fisons plc, Loughborough, UK)

Enzyme inhibitors

SKF 525-A (diethylaminoethyl 2,2-diphenylvalerate HCl) was donated by Smith, Kline & French Ltd., Herts., UK.
Carbon monoxide (99% pure; Aldrich Chemical Company Ltd., Gillingham, UK).

Enzyme inducers

Sodium phenobarbitone (PB; BDH Chemicals Ltd., Poole, UK)
Beta-naphthoflavone (5,6-benzoflavone; BNF; Sigma Chemical Company Ltd., UK)
Substrates
In addition to N-alkylformamides, the following substrates were used in microsomal incubations:
Aminopyrine (4-dimethylaminoantipyrine; Sigma Chemical Company Ltd., UK)
Acetanilide (donated by University of Washington, Seattle)

Lowry protein assay
Bovine serum albumin (fraction V), Folin and Ciocalteau's phenol reagent, potassium carbonate (Sigma Chemical Company Ltd., UK)
Copper sulphate, sodium potassium tartrate (Fisons plc., Loughborough, UK)

Aminopyrine N-demethylation assay
Paraformaldehyde (Hopkin and Williams, UK)
NaOH, ammonium acetate (Fisons plc., Loughborough, UK)
Trichloroacetic acid (Sigma Chemical Company Ltd., UK)
Acetyl acetone, glacial acetic acid (BDH Chemicals Ltd., Poole, UK)

Spectral determination of cytochrome P-450
Carbon monoxide (Aldrich Chemical Company Ltd, Gillingham, UK)
Sodium dithionite (Fisons plc., Loughborough, UK)
2.9 MISCELLANEOUS MATERIALS

Other chemicals were supplied as follows:

Bis(trimethylsilyl)-trifluoroacetamide (BSTFA; Sigma Chemical Company Ltd., UK)

Caracemide (provided by Department of Pharmaceutical Sciences, University of Aston)

Dimedone (5,5-dimethyl-1,3-cyclohexanedione; Sigma Chemical Company Ltd., UK)

Dimethyldichlorosilane solution (2% in 1,1,1-trichloroethane; BDH Chemicals Ltd., Poole, UK)

MeOH (Distol Solvent, containing <0.1% water; Fisons plc., Loughborough, UK)

Methyl isocyanate (Aldrich Chemical Company Ltd., Gillingham, UK)

$[^{18}\text{O}]$-gas (MO 2187; K&K-Greeff Ltd., Croydon, UK)

Quinoline (BDH Chemicals Ltd., Poole, UK)

Toluene (Scintillation grade; Fisons plc., Loughborough, UK)
SECTION 3

METHODS
3.1 SYNTHESIS OF RADIONON LABELLED COMPOUNDS

3.1.1 N,N-Di[\textsuperscript{14}C]methylformamide (D[\textsuperscript{14}C]MF)

DMF, labelled with \textsuperscript{14}C in either methyl group, was synthesised according to a published method (Threadgill and Gate, 1983) with some modifications. The synthesis was performed under the guidance of Dr M.D. Threadgill.

Di[\textsuperscript{14}C]methylamine hydrochloride (1mmol; specific activity 671uCi/mg) and unlabelled dimethylamine hydrochloride (80mg) were dissolved in methanol (3ml). Sodium methoxide was prepared by dissolving sodium metal (24mg) in methanol (0.5ml), and was added to the dimethylamine hydrochloride solution, followed by excess ethyl formate and excess anhydrous potassium carbonate. The mixture was stirred continuously in a stoppered flask at room temperature for two days. Additional ethyl formate and dichloromethane (approximately 30ml) were added to the flask. The contents of the flask were then filtered through cotton wool and rinsed several times with dichloromethane. Dichloromethane and ethyl formate were gently distilled off, leaving radiolabelled DMF. The basis of the chemical reaction involved in this synthesis is illustrated in Figure 3.1.

Radiochemical purity of the compound was confirmed by TLC, using a chloroform/methanol (4:1 v/v) solvent system, which demonstrated a single radioactive spot. Chemical purity was determined by GLC analysis which gave a single peak at the same retention time as commercial DMF.
\[ ^{14}\text{CH}_3\text{CH}_3\text{NH.HCl} + \text{Na}^+\text{OCH}_3 \]

Dil\(^{14}\text{C}\)methylamine hydrochloride \hspace{1cm} \text{Sodium methoxide}

\[ \text{HCl} \]

\[ \text{Ethyl formate} \]

\[ \text{Di}^{14}\text{CJMF} \]

Figure 3.1 Synthesis of \(^{14}\text{C}\)-methyl-labelled DMF
Specific radioactivity was determined by liquid scintillation counting (see Section 3.2) as 12.55uCi/ul.

3.1.2 N-(Hydroxymethyl)-N-[\textsuperscript{14}C]methylformamide (HM[\textsuperscript{14}C]MF)

HMNF, labelled with \textsuperscript{14}C in the methyl group, was synthesised using a modified method based on that published by Threadgill and Gate (1983). Dr M.D.Threadgill assisted with the synthesis.

The first stage of synthesis involved the formation of \textsuperscript{[14}C]-NMF. Unlabelled methylamine hydrochloride (134mg) and \textsuperscript{[14}C]-methylamine hydrochloride were dissolved in methanol (4ml). Ethyl formate (6ml) and excess potassium carbonate were added to the flask and the contents stirred for two days. The mixture was then filtered through cotton wool and rinsed twice with ethyl formate. Methanol and ethyl formate were removed by careful evaporation using a Buchi Rotavapor R110 rotary evaporator.

The remaining \textsuperscript{14}C-labelled NMF was then mixed with paraformaldehyde (60mg) and 10% sodium hydroxide solution (8ul) present as a catalyst. The mixture was heated in an oil bath (100°C) for 35min, with continuous stirring.

Radioactive impurities were demonstrated in the product by TLC autoradiography. These were removed by preparative chromatography and the purified final product gave a
single radioactive spot on a TLC autoradiograph. The compound gave a single peak on GLC analysis which corresponded to that of authentic HMMF or NMF. Specific radioactivity of the compound was determined as 6.0uCi/ul.

3.2 DETERMINATION OF RADIOACTIVITY BY LIQUID SCINTILLATION COUNTING

The radioactivity contained in samples was determined using a Packard TRI-CARB 2000CA liquid scintillation analyser.

An aliquot (100ul) of urine sample was mixed with scintillation fluid (3ml). A blank was prepared containing water (100ul) and scintillation fluid (3ml). In the case of synthesised radiolabelled compounds (Section 3.1), an aliquot of the compound appropriately diluted with water was similarly mixed with scintillation fluid. The radioactivity associated with TLC-separated metabolites was assessed by removing the relevant area of silica (see Section 3.4.4.4). The silica was mixed well with MeOH (3ml) and then mixed with scintillation fluid (10ml). In this case a blank was prepared using an unlabelled area of silica.

The $^{14}$C associated with each sample was expressed in disintegrations per minute (dpm) after correction for quenching effects. The quench curve for $^{14}$C was derived from an external standard source. Samples were
automatically adjusted for background activity, the value for which was derived from the first sample, the blank.

3.3 **TREATMENT OF ANIMALS**

3.3.1 **Dosing of animals**

Animals received all treatments by a single ip injection. The injection formulations were prepared in 0.9% sterile saline at the appropriate concentration. The injection volume was 0.1ml (mice) or 0.5ml (rats and hamsters). Syringes were weighed before and after delivery of the dose to enable the precise amount of compound administered to each animal to be determined.

3.3.2 **Collection of urine samples**

Animals were individually housed in metabolic cages (Jencons Ltd., UK; and Glass Blowing Section, Aston University) to allow collection of urine samples. The internal surface of all metabolic glassware was treated with dimethyldichlorosilane solution before each experiment to decrease adherence of urine to the glass surface. Animals were placed in cages for 24hr prior to dosing for collection of control urine samples. On dosing animals were immediately returned to cages and urine samples collected at 12-hr intervals up to 72hr. At each collection, the inside surface of each cage was rinsed with distilled water (5ml) with the objective of maximising recovery of the administered dose. Individual urine samples were transferred to vials, weighed and
stored at -20°C until analysis. Animals were transferred to clean cages at each 24hr collection.

3.4 STUDIES OF THE IN VIVO METABOLISM OF D[14C]MF

3.4.1 Dosing of animals
Mice received D[14C]MF (0.7 or 7.0mmol/kg) as described in Section 3.3.1. Each treatment group comprised six animals. The injection formulation was prepared using unlabelled DMF where necessary to attain a radioactive dose of 8 to 10uCi for each animal. The radioactivity of the injection formulation was determined as described in Section 3.2 and the exact amount of radioactivity administered to each animal calculated.

3.4.2 Collection of urine samples
Urine samples were collected as described in Section 3.3.2. At each 24-hr collection, the interior surface of each metabolic cage was thoroughly rinsed with water and the radioactivity of the cage-washings determined (see Section 3.2).

3.4.3 Excretion of radioactivity
An aliquot of each urine sample (100ul) and water (400ul) were mixed with scintillation fluid (3ml) and assessed for radioactivity as described in Section 3.2. The total radioactivity of each sample was then calculated and expressed as a percentage of the administered dose.
3.4.4 Analysis of radiolabelled metabolites

3.4.4.1 HMMF, "F" and unchanged DMF

Aliquots of each urine sample (5 or 10μl) were spotted onto TLC plates and run in a chloroform/methanol (4:1 v/v) solvent system. Autoradiographs were then prepared, the period of exposure depending on the amount of radioactivity applied to each TLC plate. The identity of each radioactive spot was confirmed by comparison with reference compounds, synthetic \(^{14}C\)-labelled DMF, and unlabelled HMMF and F. Unlabelled reference compounds were detected as follows:

(a) HMMF was detected using a method that detects HCHO-releasing compounds (Nair and Francis, 1980). Reagent A was prepared by adding phenylhydrazine (1ml) to a solution of water (70ml) and concentrated sulphuric acid (30ml). Reagent B contained ferric chloride (0.1g) dissolved in water (100ml). The TLC plate was sprayed with reagent A and allowed to dry. On spraying with reagent B, HMMF could be detected by the appearance of a yellow/pink spot.

(b) Authentic F was visualised, after conversion to hydroxamate, using ferric chloride (Kelly, 1964). Reagent A contained methanol (80ml) mixed with a solution of sodium hydroxide (3g) in water (20ml). Hydroxylamine hydrochloride was added until the solution was saturated. Reagent B contained ferric chloride hexahydrate (1g) dissolved in a solution of concentrated hydrochloric acid

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(10ml) and methanol (90ml). The TLC plate was sprayed with reagent A, allowed to dry, and sprayed with reagent B. The immediate appearance of a pink spot enabled location of F.

3.4.4.2 AMCC
Urine samples were applied to a TLC plate and developed in a Bu-1-OH/water/MeOH (8:2:1 v/v) solvent system. Radioactive urinary metabolites were investigated using TLC autoradiography. The Rf value of authentic AMCC on the TLC plate was established by a method which detects sulphydryl-containing compounds (Glaser et al, 1970). This technique was also used to investigate urinary AMCC. The solutions were prepared as follows:

**Reagent A (hydrolysis reagent)** - sodium hydroxide (8g) dissolved in a mixture of ethanol (50ml) and water (50ml).

**Reagent B (Ellman's reagent)** - a 0.1% solution of DTNB prepared in a 1:1 v/v mixture of EtOH and 0.45M Tris buffer (pH 8.2).

**Tris buffer (0.45M; pH 8.2)** - Tris base (6.057g) was dissolved in water (50ml). The pH was adjusted to 8.2 with HCl (1M) and made up to a volume of 100ml with water. An aliquot (90ml) of this solution was made up to a volume of 100ml.

The TLC plate was sprayed with reagent A, allowed to dry for approximately 3min, followed by reagent B. Thioesters could be located by the appearance of a yellow spot.
The limit of detection of thioesters using this method was determined by applying a range of concentrations of authentic AMCC to a TLC plate and treating as above. This was established as 11.4nmol AMCC.

As AMCC was not detectable by these methods in preliminary urine samples, other samples were subsequently concentrated by freeze-drying in an attempt to improve the chance of detecting what might be small amounts of AMCC. The urine samples from six animals were pooled, acidified to pH 1 with HCl (1M), and dried using a Modulyo freeze-drying unit plus vacuum pump (Edwards High Vacuum, Crawley, UK). The residue was resuspended in MeOH, applied to a TLC plate, and analysed for AMCC as described above.

3.4.4.3 Methyl- and dimethy lamine

Methylamine and dimethylamine, which are both endogenous constituents of urine, are detectable after conversion to their 2,4-dinitrophenyl (DNP) derivatives (Baba et al, 1975; Kestell et al, 1985b). After conversion to the neutral form under alkaline conditions, the amines react with DNFB by electrophilic displacement. The resulting DNP-derivatives are easily visualised, following TLC separation, by their yellow colour:

\[
\begin{align*}
\text{O}_2\text{N} & \quad \text{F} & + & \text{NH}_2\text{R} & \rightarrow & \text{O}_2\text{N} & \quad \text{NH}_2\text{R} & + & \text{HF} \\
\text{DNFB} & & & & & & \text{DNP-derivative of alkylamine}
\end{align*}
\]
Urine (1ml) was diluted with water (3ml) and NaHCO₃ added until pH 9 was reached. A solution of DNFB (250ul) in EtOH (5ml) was added to each sample and, after mixing, was left for 2hr in a stoppered test-tube with occasional agitation. The DNP-derivatives formed were extracted into ether. Any water present was removed by the addition of anhydrous sodium sulphate to the ether extract. Ether was removed by rotary evaporation. The resultant residue was analysed for DNP-derivatives by TLC autoradiography and HPLC.

Samples were analysed by TLC using a toluene/ethyl acetate (2.5:1 v/v) solvent system. Rf values of the distinctive yellow spots were compared with those of authentic DNP-methylamine and -dimethylamine. Autoradiographs were prepared to detect any radioactivity associated with the compounds, and this was quantified by the method described in Section 3.4.4.4.

As this method does not differentiate between the derivatives of methylamine and dimethylamine, samples were additionally analysed by HPLC. The HPLC system (Waters Associates, Northwich, UK) consisted of model 510 pumps, system controller (model 720), data module (M730), WISP 710B injector and LC spectrophotometer (model 480, Lambda-Max; set at 346nm). A C₁₈, 5um reverse-phase column was used (Waters, Northwich, UK). The mobile phase was MeOH/water (60:40 v/v) set at a flow rate of 1.5 ml/min. HPLC-separated fractions were collected at 30 sec
intervals using a Frac-100 fraction collector (Pharmacia Fine Chemicals) and were analysed for radioactivity as described in Section 3.2.

3.4.4.4 Quantitation of radiolabelled metabolites
Following TLC analysis and location of the radiolabelled metabolites by autoradiography, the TLC plate was divided into segments. The segment of silica corresponding to a particular metabolite was scraped from the plate, mixed with MeOH and the radioactive content determined by liquid scintillation counting (see Section 3.2).

3.5 STUDIES OF THE IN VIVO METABOLISM OF DMF

3.5.1 Dosing of animals
Mice, rats and hamsters received DMF (0.1, 0.7 or 7.0mmol/kg) as outlined in Section 3.3. Each treatment group comprised six animals.

3.5.2 Collection of urine samples
See Section 3.3.2.

3.5.3 Derivatisation of urine samples
(a) Each urine sample was derivatised according to the method published by Mraz, 1988. Urine (1ml) was mixed with EtOH (2ml), containing 0.5mM quinoline as the internal standard, in a stoppered test-tube. Anhydrous potassium carbonate (1.5g) was added and the mixture was shaken vigorously for 2min. Centrifugation of the mixture at 2,000rpm for 2min (500 g) in a Heraeus Labofuge 6000
centrifuge caused the separation of aqueous and ethanolic layers. The EtOH fraction was removed for GLC analysis. The basis of this derivatisation procedure involves the conversion of AMCC to ethyl N-methyl carbamate (EMC), which is stable enough to be detected by GLC (see Figure 3.2).

Other urinary metabolites which were present and detectable by GLC, HMMF or "F", and unchanged DMF, were extracted into the EtOH unaltered and simultaneously measured by GLC together with the derivatised AMCC.

(b) Removal of endogenous urinary constituents which interfered with GLC analysis of very low concentrations of AMCC was achieved by modifying the above derivatisation procedure. The EtOH extract was concentrated down to a volume of 200ul at room temperature using a Gyrovap centrifugal evaporator, mixed first with water (1ml), then with ethyl acetate (1ml). The ethyl acetate fraction was removed, concentrated to 200ul, and injected onto the GLC column. The necessary inclusion of two evaporation steps in this modified method meant that GLC analysis of DMF, HMMF or "F" was not possible in ethyl acetate-derivatised samples. Figure 3.3 illustrates how extraction into ethyl acetate improved the measurement of a low urinary concentration of AMCC.
Figure 3.2
Basis of the method used to derivatise AMCC with either ethanol or propanol under alkaline conditions. The resulting N-methyl carbamic acid ester can be analysed by GLC.
Figure 3.3
GLC chromatograms of derivatised urine samples following extraction into (A) EtOH and (B) ethyl acetate
Standard concentrations of authentic compounds (DMF, NMF, F and AMCC) were prepared in control urine and derivatised in the same manner as the test urine samples.

3.5.4 GLC analysis of urine samples

Derivatised samples were analysed on a Pye Unicam Series 204 Gas Chromatograph fitted with a Nitrogen-Phosphorus detector. A glass column, 2m length, 1/4 inch diameter, was used, containing Carbowax 20M + 5% KOH, mesh range 80-100. Nitrogen was the carrier gas, at a flow rate of 50ml/min. Hydrogen and air pressures were set at 0.8kg/cm² and 0.9kg/cm² respectively. Samples were injected onto the column in 1.0ul volumes. Amplifier attenuation was set according to the metabolite concentration range to be measured. Analyses were recorded by a Gallenkamp Euroscribe chart recorder set at a speed of either 2.5cm/min (DMF, AMCC and HMMF metabolites) or 0.5cm/min ("F" and internal standard).

Quantitation of the detected metabolites was achieved by calculating the ratio of metabolite peak height to that of the internal standard. Values obtained from the standard solutions were used to construct a calibration curve, and this was used to determine the concentration of metabolites in the urine samples.

3.5.5 Evaluation of derivatisation procedure

The derivatisation procedure and the GLC analytical method used for measuring AMCC was assessed for reproducibility and variation. Two concentrations of AMCC
were prepared in urine samples (20uM and 100uM), derivatised (see Section 3.5.3) and analysed by GLC (see Section 3.5.4). Each concentration was used to assess detector reproducibility, within-day variation, and between-day variation.

3.6 STUDIES OF THE IN VIVO METABOLISM OF HM[\textsuperscript{14}C]MF

3.6.1 Dosing of animals

Mice received HM[\textsuperscript{14}C]MF (0.7 or 7.0mmol/kg) as described in Section 3.3.1. Each treatment group comprised 6 animals. The injection formulation was prepared using unlabelled HMMF where necessary to attain a radioactive dose of 8 to 10uCi for each animal. The radioactivity of the injection formulation was determined by liquid scintillation counting (see Section 3.2) and the exact amount of radioactivity administered to each animal calculated.

3.6.2 Collection of urine samples

See Section 3.3.2.

3.6.3 Excretion of radioactivity

Urinary excretion of radioactivity from HM[\textsuperscript{14}C]MF-dosed animals was determined by liquid scintillation counting as previously described in Section 3.2.

3.6.4 Analysis of radiolabelled metabolites

Radiolabelled HMMF and "F" were detected by TLC autoradiography (see Section 3.4.4.1).
3.7 STUDIES OF THE IN VIVO METABOLISM OF HMMF

3.7.1 Dosing of animals
Hamsters received HMMF (0.7 or 7.0 mmol/kg) as outlined in Section 3.3.1. Each treatment group contained six animals.

3.7.2 Collection of urine samples
Individual urine samples were collected at 12-hr intervals as previously described in Section 3.3.2.

3.7.3 Derivatisation and GC analysis of urine samples
Urine samples were derivatised and extracted into ethyl acetate (see Section 3.5.3). Derivatised samples were analysed by GLC (see Section 3.5.4).

3.8 INVESTIGATION OF THE IDENTITY OF THE METABOLITE, "F"

3.8.1 Separation of "F" metabolite
Urine samples from four mice which had received D\(^{14}\)C)MF (0.7 mmol/kg) were each treated as follows. Urine (100 μl) was applied to each of two TLC plates and the plates were developed using a chloroform/MeOH (4:1 v/v) solvent system. Autoradiographs were prepared and the band of silica corresponding to "F" was removed. The silica from the two plates was pooled, washed with four volumes of MeOH, and the MeOH evaporated to dryness using a Gyrovap evaporator. The residue from each of the four samples, containing "F", was resuspended in MeOH (1.0 ml) and treated by the following methods:
3.8.2 GLC analysis
An aliquot (300ul) of the sample was mixed with MeOH containing internal standard (150ul). Samples were then analysed for "F" by GLC, using the conditions previously described (see Section 3.5.4). The amount of "F" in each sample was determined using a calibration curve which had been prepared from standard concentrations of authentic F. Relating this back to the amount of DMF and radioactivity administered to the mice, the maximum radioactivity present in "F" was calculated. This was based on the assumption that each molecule of "F" contained one [14C]-methyl group.

3.8.3 HCHO-release
The following method is a modification of that described by Gate (1985). An aliquot (300ul) of the sample was mixed with an equal volume of water in a stoppered test-tube. A blank was prepared using MeOH (300ul) and water (300ul). The pH was adjusted to 10 or 11 using 0.5M NaOH. After mixing, the solution was allowed to stand at room temperature for 30min. The pH was then adjusted to 4.5 or 5 using HCl (5.0M). An aliquot (2.0ml) of dinedone, prepared at a concentration of 0.4M in 50% EtOH, was added to the solution and mixed. The contents of the test-tube were heated in a boiling water-bath for 5min, with a glass marble replacing the stopper. The mixture was allowed to cool. Toluene (Scintillation grade; 3ml) was added and the solution shaken for 1min. The organic fraction was removed and analysed for radioactivity by
liquid scintillation counting as described in Section 3.2. The toluene extraction procedure was repeated until the amount of radioactivity present reached negligible levels. The total radioactivity extracted from each sample was calculated and compared with the results from GLC analysis.

In the above experiment, dimedone (below) acts as a trapping agent for any HCHO which is released from "F" under alkaline conditions, forming a compound which is extracted into toluene.

\[
\begin{align*}
\text{CH}_3 & \quad \text{H}_2 \quad \text{C} \quad \text{C} \quad \text{C} \quad \text{C} \quad \text{CH}_2 \\
\text{CH}_3 & \quad \text{C} \quad \text{C} \quad \text{C} \quad \text{C} \quad \text{H}_2 \\
& \quad \text{5,5-dimethyl-1,3-cyclohexanedione (dimedone)}
\end{align*}
\]

3.9 STUDIES OF THE IN VITRO METABOLISM OF N-ALKYLFORMAMIDES USING HEPATIC MICROSONES

3.9.1 Preparation of microsomes

In order to minimise loss of enzyme activity, all procedures, where possible, were performed on ice. Buffer and equipment were all pre-cooled. Unless otherwise stated BALB/c mice were used in the in vitro experiments. Animals were killed by cervical dislocation. Livers were immediately removed, rinsed in phosphate buffer (50mM; pH 7.4; see Section 3.9.2) and weighed. Tissue was chopped with scissors and transferred to a glass homogenising tube. The tissue was then homogenised with phosphate buffer by a Camlab homogeniser (model S63C; TRI-R
Instruments, New York, USA) using six downward strokes of the teflon pestle at a speed setting of five. The homogenate was transferred to Beckman polycarbonate centrifuge tubes and spun in a Beckman L8-60M ultracentrifuge. The ultracentrifuge, rotor and tubes had all been previously cooled to 4°C. The first spin was at 12,000rpm for 20min (9,000 g). The post-mitochondrial supernatant was carefully removed and spun at 42,000rpm for 1hr (100,000 g), separating the microsomal fraction from the soluble cell fractions. The microsomal pellet was then resuspended in phosphate buffer by gentle hand homogenisation using a teflon pestle, and then spun for a further 1hr at 100,000 g. This final spin removes any haemoglobin present. The microsomal pellet was again resuspended in phosphate buffer by gentle homogenisation. The volume of buffer used in this final suspension was calculated to give a concentration of 0.5g original liver wet weight/ml buffer. The protein content of the microsomal preparation was determined by the Lowry method (see Section 3.9.5).

3.9.2 Microsomal incubations
Microsomal incubations, unless otherwise stated, were performed in open vials in a water-bath set at a temperature of 37°C, with continuous gentle agitation. All solutions were prepared in phosphate buffer (50mM; pH 7.4). The total volume of each preparation was 2ml and contained glutathione (GSH), NADPH or a NADPH-generating
system, microsomal protein (equivalent to 250mg liver) and substrate. Solutions were prepared as follows:

**Phosphate buffer (50mM; pH 7.4)**

A solution of potassium dihydrogen orthophosphate (anhydrous; 1.36g) in water (200ml) was added to disodium orthophosphate (3.54g) dissolved in water (500ml) until pH 7.4 was attained.

**GSH (10mM final concentration in incubation)**

GSH (0.3073g) was dissolved in buffer (5ml). The pH was adjusted to 7.4 using 10M NaOH and an aliquot (100ul) was added to each incubation.

**NADPH-generating system (1 mM final concentration in incubation)**

Glucose-6-phosphate (56.42mg), NADP (15.3mg), 100mM MgCl₂ (2ml) and glucose-6-phosphate dehydrogenase (40 units) were dissolved in buffer, made up to a volume of 10ml. An aliquot (1ml) was added to each incubation.

Samples were incubated for 5min prior to the addition of substrate in order to attain the incubation temperature and, in incubations where an NADPH-generating system was employed, to preform NADPH. One of the following substrates was added at the given final concentration: NMF (1, 5 or 10mM); DMF (5 or 10mM); HMMF (5 or 10mM). In each experiment control incubations were prepared in which the substrate had been omitted. Samples, which were all prepared in duplicate, were incubated for periods of
up to 60 min. Incubations were terminated by the addition of a volume of incubate (1.5 ml) to ice-cold EtOH (3 ml), containing 0.5 mM quinoline as the internal standard.

3.9.3 Derivatisation of samples

Microsomal incubations were derivatised prior to GLC analysis according to the published method (Mraz, 1988) with some modifications. This method was used for detecting AMCC, and the basis of it was described in Section 3.5.3. In the same manner as AMCC is derivatised, any SMG formed in these incubations is converted to ethyl N-methyl carbamate (EMC).

After mixing the incubate with EtOH, containing 0.5 mM quinoline, the solution was vortexed and centrifuged at 500 g for 5 min in a Heraeus Labofuge 6000 centrifuge to precipitate out the protein. The supernatant was then shaken vigourously with anhydrous K$_2$CO$_3$ (2.25 g) in a stoppered test-tube for 2 min. The mixture was treated as previously described in Section 3.3.3. The ethyl acetate extract was evaporated to approximately 300 ul.

Standard concentrations of synthetic SMG were prepared using microsomes diluted to the same extent as in the test incubations. These samples were derivatised in the same manner as test samples.

In the case of incubations where DMF was used as the substrate, an aliquot (1.5 ml) of the incubate was mixed
with propan-1-ol (3ml) instead of EtOH. SMG formed in these incubations would thus, on derivatisation, be converted to propyl N-methyl carbamate (PMC) instead of EMC. The longer retention time of this compound on GLC analysis compared to EMC improved its separation from the DMF peak.

3.9.4 GLC analysis of microsomal metabolites
Derivatised samples were analysed for EMC or PMC by GLC using the same conditions as described in Section 3.5.4. Peak height ratios for the standard SMG concentrations were used to construct a calibration curve. The amount of SMG formed in each incubation was expressed as nMol SMG/mg microsomal protein.

3.9.5 Lowry protein assay
The protein content of each microsomal fraction prepared was determined by the method of Lowry et al (1951). Standard protein concentrations, ranging from 0 to 500ug protein/ml, were prepared using a stock solution of bovine serum albumin (BSA; 50mg) in 0.5N NaOH (50ml). The sample containing no protein was used as the blank. Lowry reagent was prepared by adding a 1% solution (w/v) of copper sulphate (2ml) and a 2% solution (w/v) of potassium sodium tartarate (2ml) to a 2% (w/v) sodium carbonate solution (200ml) and mixing. Folin and Ciocalteau's phenol reagent (2.0N) was diluted with an equal volume of water. All solutions were freshly
prepared. The microsomal suspension was diluted with water to give a dilution factor of 20 and 40.

The standard solution or the diluted microsomal sample (500ul) was mixed with Lowry reagent (2.5ml) and the resultant mixture allowed to stand for 10min. The diluted Folin and Ciocalteau phenol reagent (0.25ml) was added to each sample, vortexed and allowed to stand for 30min. All samples were prepared in duplicate. The absorbance of the samples was then read at 750nm on a Cecil CE594 Double Beam UV spectrophotometer, using the blank sample to obtain zero. Absorbance values from the standard protein concentrations were used to construct a calibration curve. The protein content of the diluted microsomal samples was determined from the calibration curve and the protein concentration of the original microsomal samples calculated.

3.9.6 Aminopyrine N-demethylation assay

Aminopyrine undergoes N-demethylation via an unstable carbinolamine generating monomethyl-4-aminoantipyrine and formaldehyde (Gibson and Skett, 1986):

\[ \text{Aminopyrine} \xrightarrow{\text{mixed function oxidation, NADPH, O}_2} \text{Unstable carbinolamine} \rightarrow \text{Monomethyl-4-aminoantipyrine} \rightarrow \text{Formaldehyde} \]
The HCHO released may be measured by a method based on the Hantzsch reaction (Nash, 1953):

\[
\text{Acetylacetone} + \text{NH}_3 + \text{HCHO} \xrightarrow{\text{Hantzsch reaction}} \text{3,5-Diacetyl-1,4-dihydrouridine (A max 415 nm)} + 3\text{H}_2\text{O}
\]

As aminopyrine N-demethylation is dependent on cytochrome P-450, determination of the amount of HCHO released from aminopyrine incubated with microsomes provides a measure of enzyme activity in the microsomal sample (Gibson and Skett, 1986).

The solutions used in this assay were prepared as follows:

**Aminopyrine** (5mM final concentration in incubation)
Aminopyrine (115.7mg) was dissolved in 50mM phosphate buffer (5ml; pH 7.4) to give a 100mM solution. An aliquot (100ul) was added to the incubation to give a final concentration of 5mM.

**Trichloroacetic acid**
Trichloroacetic acid was prepared as a 12.5% w/v solution with distilled water.

**Nash reagent**
Ammonium acetate (45g), acetylacetone (0.6ml) and acetic
acid (0.9ml) were mixed with distilled water (100ml). The pH of the solution was adjusted to 6.7.

Standard HCHO solution

Paraformaldehyde (0.15g) was dissolved in 0.1N sodium hydroxide solution (100ml). This solution (1ml) was then made up to 100ml with distilled water.

Standard concentrations of HCHO were prepared from 0 to 250µM, using the zero concentration as the blank. Microsomal incubations were prepared as previously described in Section 3.9.2, with aminopyrine as the substrate, and incubated for 30min. Control incubations were similarly prepared, but omitting the substrate. Incubations were terminated by adding a 200ul aliquot of the incubate to 12.5% trichloroacetic acid (800ul) and vortexing. Samples were spun in a MSE MicroCentaur microfuge for 3min to precipitate out the protein and the supernatant (800ul) was mixed with Nash reagent (400ul). Standard solutions were, likewise, mixed with 12.5% trichloroacetic acid and Nash reagent in these proportions. Samples and standards were all prepared in duplicate.

On the addition of Nash reagent, samples and standards were incubated in a water bath at 60°C for 30min. After cooling, the absorbance of samples was read on a Cecil CE594 Double Beam UV spectrophotometer at 412nm, using the blank sample to attain zero. Absorbance readings from
the standard concentrations were used to construct a calibration curve. The amount of HCHO produced in each microsomal incubation was determined after subtracting the control values (minus substrate). The extent of aminopyrine N-demethylation was expressed as nmol HCHO/mg protein.

3.9.7 Cytochrome P-450 assay
The assay is based on the ability of cytochrome P-450, in its reduced form, to form a complex with CO, giving a characteristic absorbance spectrum at 450nm (Gibson and Skett, 1986).

Microsomal samples were diluted 1:4 with phosphate buffer (50mM; pH 7.4) and divided between two cuvettes, a reference and a sample cuvette. Using a Cecil CE594 Double Beam UV spectrophotometer, the baseline for the samples was recorded between 400 and 500nm. Sodium dithionite (several grains) was added to both cuvettes and the cuvettes inverted several times. CO was gently bubbled through the sample cuvette for 1min. Both samples were rescanned between 400 and 500nm. The absorbance difference for each pair of samples was measured between 450 and 490nm. Using the following equation, which is based on Beer's Law, the cytochrome P-450 concentration was calculated:

\[
\text{Cyt.P-450 conc'n} = \frac{\text{Absorbance diff'ce (420-490nm)}}{\text{Extinction coefficient (450-490nm)}} \times 1000
\]

Extinction coefficient = 91mM\textsuperscript{-1}cm\textsuperscript{-1}
Cuvette path length = 1cm.
The dilution factor and the protein content of the microsomes were incorporated into the result. The cytochrome P-450 content of the original microsome sample was expressed as nmol/mg protein.

3.10 CHARACTERISATION OF THE MICROSOMAL GENERATION OF SMG

3.10.1 Effect of heat-inactivation of microsomes
Incubations were performed as described in Section 3.9.2, substituting fresh microsomes with ones that had been inactivated by heating in boiling water for 10 min and then cooled. Control incubations were conducted using fresh microsomes. NMF was added to incubations as the substrate, at a final concentration of 10 mM. Samples were derivatised and analysed by GLC as described in Sections 3.9.3 and 3.9.4 respectively.

3.10.2 Investigation of cofactor requirements
Incubations were performed as described in Section 3.9.2, but omitting either GSH or the NADPH generating system. The involvement of oxygen in the microsomal metabolism of NMF was assessed by conducting the incubations in stoppered vials supplied with a continuous flow of nitrogen gas. Samples were derivatised (see Section 3.9.3) and analysed by GLC (see Section 3.9.4).
3.11 IN VITRO METABOLISM OF N ALKYLFORMAMIDES BY BALB/C MOUSE HEPATIC MICROSONES

3.11.1 Microsomal incubations
Microsomes were prepared (see Section 3.9.1) and incubations conducted as described in Section 3.9.2, in the presence of GSH and NADPH. NMF (1, 5 and 10mM), DMF (5 and 10mM) and HMMF (5 and 10mM) were added as the substrates. Samples were incubated for periods of 0, 20, 40 and 60 min.

3.11.2 Derivatisation of samples and GLC analysis
See Sections 3.9.3 and 3.9.4.

3.12 IN VITRO METABOLISM OF N-ALKYLFORMAMIDES BY HUMAN HEPATIC MICROSONES

3.12.1 Preparation of microsomes
Two samples of human liver were obtained through Dr K. Chipman, University of Birmingham from the Queen Elizabeth Hospital. The donors were both healthy young adults, one male and one female, both victims of road traffic accidents. Tissue had been stored in buffer on ice immediately following removal. The time-interval between removal of the liver sample and the commencement of microsome preparation was in both cases estimated as 10 hr. Samples were weighed and microsomes were prepared essentially as described in Section 3.9.1.
3.12.2 Microsomal incubations

Incubations were conducted as described in Section 3.9.2, in the presence of GSH and NADPH, for 30 and 60 min. The substrates used were NMF (10 mM), DMF (5 and 10 mM) and HMMF (10 mM).

3.12.3 Derivatisation of samples and GLC analysis

Samples were derivatised and analysed for EMC or PMC as described in Section 3.9.3 and 3.9.4.

3.13 INVESTIGATION OF SPECIES AND STRAIN DIFFERENCES IN THE MICROSONAL METABOLISM OF NMF

3.13.1 Microsomal incubations

Hepatic microsomes were prepared from BALB/c mice, CBA/CA mice and Sprague Dawley rats (see Section 3.9.1). Incubations were conducted in the presence of GSH and NADPH (see Section 3.9.2) for 0, 20, 40 and 60 min, using NMF (10 mM) as the substrate.

3.13.2 Derivatisation of samples and GLC analysis

See Section 3.9.3 and 3.9.4.

3.14 INVESTIGATION OF CYTOCHROME P-450 INVOLVEMENT IN THE METABOLIC ACTIVATION OF N-ALKYLFORMAMIDES

3.14.1 Inhibition of microsomal enzymes

3.14.1.1 Carbon monoxide (CO)

Incubations were prepared in 4 individual flasks attached to a sealed apparatus so that the incubation atmosphere could be controlled (see Figure 3.4). The internal volume
Figure 3.4
Apparatus used for conducting microsomal incubations within a controlled atmosphere
of the apparatus had been previously measured. Before the addition of substrate to the incubations, the apparatus was evacuated under vacuum, filled with nitrogen gas and evacuated again. This procedure was repeated three times, ensuring removal of all oxygen. The apparatus was then filled with CO and sealed. The tap separating a connecting flask containing a known volume of air was then opened. The apparatus thus contained a known volume of CO and air. After incubating for 5 min, substrates were introduced into the incubation flasks by syringe through the rubber stoppers. Aminopyrine (final concentration of 5 mM) was added as the substrate to the first two flasks. NMF was added to the remaining two flasks (final concentration 10 mM). Microsomal preparations were incubated for 30 min at 37 °C. At the end of this time samples were treated as described in Sections 3.9.3 and 3.9.4 (NMF samples) and Section 3.9.6 (aminopyrine samples). The ratio of CO to air used in this experiment was 1:1 v/v. In a control experiment, CO was substituted with nitrogen gas in a ratio of 1:1 v/v with air.

3.14.1.2 SKF 525-A

A solution of SKF 525-A in buffer was added to microsomal incubations and incubated at 37 °C for 5 min prior to the addition of substrate. The effect of a range of SKF 525-A concentrations (0, 0.5, 1.0, 2.0 and 3.0 mM) on the activity of microsomal enzymes was assessed by measuring the extent of aminopyrine N-demethylation (see Section 3.9.). SKF 525-A was dissolved in 50 mM phosphate buffer
(pH 7.4) to give a final concentration in the incubation, for example, as follows:

0.5mM
SKF 525-A (77.7mg) was mixed with buffer (20ml). An aliquot of the solution (100ul) was added to the incubations (total volume 2ml).

3.0M
SKF 525-A (51.80mg) with buffer (20ml) to obtain a 6.67mM solution. This solution (900ul) was added to microsomal incubations (final volume 2ml). The larger volume of SKF 525-A added in this instance necessitated that concentration of the NADPH-generating system be adjusted accordingly, so that the final volume of the incubation remained at 2ml.

3.14.2 Induction of metabolising enzymes
3.14.2.1 Phenobarbitone (PB) treatment
Mice received PB by 4 daily ip injections. Each injection contained either 50 or 80mg PB/kg bodyweight, prepared in 0.9% sterile saline. The volume of each injection was 0.2ml. Animals were injected at approximately 9am each day, and were killed at approximately the same time on the fifth day. Control mice were injected with 0.9% sterile saline (0.2ml) for 4 days and similarly killed on the fifth day. The cytochrome P-450 content of the treated and control microsomes was determined as described in Section 3.9.7.
3.14.2.2 Beta-naphthoflavone (BNF) treatment
BNF was administered to mice by 4 daily ip injections. Each injection contained 50mg BNF/kg, prepared in corn-oil. The injection volume was 0.3ml. Mice were injected at approximately 9am each day, and were killed at the same time on the fifth day. Control animals were injected ip with corn-oil (0.3ml) for 4 days and killed on the fifth day. The cytochrome P-450 content of the treated and control microsomes was determined as described in Section 3.9.7.

3.14.3 Microsomal incubations
Unless otherwise stated, incubations were conducted as described in Section 3.9.2. NMF (10mM) and HMMF (10mM) were used as the substrates, and incubations were continued for 20, 40 and 60min. The effect of each treatment on enzyme activity was assessed by measuring the extent of aminopyrine N-demethylation (see Section 3.9.6).

3.14.4 Derivatisation of samples and GLC analysis
Samples were derivatised and analysed for EMC as described in Section 3.9.3 and 3.9.4 respectively.

3.15 INVESTIGATION OF $^{18}$O INCORPORATION INTO SMG
By conducting microsomal oxidations, such as occur in the metabolism of NMF, in an atmosphere containing oxygen-18 gas ($^{18}$O), the incorporation of an oxygen atom into the resulting metabolite can be traced using gas
chromatography-mass spectrometry (GC-MS). The metabolic oxidation of acetanilide to acetaminophen, which is catalysed by cytochrome P-450, serves as a positive control.

3.15.1 Microsomal incubations
Hepatic microsomes were prepared from BALB/c mice essentially as described in Section 3.9.1, and diluted to give a concentration of 0.8g original liver wet weight/ml buffer. Incubations were performed in four separate glass flasks, which formed part of the sealed apparatus previously described in Section 3.14.1.1 and illustrated in Figure 3.4. Gases could to be introduced or removed and the apparatus then sealed, thus allowing the gaseous atmosphere in which incubations took place to be strictly controlled. Incubations were prepared in 2ml volumes as described in Section 3.9.2, with the addition of GSH and NADPH. Before addition of the substrate, with flasks still on ice, the apparatus was subjected to a vacuum. The apparatus was then sealed, filled with nitrogen followed by another evacuation. This performance was repeated twelve times finishing with an evacuation and then sealed, thus ensuring that any oxygen present had been effectively removed. With the apparatus under vacuum, the substrate was added to each flask by means of a syringe inserted through the rubber septum. In the first three flasks NMF was added as the substrate, at a final concentration of 10mM. Acetanilide was added to the remaining flask at a final concentration of 10mM. The
apparatus was flushed through with nitrogen and evacuated a further two times. Whilst the apparatus was under vacuum, the neck of the connected $[^{18}O_2]$-flask was carefully broken using a magnet and metal rod. This allowed entry of $^{18}O_2$ into the sealed apparatus. The apparatus was then suspended in a 37°C shaking water-bath and incubated for 60min.

At the end of the incubation period, the incubation preparations containing NMF were mixed with ice-cold ethanol (4ml). The incubation containing acetaldehyde was mixed with acetone (2ml).

To obtain control samples, the entire experiment was repeated exactly as above, but substituting $^{16}O_2$ for $^{18}O_2$. The incubations thus took place in an atmosphere of $^{16}O_2$.

3.15.2 Derivatisation of samples

NMF incubations were separately derivatised as described in Section 3.9.3. GLC analysis of the ethyl acetate extracts confirmed the presence of EMC in both control and test samples. Samples were then evaporated to dryness.

After mixing the acetaldehyde sample with acetone, the solution was spun at 2,000rpm in a Heraeus Labofuge 6000 centrifuge to precipitate out the protein. The supernatant was acidified with 1N HCl (1 drop) and
extracted twice with ethyl acetate (4ml). The extract was then dried with magnesium sulphate (anhydrous) and concentrated to dryness using a Gyrovap centrifugal evaporator. BSTFA (1ml) was added to the residue and heated at 70°C for 2hr. The mixture was evaporated to dryness, stored at -20°C and resuspended in ethyl acetate (40ul) on analysis.

A standard solution of paracetamol (APAP) was prepared from 9.5mg of compound dissolved in ethyl acetate (5ml). This was evaporated to dryness and treated in the same way as the acetaldehyde sample.

3.15.3 Analysis of samples
All eight samples (three NMF/\textsuperscript{18}O\textsubscript{2}; three NMF/\textsuperscript{16}O\textsubscript{2}; one acetaldehyde/\textsuperscript{18}O\textsubscript{2}; one acetaldehyde/\textsuperscript{16}O\textsubscript{2}) were sent to Dr Paul Pearson, University of Washington, Seattle for analysis. Samples from the NMF incubations were analysed by GC-MS (VG 7070H). Separations were carried out on a carbowax capillary column, with a temperature programme as follows: 80°C (1min); 80 - 190°C (10°C per min); 190°C (10min). Authentic EMC had a retention time of 12min and 54 sec. MH\textsuperscript{+} ions following chemical ionisation were detected by selective ion monitoring in the range m/z 103 to 107 inclusive.

The derivatised samples from the acetaldehyde incubations and the standard APAP sample were also analysed by GC-MS, using electron ionisation. Separations were performed on
a capillary column (DBS; 30m) with a temperature programme as follows: 50°C (3min); 50 - 200°C (15°C per min); 200 - 250°C (30°C per min); 250°C (10min).

3.16 INVESTIGATION OF THE IDENTITY OF THE REACTIVE METABOLITE OF N-ALKYLFORMAMIDES

The possible release of methyl isocyanate (MIC) from SMG was investigated using a method based on a published experiment where the release of MIC from the antitumour drug caracemide was studied (Newman and Farquhar, 1987). Caracemide [N-acetyl-N-(methylcarbamoyloxy)-N'-methylurea] incubated at 37°C in buffer (pH 7.4) degrades to N-(methylcarbamoyloxy)-acetamide and MIC:

![Chemical Structure]

The published method provides a means of trapping the released MIC. A modification to this method, which was suggested by Dr I. Linhart of the Institute of Hygiene and Epidemiology, Prague, was used to determine whether any MIC was released from synthetic SMG.

3.16.1 Detection of MIC released from caracemide

The apparatus used in the following experiments is illustrated in Figure 3.5. Flask A contained caracemide (37.8mg; 0.2mmol) dissolved in 0.2M phosphate buffer (pH 8.0). Flask B contained potassium hydroxide (19.8mg)
Nitrogen was passed through the reaction mixture for 30 min. The contents were then extracted with chloroform.

3.15.2 Releasing MIC from Caracemide

(i) MIC (flask A) was added to an excess of buffer immediately before use. Room temperature.

(ii) MIC (flask A) was added to an excess of buffer previously extracted with GLC. See...

Figure 3.5
Apparatus used for releasing and trapping MIC from caracemide, and for investigating MIC-release from SMG.
dissolved in Pr-1-OH (6ml), acting as the trapping solution. Flask A was suspended in a 37°C water bath and nitrogen was gently bubbled through the solution for 30min. The contents of flask B were then shaken with a saturated solution of sodium chloride in distilled water (6ml). The propanol layer was removed and analysed for PMC by GLC using the conditions described in Section 3.5.4.

3.16.2 Estimation of the trapping efficiency for MIC
(i) MIC (12ul; 0.2mmol) was added to 0.2M phosphate buffer (pH 8.0; 2ml) in flask A. The solution was immediately bubbled through with nitrogen for 30 in at room temperature, with the same trapping solution as previously used in flask B. The contents of flask B were extracted as described in Section 3.16.1 and analysed by GLC.

(ii) MIC (12ul; 0.2mmol) only was carefully added to flask A which was stood on ice. The apparatus, with flask B contents as in the previous experiment, was immediately assembled and nitrogen gently blown through. After approximately 10min, the solution in flask B was extracted as previously described and analysed by GLC.

3.16.3 Investigation of MIC release from SMG
Flask A contained a solution of SMG (72.8mg; 0.2mmol) in 0.2M phosphate buffer (pH 8.0; 2ml). Flask B contained potassium hydroxide (19.8mg) dissolved in Pr-1-OH (6ml).
Flask A was suspended in a water-bath set at 37°C, and nitrogen gently bubbled through for 30, 60 or 90min. At the end of each time-point, the contents of flask B were treated by the same method outlined in Section 3.16.1. Samples were similarly analysed for PMC by GLC.

A negative control experiment was additionally performed. 0.2M phosphate buffer (pH 8.0; 2ml) only was added to flask A. The contents of flask B were as described in Section 3.16.1. Nitrogen was bubbled through as before for 30min, and the sample similarly extracted into Pr-1-OH and analysed by GLC.

3.16.4 Investigation of MIC generation in microsomal incubations

A microsomal incubation was prepared in flask A as described in Section 3.9.2, containing a NADPH-generating system but omitting GSH. NMF was added as the substrate at a final concentration of 10mM. The total volume of the incubation was 2ml. Flask B contained the trapping solution, described in the previous three experiments. Flask A was suspended in a 37°C shaking water-bath, with a gentle stream of air bubbling through the incubate for a period of 60min. At the end of the incubation period, the contents of flask B were extracted as described previously in Section 3.16.1 and analysed by GLC.
4.1 THE ACUTE RESPONSE

4.1.1 INTRODUCTION

The toxicity of occupationally exposed levels of methylcarbarnine and its metabolites have been described in detail. This feature of the toxicity of 

SECTION 4

RESULTS

The acute toxicity of the metabolite of these compounds, which have been previously reported in the literature, is also considered.

It is known that the hepatotoxicity of these compounds may be due to their metabolism.

MME and MME respectively (Mehl) leading to the formation of compounds that have been implicated in the acute toxicity.

Recent investigations have described the detection of these metabolites (Egan, 1989).

Studies of the metabolism of these compounds have reported on the major metabolites and their formation. However, information on the metabolism of these compounds in animals is limited.
4.1 THE IN VIVO METABOLISM OF DMF IN THREE RODENT SPECIES

4.1.1 INTRODUCTION

The toxicity of the industrial solvent, DMF, in occupationally exposed humans and in experimental animals have been described in Sections 1.2 and 1.3. A major feature of the toxicity of DMF is its ability to cause liver damage. Studies of DMF and other N-alkylformamides, which have been reported in Section 1.5, have suggested that the hepatotoxicity of these compounds is linked to their metabolism.

NMF and NEF are metabolised in mice to N-(methylcarbamoyl) and N-(ethylcarbamoyl) mercapturates respectively (Kestell et al, 1987). The metabolic pathway leading to the urinary excretion of mercapturic acids has been implicated in the hepatotoxicity of these compounds. Recent investigations of humans exposed to DMF have similarly reported the presence of AMCC in the urine and have described a sensitive analytical method for the detection of this metabolite (Mraz and Turecek, 1987; Mraz, 1988).

Studies of the metabolism of DMF in experimental animals reported so far (reviewed in Section 1.4) have shown the major metabolic pathway to proceed by C-hydroxylation to form HMMF. However, there has been no evidence of the metabolism of DMF to a N-(methylcarbamoyl) mercapturate in animals.
We wished to investigate and compare the in vivo metabolism of DMF in three different rodent species, mice, rats and hamsters, with the aim of addressing the following questions:

(a) is HMMF a major metabolite of DMF similarly in all three rodent species?
(b) is DMF metabolised to AMCC in any of these rodents?
(c) is the metabolic pathway of DMF dependant on the dose?

4.1.2 RESULTS
The time-course of the excretion of DMF was established in BALB/c mice by measuring the recovery of radioactivity from the urine after administering radiolabelled DMF at three different doses. Urine samples collected at 12-hr intervals were analysed for radioactivity by liquid scintillation counting as described in Section 3.2. The pattern of excretion of radioactivity over 72hr (Figure 4.1.1) shows rapid elimination of species derived from DMF, with most of the excreted drug and its metabolites appearing in the urine within 24hr of treatment.

As the total radioactivity recovered was less than 60%, in the case of the two lower doses (Figure 4.1.1), the location of the unrecovered radioactivity was investigated. In one experiment activity was searched for in cage-washings and in another the content of radiolabelled material in livers of mice was studied. A small amount (3.6 ± 1.5%) of the administered
Figure 4.1.1

The excretion of radioactivity in the urine of male BALB/c mice following a single ip injection of radiolabelled DMF.

(A) Time course of excretion  (B) Total radioactivity excreted in 72hr. Values are the mean ± SD of 6 animals.
radioactivity was recovered in the cage-washings of animals dosed with 0.1mmol/kg DMF. In the livers 1.3% of the dose was found in mice which had received 0.7mmol/kg DMF.

The excretion of species derived from unlabelled DMF, measured by GLC, was investigated in mice, rats or hamsters (Figure 4.1.2). DMF and its metabolites were rapidly excreted in all three species. The proportion of the dose recovered within 72 hr ranged from 36.0 ± 6.5% (mice; 0.1mmol/kg) to 79.5 ± 5.8% (rats; 0.1mmol/kg) (Figure 4.1.3). In mice which had received 0.1 or 0.7mmol/kg, less than 50% was recovered, which matches the low recovery of radiolabelled DMF at these dose levels.

Using TLC autoradiography and spray detection (see Section 3.4.4.1), the main radiolabelled metabolites of DMF in BALB/c mice were found to have the same Rf values as authentic HMMF and F (see Figure 4.1.4). A small amount of unchanged DMF was detected in the case of mice which had received the highest dose.

Methylamine and dimethylamine, after conversion to their DNP-derivatives, were also confirmed as urinary metabolites of ¹⁴C-DMF in mice using a combination of analytical techniques (see Section 3.4.4.3). An example of a TLC autoradiograph is shown in Figure 4.1.5.
(A) BALB/c mouse

(B) Sprague Dawley rat

- □ 0.7mmol/kg
- ■ 7.0mmol/kg

(C) Syrian hamster

Figure 4.1.2
The excretion of total unlabelled urinary metabolites measured by GLC in rodents following a single ip injection of DMF. Values are the mean ± SD of 6 samples.
Figure 4.1.3
Total of all metabolites measured by GLC, excreted in the urine of rodents within 72hr of a single ip injection of DMF. Values are the mean + SD of 6 animals.
Solvent system: Chloroform/methanol (4:1 v/v)

Figure 4.1.4
TLC autoradiographs of urine samples from BALB/c mice following a single ip injection of radiolabelled DMF as (A) 0.7mmol/kg and (B) 7.0mmol/kg
SAMPLES:

1 Authentic DNP-methylamine
2 Authentic DNP-dimethylamine
3 Two urine samples derivatised with DNFB
4

Solvent system: ethyl acetate/toluene (1:2.5 v/v)

Figure 4.1.5
TLC autoradiograph demonstrating the DNP-derivatives of alkylamines in two urine samples collected from BALB/c mice which had received D\(^{14}\)C]MF (7.0mmol/kg)
Attempts to detect urinary N-(alkylcarbamoyl) mercapturates by employing TLC autoradiographic techniques in conjunction with spray detection methods (see Section 3.4.4.2) were inconclusive. When urine samples were concentrated by freeze-drying to increase what might have previously been undetectable levels of AMCC, the presence of large amounts of material caused poor separation of the radioactive metabolites (see Figure 4.1.6). The limit of detection of thioesters by TLC with the spray method employed (11.4nmol AMCC) meant that small amounts of mercapturates in the urine might have escaped detection.

Unlabelled metabolites of DMF were detected by GLC, in the case of thioesters following derivatisation (see Sections 3.5.3 and 3.5.4). GLC peaks for the metabolites were found to correspond to authentic DMF, AMCC, NMF and F. Typical chromatograms are illustrated in Figure 4.1.7. As described before (Section 1.4), HMMF undergoes thermal decomposition to NMF on the GLC column (Scailteur et al, 1984; Kestell et al, 1986). In these analyses the peak which corresponded to NMF was therefore assumed to be HMMF and was referred to as "NMF". Previous studies have suggested that a small proportion of DMF is excreted as NMF, amounting to about 4% of the dose (Scailteur and Lauwerys, 1984a). The analytical method employed in these studies, however, does not distinguish between HMMF and NMF.
Solvent system: Butanol/water/methanol (8:2:1 v/v)

Figure 4.1.6
TLC autoradiograph of a freeze-dried urine sample from a mouse which had received 7.0mmol/kg D[14C]MP, demonstrating the difficulties encountered in the location of thioesters
Syrian hamster

(A) 0.7 mmol/kg

(B) 7.0 mmol/kg

Internal standard

"NMF"

AMCC

"F"

"NMF"

AMCC

"F" IS

Figure 4.1.7
GLC chromatograms of derivatised urine samples from rodents which had received a single ip injection of DMF
The GLC peak with the same retention time as that of F was similarly considered to be a thermal degradation product of HMF, and will be referred to as "F". Investigations into the identity of this metabolite are described in Section 4.4.

The derivatisation method which involves the conversion of urinary AMCC to EMC and extraction into ethyl acetate (see Section 3.5.3) was evaluated for accuracy and reproducibility (see Section 3.5.5). The results of this evaluation are shown in Table 4.1.1.

The amounts of DMF metabolites measured by GLC in the urine of these rodents are summarised in Figures 4.1.8 to 4.1.15. Unchanged DMF was detectable in all three species, but only at the two higher doses (Figure 4.1.8). More was excreted unchanged in the rat than in mice or hamsters (Figure 4.1.9). In all cases, the amount of DMF excreted unchanged never exceeded 6% of the administered dose.

AMCC was found in the urine of all rodents at each dose (Figure 4.1.10). In all cases the amount measured was small, ranging from $1.1 \pm 0.3\%$ in the mouse ($7.0\text{mmol/kg}$) to $5.2 \pm 1.1\%$ in the rat ($0.1\text{mmol/kg}$). The amount of AMCC excreted in the rat showed an inverse relationship to the dose. This relationship was not apparent in the two other species. A species difference was only discernible at the lowest dose where rats excreted more AMCC than the other
<table>
<thead>
<tr>
<th>AMCC concentration (μM)</th>
<th>Variation on repeated injection</th>
<th>Within-day variation</th>
<th>Between-day variation</th>
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<tr>
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<td>(a)</td>
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<td>50</td>
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<td></td>
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<tr>
<td>20</td>
<td>6.12</td>
<td>8.25</td>
<td>3.78</td>
</tr>
<tr>
<td>100</td>
<td>0.85</td>
<td>2.84</td>
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</tr>
</tbody>
</table>

AMCC was shown to be linear over the concentration range 10 to 250μM.

Table 4.1.1
Accuracy and reproducibility of the measurement of AMCC in urine samples following derivatisation and extraction into (a) ethanol, or (b) ethyl acetate. The number of samples was 6 in each case.
Figure 4.1.8

The effect of dose on the urinary excretion of unchanged parent compound in rodents following a single ip injection of DMF. Values are the mean ± SD of 6 animals.
Figure 4.1.9
Species variation in the urinary excretion of unchanged parent compound following a single ip injection of DMF at two different doses. Values are the mean ± SD of 6 animals.
Figure 4.1.10
Effect of dose on the urinary excretion of AMCC in rodents following a single ip injection of DMF. Values are the mean ± SD of 6 animals.
two species (Figure 4.1.11).

Quantitatively, "NMF" was the major urinary metabolite of DMF, accounting for between 29.8 ± 6.8% and 47.3 ± 8.0% of the dose in rats and hamsters (Figures 4.1.12 and 4.1.13). Mice which had received 7.0mmol/kg similarly excreted a large amount (45.5 ± 14.3%) as "NMF". The proportion of the dose excreted as "NMF" in mice, but not in rats and hamsters, showed dose-dependence. At the two lower doses, 0.1 and 0.7mmol/kg, mice excreted 8.4 ± 3.5% and 18.6 ± 4.4% as "NMF" respectively.

"F" was a minor metabolite when DMF was administered at the high dose (Figures 4.1.14 and 4.1.15). However, at the lower doses the amount of urinary "F" increased in all three species. In some cases (mice - 0.1 and 0.7mmol/kg; rats - 0.1mmol/kg), it was quantitatively more important than "NMF". The excretion of "F" showed an inverse relationship to the dose in rats.

The time course of urinary excretion of the metabolites demonstrates some dependence on the dose of DMF administered. In rats and hamsters which had received 7.0mmol/kg DMF, AMCC appeared in the urine later than in the case of the lower dose (Figure 4.1.16). In rats, AMCC was measured in the urine for longer periods at 7.0mmol/kg than at the lower dose. It was detectable for 60hr after dosing. Another metabolite, "NMF", was similarly detectable for longer periods in the urine of
Figure 4.1.11
Species variation in the urinary excretion of AMCC following a single ip injection of DMF at three different doses. Values are the mean ± SD of 6 animals.
Figure 4.1.12

Effect of dose on the urinary excretion of "NMF" in rodents following a single ip injection of DMF. Values are the mean ± SD of 6 animals.
Figure 4.1.13
Species variation in the urinary excretion of "NMF" following a single ip injection of DMF at three different doses. Values are the mean ± SD of 6 animals.
Figure 4.1.14
Effect of dose on the urinary excretion of "F" in rodents following a single ip injection of DMF.
Values are the mean ± SD of 6 animals.
Figure 4.1.15
Species variation in the urinary excretion of "F" following a single ip injection of DMF at three different doses. Values are the mean ± SD of 6 animals.
Figure 4.1.16

Time-course in the urinary excretion of (A) AMCC in the rat; (B) AMCC in the hamster; and (C) "NMF" in the rat following a single ip injection of DMF at two different doses. Values are the mean ± SD of 6 animals.
rats at the higher dose.

The amount of methylamine and dimethylamine were measured in the urine of mice which had received 0.7mmol/kg DMF. Following conversion to their DNP-derivatives and separation by HPLC, the radioactivity associated with methylamine and dimethylamine was found to be $3.3 \pm 0.03\%$ and $2.7 \pm 0.3\%$ of the dose respectively. Given that only $50\%$ of the methylamine derived from DMF will contain a $^{14}$C-labelled methyl moiety, the amount of this metabolite is likely to be twice this value.

4.1.3 DISCUSSION

The pattern of urinary excretion of DMF in rodents, administered as a single dose in either the labelled or unlabelled form, demonstrates that DMF was rapidly eliminated from the body, extensively metabolised, and that the urine was the main route of excretion. The speed of elimination of DMF and the importance of urinary compared to other pathways of excretion is in agreement with previous animal studies of acute exposure to DMF (Kimmerle and Eben, 1975; Brindley et al, 1983; Scailteur and Lauwerys, 1984a).

In some treatment groups the proportion of the dose which was not recovered from the urine amounted to between 40 and 65%. This result could be due to one or more of several reasons. Some DMF was metabolised and probably exhaled as CO$_2$, which has been shown to be a significant
metabolic pathway for NMF (Kestell et al, 1985b). Some drug-derived species may have been excreted via the faeces. Measurement of urinary metabolites by the GLC method excluded methylamine and dimethylamine, both of which have been shown to be minor metabolites of DMF in separate experiments. Another possibility is that a proportion of the dose may have been retained in the body. However, investigations of the radioactive content of mouse livers at the completion of one experiment demonstrated that only approximately 1% of the dose remained in this tissue. A further feasible explanation for the reduced recovery of DMF, which was most apparent in the mice treated with 0.1 or 0.7 mmol/kg, is that in the case where small volumes of urine are produced the potential loss of considerable amounts of drug-derived species during urine collection is possible. This was minimised by using silanised glassware and rinsing the metabolic cages with a small volume of water when collecting samples. Indeed, radioactivity recovered from cage-washings at the end of one experiment showed that less than 4% of the administered radioactivity was lost in this manner.

There remains, therefore, the possibility that in some species treated with the lower doses of DMF different routes of excretion may have been quantitatively more important than in the animals treated with the higher doses.
All three species studied metabolised DMF in a qualitatively similar manner. "NMF", "F", AMCC and, at high doses, unchanged DMF were detectable in the urine in all cases. Methylamine and dimethylamine were excreted in mice, although in the other rodents these metabolites were not investigated. The identification of "NMF", "F", methylamine and dimethylamine as urinary metabolites of DMF, plus the excretion of some unchanged parent compound, is in accordance with the findings of other studies which have been described in Section 1.4.

Unchanged DMF was detected in the urine only when higher doses were administered. This has also been reported in other studies (Lundberg et al, 1983; Scailteur et al, 1984) and lends support to the proposal that at high levels of exposure DMF inhibits its own metabolism.

In most cases, "NMF" was found to be quantitatively the major urinary metabolite. On the assumption that "NMF" was derived by thermal degradation from HMMF, it seems that DMF is extensively metabolised to HMMF in rodents.

It is interesting that in the case of low doses of DMF, there was a large percentage of the dose excreted as "F"; in the rat it exceeded that of "NMF". This observation has not been made previously and it suggests that at high doses of DMF its metabolism to "F" is saturable or inhibited by other metabolites.
This is the first time that AMCC has been identified and quantitated as a metabolite of DMF in rodents. The percentage of DMF excreted as AMCC amounted to never more than 5.2% of the dose, and in most cases was below 2%. This suggests that in rodents the metabolic pathway from DMF to AMCC is quantitatively of minor importance. In accordance with this finding, a recent study reports the existence of AMCC as a urinary metabolite of DMF in rats (Tulip et al, 1989).

The inverse relationship between AMMC excretion and dose in rats indicates that in this species metabolism of DMF to AMCC may be saturated or inhibited by higher doses. Indeed the delay in the appearance and the prolonged excretion of urinary AMCC in rats dosed with 7.0mmol/kg supports this suggestion.

The results described here on the metabolism of DMF to AMCC in rodents, and the extent of this pathway, are consistent with current proposals for the mechanism of N-alkylformamide toxicity (Kestell et al, 1987; Threadgill et al, 1987; Tulip and Timbrell, 1988). The metabolic pathway for N-alkylformamides which leads to the excretion of N-(alkylcarbamoyl)mercapturates is considered to be a bioactivation pathway. The extent to which these compounds are metabolised along this route is thought to reflect their ability to cause liver damage (Kestell et al, 1987). The significant extent to which NMF and NEF are metabolised to N-(alkylcarbamoyl)
mercapturates is accompanied by marked hepatotoxicity compared to that of DMF or F. Other animal studies have similarly demonstrated DMF to be a less severe hepatotoxicant than NMF (Lundberg et al, 1981; Scailteur et al, 1981; Scailteur and Lauwerys, 1984a). The results presented here show that in rodents the metabolic pathway from DMF to AMCC is quantitatively a minor one, that is DMF is bioactivated to a minute extent. This provides an explanation as to why DMF is less hepatotoxic in animals than NMF or NEF. DMF did not cause depletion of hepatic GSH levels in rats in vivo (Scailteur and Lauwerys, 1984a) or in isolated mouse hepatocytes (Shaw et al, 1988). As only a little DMF undergoes metabolism involving conjugation with GSH, the level of GSH depletion may have been so low as to escape detection.

The derivatisation method employed for trapping and detecting AMCC in the urine measures all carbamoylating compounds present. It should therefore be considered that the resulting EMC might in fact have originated from carbamoylating species present in the urine in addition to AMCC. However, an earlier study has shown that the N-methylcarbamoylating activity of the urine of humans exposed to DMF is solely due to AMCC (Mraz and Turecek, 1987). Also, studies conducted on the metabolism of NMF indicate that AMCC is the only urinary carbamoylating metabolite formed (Kestell et al, 1986b; Tulip et al, 1986). Thus the total amount of EMC measured in these experiments is assumed to originate entirely from AMCC.
Nevertheless, the presence of small amounts of other carboxamoylating thioesters or their reactive precursor cannot be entirely ruled out. The amount of methylamine and dimethylamine measured in these experiments was small, amounting to <5% of the dose. The generation of alkylamines from N-alkylformamides is thought to involve oxidation of the formyl moiety (Kestell et al., 1987; Threadgill et al., 1987). This oxidation step is implicated in hepatotoxicity of formamides. The actual precursors of N-alkylamines are unknown. It has been suggested by the above authors that they may be formed from the reactive intermediate, or as degradation products of the various conjugates that are generated along this bioactivation pathway. NMF and NEF are metabolised to both alkylamines and mercapturates, reflecting the extent to which they are oxidised in the formyl group.

It is possible that the alkylamines measured in this study resulted from the degradation of AMCC under the alkaline conditions that were required for derivatisation. Nevertheless, their existence provides additional evidence that DMF undergoes oxidative biotransformation in the formyl moiety - a metabolic event that might well be linked with hepatotoxicity.

The in vivo investigations reported here demonstrate that the rodent species studied metabolise DMF in a qualitatively similar manner. This results in the urinary
excretion of AMCC, HMMF, "F", methylamine, dimethylamine, and at high doses unchanged DMF. HMMF is generally found to be the major metabolite of DMF in rodents. The metabolism of DMF to AMCC in rodents is a pathway that was previously unknown. The existence of this pathway suggests that the hepatotoxicity of DMF is the result of metabolic activation. The extent of this biotransformation route supports the hypothesis that DMF lacks marked hepatotoxic properties in these species because it is only bioactivated at a very slow rate.
4.2 COMPARISON BETWEEN RODENTS AND HUMANS IN THE IN VIVO METABOLISM OF DMF

4.2.1 INTRODUCTION

Rodents are commonly used as models for studying the metabolism and toxicity of industrial chemicals. However, extrapolation of data from animal experiments to humans is often problematic due to species variation in the absorption, distribution, metabolism and elimination of chemicals (Travis, 1987).

In order to maximise the usefulness of data derived from animal studies, there is a need to identify any species differences in the biological fate of a chemical. The limitations of a particular animal model may thus be recognised, or a more appropriate model sought.

In the second part of this study potential differences between rodents and humans in the metabolism of DMF were examined. The comparative investigation reported in Section 4.1 was extended to include human data obtained in a collaborative study conducted by Dr Jaroslav Mraz of the Institute of Hygiene and Epidemiology, Prague, Czechoslovakia.

The questions addressed in this part of the study are:
(a) are there any qualitative differences between rodents and humans in the metabolism of DMF?
(b) are there any interspecies quantitative differences in the urinary metabolites of DMF?
(c) is there a difference between rodents and humans in the time-course of excretion of the metabolites?

4.2.2 RESULTS
Urine samples were collected from human volunteers exposed to airborne DMF (60mg/m³) for 8hr. The samples were analysed for metabolites using the same methods employed in this study. The actual amount of DMF absorbed by the individuals was calculated as 49.3 ± 11.6μmol/kg, which was just less than half the lowest dose administered to rodents in our study.

A comparison of the human data obtained by Dr Mraz with the rodent data previously reported in Section 4.1.2, using values from the lowest exposure group (0.1mmol/kg DMF), is represented in Figures 4.2.1 and 4.2.2.

The metabolites of DMF detected in human urine were qualitatively the same as those found in rodents - "NMF", "F", AMCC and some unchanged DMF. In humans "NMF" was the major metabolite, accounting for 25.9 ± 8.5% of the absorbed dose. "F" and AMCC were also major metabolites, representing 14.2 ± 5.2% and 14.5 ± 3.6% of the dose respectively. A small amount (0.7 ± 0.4%) was excreted as unchanged DMF.

The most obvious difference between the results from humans and those from rodents is the proportion of the dose excreted as AMCC (Figure 4.2.1B). In humans the
Figure 4.2.1
The urinary excretion of unchanged parent compound and AMCC in humans and rodents following either inhalation of 0.049mmol/kg (humans) or ip injection of 0.1mmol/kg DMF (rodents).
Values are the mean ± SD of 10 humans or 6 animals.
Figure 4.2.2
The urinary excretion of "NMF" and "F" in humans and rodents following either inhalation of 0.049 mmol/kg (humans) or ip injection of 0.1 mmol/kg DMF (rodents). Values are the mean ± SD of either 10 humans or 6 animals.
metabolism of DMF to AMCC was quantitatively a major pathway, whereas in rodents it amounted to 5.2% or less of the dose.

As different routes of exposure were used in the animal and human studies, ip injection and exposure to airborne DMF, an additional experiment was conducted by Dr Mraz in which he ingested DMF (0.02 or 0.1mmol DMF/kg). AMCC was measured in the urine accounting for 8.5% and 9.1% of the dose, respectively (Table 4.2.1A). After exposure to airborne DMF 15.8% was excreted as AMCC. "NMF" was also detected in the urine after oral ingestion and comprised between 17 and 18% of the dose, compared to 25.6% following inhalation. The pattern of urinary excretion of AMCC and "NMF" after oral ingestion of DMF (Figures 4.2.3A and 4.2.4A) was found to be very similar to the pattern following inhalation of DMF (results not shown). AMCC was detectable in the urine for up to 72hr after the end of exposure, with the maximum urinary concentration of this metabolite appearing 24hr after exposure.

The time-course of the urinary excretion of AMCC and "NMF" in the rat was compared with the human results (Figures 4.2.3B and 4.2.4B). In accordance with the human excretion of these metabolites, at the lower dose (0.7mmol/kg) "NMF" and AMCC appeared in the urine rapidly, reaching peak levels within 12hr and 24hr respectively. However, in contrast to the volunteer, 48hr after exposure to 0.7mmol/kg AMCC was not detectable in
(A) The excretion of AMCC and "NMF" in the urine of one male volunteer following the ingestion or inhalation of DMF.

(B) The excretion of AMCC and "NMF" in the urine of humans who were exposed to DMF in the workplace.

## URINARY METABOLITES (% of dose)

<table>
<thead>
<tr>
<th>Route of exposure</th>
<th>DMF dose (mmol/kg)</th>
<th>AMCC</th>
<th>&quot;NMF&quot;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oral</td>
<td>0.02</td>
<td>8.5</td>
<td>17.7</td>
</tr>
<tr>
<td>Oral</td>
<td>0.10</td>
<td>9.1</td>
<td>17.0</td>
</tr>
<tr>
<td>Inhalation</td>
<td>0.049</td>
<td>15.8</td>
<td>25.6</td>
</tr>
</tbody>
</table>

## Concentration of metabolites measured in urine

<table>
<thead>
<tr>
<th></th>
<th>AMCC (uM)</th>
<th>&quot;NMF&quot; (uM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>109</td>
<td>45</td>
</tr>
<tr>
<td>Range</td>
<td>10 - 590</td>
<td>14 - 112</td>
</tr>
</tbody>
</table>

Table 4.2.1
Figure 4.2.3
Time-course in the urinary excretion of AMCC in one male volunteer and in male Sprague Dawley rats following:
(A) ingestion of 0.02 or 0.1 mmol/kg DMF (human)
(B) ip injection of 0.1, 0.7 or 7.0 mmol/kg DMF (rat)
Values in (B) are the mean ± SD of 6 animals.
Figure 4.2.4
Time-course in the urinary excretion of "NMF" in one human volunteer and Sprague Dawley rats following:
(A) ingestion of 0.02 or 0.1 mmol/kg DMF (human)
(B) ip injection of 0.1, 0.7 or 7.0 mmol/kg DMF (rat)
Values in (B) are the mean ± SD of 6 animals.
rat urine. The time-course of both "NMF" and AMCC excretion in the rat was clearly affected by the dose, another feature which was absent in the human results.

In an additional study Dr Mraz analysed urine samples which had been collected from humans exposed to DMF in the workplace. AMCC and "NMF" were measured in the samples and values are shown in Table 4.2.1B. The levels of DMF in the workplace was not determined in this investigation.

4.2.3 DISCUSSION

When the comparative part of this study was extended to include the human data provided by Dr J Mraz, qualitative similarities were observed in the metabolism of DMF. In both human and rodent studies, "NMF", "F", AMCC and unchanged DMF were all measured as metabolites of DMF.

Quantitatively "NMF" and "F" were both found to be major urinary metabolites in humans and rodents. However, AMCC was also a major metabolite in humans but not in rodents. Nearly 15% of the absorbed dose was excreted as AMCC in humans compared to 5.2% or less in rodents. This suggests that in humans the metabolism of DMF to AMCC is more significant than in rodents.

The implications of this finding are profound. Previous investigations, already discussed in Section 4.1.3, have
led to the proposal that the metabolism of N-alkylformamides to mercapturates is a bioactivation pathway responsible for their hepatotoxic properties (Kestell et al, 1987). If this metabolic pathway is quantitatively more important in humans than in rodents, as has been indicated by the results presented here, then the hepatotoxic risk of DMF for humans is likely to be greater than that suggested from animal studies.

Caution should be exercised, however, when drawing conclusions from this comparative study. The amount of DMF absorbed by the human volunteers was calculated as 49.3umol/kg. In addition, exposure to DMF vapours would result in not only inhalation but also some skin absorption of the solvent. In the rodent studies used for comparison, the dose was 0.1mmol/kg, and was administered by ip injection. It is possible that these important variations between the treatments, that is the size of dose and the route of absorption, might have contributed to any differences in DMF metabolism.

These issues can be partly resolved by the results of the additional study performed by Dr Mraz, where he ingested DMF at 0.02 or 0.1mmol/kg. Whereas 15.8% of the dose was excreted as AMCC following exposure to DMF vapours, 8 to 9% of the orally administered dose was excreted as AMCC. This suggests that DMF metabolism to AMCC in humans is influenced to some extent by the route of absorption. Nevertheless, independent of the absorption route this
biotransformation route seems to be still quantitatively more important in humans than in rodents.

There is no apparent dose-dependence in the amount of AMCC excreted following oral absorption in the human volunteer. This leads us to assume that the different doses used in the rodent (0.1mmol/kg) and human studies (0.049mmol/kg) would not have significantly affected the extent of this metabolic pathway. Additionally, calculation of the amount of DMF vapour absorbed in the human study did not include an estimation for skin absorption. There is the possibility that the dose absorbed in the human study was somewhat in excess of 0.049mmol/kg.

The evidence presented here suggests that there are important species differences between rodents and humans in the metabolism of DMF. Although qualitatively similar, the existence of the metabolic pathway from DMF to AMCC as a major pathway in humans, but a minor pathway in rodents, indicates that the animal models investigated in these studies may well be inappropriate for estimating the risks for humans exposed to DMF.

The analysis of urine samples from humans who were exposed to DMF in the workplace clearly shows that AMCC was excreted in measurable amounts, generally exceeding the amount excreted as "NMF". This prompts the suggestion that urinary levels of AMCC might be a useful indicator
of exposure to DMF, an issue which is discussed in some detail in Section 5.

4.3.1 INTRODUCTION

DMF, measured at quantitatively the higher contents in experimental animals and in humans. Studies conducted have not been in agreement with this finding. It was noted that in some species had that "p" was excreted than "x".

As HDMF is the warrant some conclusions have hardly have been conducted by. Studies that examined the metabolism of DMF in rats. HDMF was found to be a major metabolite of DMF was largely excreted in urine. Toxic properties in these metabolites were those of DMF but less severe. HDMF was therefore concluded to be a toxicity of DMF.

Some attention has been paid to the (hydroxynaphth) of the precursors of DMF. (Overton et al., 1970).
4.3 IN VIVO METABOLISM OF HMMF, A MAJOR METABOLITE OF DMF, IN RODENTS

4.3.1 INTRODUCTION

HMMF, measured as "NMF" by GLC, has been shown to be quantitatively the major metabolite of DMF in experimental animals and in humans (see Section 1.4). The studies conducted here and reported in Section 4.1 are in agreement with this suggestion, although it was observed that in some species treated with low doses of DMF more "P" was excreted than "NMF".

As HMMF is the major metabolite of DMF its properties warrant some consideration. Its toxicity and metabolism have hardly been studied. A comparative study was conducted by Scailteur and Lauwerys (1984a) which examined the metabolism and toxicity of HMMF, NMF and DMF in rats. HMMF was found to be a stable compound and it was largely excreted unchanged in the urine. It possessed toxic properties in the rat that were less severe than those of NMF but more pronounced than in the case of DMF. HMMF was therefore concluded not to play a role in the toxicity of DMF.

Some attention has previously been focussed on N-(hydroxyalkyl) metabolites of some compounds as potential precursors of reactive, electrophilic iminium species (Overton et al, 1985). This possible bioactivation pathway, outlined below, was considered to be important
in the case of carbinolamines or carbinolamides which are relatively stable compounds.

As HMMF is a stable compound, it is conceivable that it may be bioactivated in this manner. Metabolic activation by the formation of iminium intermediates is understood to account for the toxicity of several compounds, for example the anti-tumour agent hexamethylmelamine (Nelson and Harvison, 1987). However there is no evidence which suggests that hydroxymethylformamides generate reactive iminium species via which NMF or DMF exert their toxicities.

The objective of these experiments was to clarify the role of HMMF in the hepatotoxicity of DMF. To this end the in vivo metabolic fate of HMMF was investigated in rodents. The questions addressed in these studies were:

(a) what is the metabolic fate of HMMF in rodents?
(b) is HMMF metabolised to AMCC in this species?
(c) does the metabolism of HMMF differ qualitatively or quantitatively to that of DMF?
4.3.2 RESULTS
The pattern of excretion of HMMF was established by measurement of the radioactivity recovered from the urine of BALB/c mice treated with HMMF labelled with $^{14}$C in the methyl group (Figure 4.3.1). At both doses most of the excreted radioactivity appeared in the urine within 24hr of treatment. The proportion of the dose excreted in the urine was $25.7 \pm 8.3\%$ (0.7mmol/kg) and $71.4 \pm 12.7\%$ (7.0mmol/kg). The poor recovery of the low dose was partly attributed to inadequate collection of urine samples as $15.7 \pm 4.7\%$ of the radioactivity was recovered from cage-washings on completion of this experiment. Even if this was taken into consideration, over 50% of the dose remained unaccounted for.

Identification of the radiolabelled metabolites was achieved by TLC autoradiography, described in Section 3.4.4.1. An example of an autoradiograph is shown in Figure 4.3.2. The metabolites had the same Rf values as authentic HMMF and F. The intensity of the radioactive spots suggests that at the higher dose most of the dose was excreted unchanged with only a small proportion metabolised to "F". The radioactivity remaining at the origin of the TLC autoradiograph indicated the presence of other metabolites.

GLC analysis of urine samples from animals treated with unlabelled HMMF was in agreement with the findings from the radioactivity studies. HMMF administered to hamsters
Figure 4.3.1
The excretion of radioactivity in the urine of BALB/c mice following a single ip injection of radiolabelled HMMF:
(A) time-course of excretion over 72hr
(B) total radioactivity recovered in the urine in 72hr.
Values are the mean ± SD of 6 animals
Radiolabelled HMMF  "NMF"  "F"

Authentic HM$[^{14}$C]MF  6 x urine samples

Solvent system: Chloroform/methanol (4:1 v/v)

Figure 4.3.2
TLC autoradiograph of urine samples from BALB/c mice which had received a single ip injection of HM$[^{14}$C]MF (7.0mmol/kg)
at either 0.7 or 7.0 mmol/kg was mainly excreted as "NMF" and "F" (Figure 4.3.3). At the higher dose, more was excreted as "NMF" (52.2 ± 8.5%) than as "F" (8.8 ± 1.4%). In the case of the low dose more "F" was excreted than with the high dose, accounting for 19.1 ± 2.7%. At this dose 38.8 ± 4.7% was excreted as "NMF". AMCC, following its derivatisation to EMC, was measured as a metabolite of HMMF at either dose. The amount excreted as AMCC in hamsters was 3.6 ± 0.4% (0.7 mmol/kg) or 4.6 ± 0.5% (7.0 mmol/kg). BALB/c mice, which had received 7.0 mmol/kg HMMF, excreted 1.2 ± 0.2% as AMCC within 48 hr. In the latter experiment, the other urinary metabolites were not quantified.

The excretion of HMMF and its metabolites in hamsters and mice was compared with the results from matched experiments with DMF, previously reported in Section 4.1.2. The results are represented in Figure 4.3.4. The proportion of the dose excreted as "NMF", "F" and AMCC in hamsters, whether HMMF or DMF was the parent compound, was in most cases very similar. Hamsters were observed to excrete slightly more of the higher dose as AMCC (4.6 ± 0.5%) than rats (1.5 ± 0.4%). In the case of mice, the amount of urinary AMCC formed from either compound was virtually the same.
Figure 4.3.3
Metabolites measured in the urine of male Syrian hamsters following a single ip injection of HMMF. Values are the mean ± SD of 6 animals.
Figure 4.3.4
Metabolites measured in the urine of hamsters or mice following a single ip injection of HMMF or DMF. Values are the mean ± SD of 6 animals.
4.3.3 DISCUSSION

In general HMMF was rapidly eliminated in the urine of mice and hamsters within 24hr of treatment. This is in agreement with the findings of Scailteur and Lauwerys (1984a). Mice were exceptional in that recovery of a dose of 0.7mmol/kg was less than 50%. This finding suggests that in mice, but not in hamsters, the extent of HMMF metabolism is influenced by the dose. It is of interest that when DMF was administered to mice at this and lower doses, recovery was similarly less than at high doses (see Section 4.1.2).

In both rodent species studied, "NMF", "F" and AMCC were measured in the urine. The metabolite measured as "NMF" by GLC is, as outlined in Section 1.4, assumed to be HMMF, that is unchanged parent compound. The higher dose of HMMF was largely excreted unchanged. In the case of the lower dose, a larger proportion was excreted as "F" than as unchanged parent compound. This indicates that the metabolism of HMMF to "F" is saturable. Scailteur and Lauwerys (1984a) similarly reported that administered HMMF was eliminated largely unchanged. In their study rats excreted 65% of the dose (1ml/kg) in this manner. The data presented here thus supports their conclusion that HMMF is a stable compound which is relatively resistant to further biotransformation.

It is conceivable that a proportion of the urinary "NMF" measured here actually was NMF and not HMMF. NMF could
result from breakdown or metabolism of HMMF. NMF was not detected as a metabolite of HMMF using analytical methods which allow differentiation between the two compounds (Scailteur and Lauwerys, 1984a). These authors proposed that any NMF generated during DMF metabolism does not result from further biotransformation of HMMF, but by a separate route.

In these experiments HMMF was shown to be metabolised to AMCC, although in all cases it was found to be a quantitatively minor pathway. This is the first time AMCC has been identified as a metabolite of HMMF. This finding suggests that HMMF is bioactivated in the same manner as DMF, and it follows that it may possess toxic properties. The toxicity of HMMF was not investigated here, although experiments conducted in our laboratories have reported HMMF to be non-toxic to isolated hepatocytes (R.Dayal-personal communication). In another study HMMF was found to be more toxic to rats than DMF, but less toxic than NMF. However, as in the case of DMF, HMMF did not cause depletion of hepatic GSH (Scailteur and Lauwerys, 1984a).

Comparison of results from HMMF metabolic studies with those previously obtained on DMF metabolism revealed many common features. At the same doses both compounds were excreted largely as "NMF" and "F" in approximately the same proportions. Both parent compounds were also metabolised to AMCC, and the extent of this biotransformation route was minor in both cases.
The qualitative and quantitative similarities in the metabolic fate of DMF and HMMF suggest that common biotransformation pathways exist for these two compounds. The following metabolic pathway is proposed (Figure 4.3.5) in which DMF is largely metabolised by hydroxylation of one methyl group to form HMMF. As this carbinolamine is a relatively stable compound, much of the administered dose is excreted as this metabolite. However, a proportion of the HMMF generated is further metabolised in two ways. One pathway involves hydrolysis of HMMF to NMF and oxidation of the formyl group which leads to SMG and AMCC. This is likely to be a bioactivation pathway. The other route leads to the formation of the metabolite measured as "P".

As the biotransformation route leading to AMCC formation is probably linked to DMF hepatotoxicity, data presented here provides evidence that HMMF is likely to be an intermediate in the metabolic activation of DMF. Other metabolic events in this toxification pathway remain to be elucidated and are explored in the remainder of this study.
Figure 4.3.5
Suggested biotransformation route of DMF which involves HMME as a metabolic intermediate.
4.4 INVESTIGATIONS OF THE IDENTITY OF THE DMF METABOLITE 
"F"

4.4.1 INTRODUCTION

DMF is metabolised to a species which is detected in the urine as formamide by GLC. The existence of "F" as a minor metabolite of DMF has frequently been reported in animal studies (see Section 1.4). The in vivo studies conducted here (Section 4.1) have also demonstrated the existence of "F" as a urinary metabolite of DMF. Interestingly, when DMF was administered at low doses "F" was quantitatively more important than in the case of high doses, particularly in rats (see Figures 4.1.14 and 4.1.15).

The metabolite HMMF is known to undergo thermal decomposition during GLC analysis to NMF (see Section 1.4). By analogy "F" has been considered as being derived from the metabolite HMF in the following manner:

\[
\begin{align*}
\text{HMF} & \quad \xrightarrow[170^\circ C \text{ or pH 9}]{\text{170}^\circ C} \quad \text{HCHO} \\
\text{O} & \quad \text{C} - \quad \text{N} - \quad \text{CH}_2\text{OH} & \quad \text{O} & \quad \text{C} - \quad \text{N} & \quad \text{H} \\
\text{H} & \quad \text{H} & \quad \text{H} & \quad \text{H} & \quad \text{H} & \quad \text{H} \\
\end{align*}
\]

This proposal is based entirely on indirect evidence, originating from knowledge of the thermal degradation of HMMF, and from measurement of HCHO-precursors following alkaline hydrolysis in the urine of treated animals (Brindley et al, 1983; Scailteur et al, 1984; Scailteur
and Lauwerys, 1984a). This method for detecting HCHO-precursors lacks specificity as HMF could conceivably be generated by the oxidation of any NMF produced from HMMF (Scailteur et al, 1984). Alternatively N-hydroxymethyl compounds could be formed as a result of HCHO reacting with endogenous urinary constituents (Brindley et al, 1983).

Conclusive analytical evidence in support of HMF being the precursor of "F" is so far lacking. The following experiments were conducted with the objective of clarifying the nature of this urinary metabolite of DMF.

4.4.2 RESULTS
The experiments reported here used urine from BALB/c mice which had received either 0.7 or 7.0mmol/kg of radiolabelled DMF.

The radioactivity associated with urinary metabolites of D[14C]MF which had been previously identified by TLC autoradiography (see Section 1.2) was determined by the method described in Section 3.4.4.4. This was compared with amounts of the same metabolites measured in the unlabelled form by GLC (Figure 4.4.1). The quantity of unlabelled "NMF" correlated well with its radiolabelled counterpart, as did the amount of unchanged DMF measured. However, the metabolite measured by GLC as "F" accounted for 1.5 to 2 times the value that was obtained by radioactivity counting.
Figure 4.4.1
Comparison of the amounts of metabolites measured in the radiolabelled form or the unlabelled form in the urine of BALB/c mice following a single ip injection of DMF

- Radiolabelled metabolites were measured by the method described in Section 3.4.4.4
- Unlabelled metabolites were measured by GLC (Section 3.4.5)

Values are the mean ± SD of 6 animals
This finding indicates that between one third and one half of what was measured as "F" by GLC did not exist in the radioactive form. That is, it did not contain the $^{14}$C-label derived from the parent compound. Therefore the "F" appears to contain only the $^{14}$C atom derived from half to two-thirds of the total methyl moieties in the parent DMF molecule. If the metabolite contained 50% of the DMF-derived radioactivity, this would suggest that it possessed one methyl moiety and is probably HMF. If the metabolite contained 100% of the radioactivity derived from the parent compound, this would indicate that it possessed two methyl moieties. This latter compound could possibly be bis-HMF.

In the second part of this investigation the amount of radioactive formaldehyde released from a known quantity of isolated "F" metabolite was determined (see Section 3.8.3). This was compared with the maximum radioactivity that would be contained in the "F" that was measured by GLC (see Section 3.8.2) should each molecule contain two methyl moieties, that is should it be bis-HMF. The results from four different urine samples are shown in Table 4.4.1. The amount of radiolabelled formaldehyde that was generated was $50.8 \pm 7.6\%$ of the maximum radioactivity of calculated for "F".
<table>
<thead>
<tr>
<th>Sample No</th>
<th>[¹⁴C]-HCHO recovered (dpmi)</th>
<th>&quot;F&quot; measured by GLC (μM)</th>
<th>&quot;F&quot; measured by GLC (dpmi)</th>
<th>(A) as % of (B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>83441</td>
<td>0.127</td>
<td>173234</td>
<td>48.2</td>
</tr>
<tr>
<td>2</td>
<td>72212</td>
<td>0.093</td>
<td>127078</td>
<td>56.8</td>
</tr>
<tr>
<td>3</td>
<td>19777</td>
<td>0.036</td>
<td>33741</td>
<td>58.6</td>
</tr>
<tr>
<td>4</td>
<td>8057</td>
<td>0.022</td>
<td>20443</td>
<td>39.4</td>
</tr>
</tbody>
</table>

Mean ± SD = 50.8 ± 7.6

The amount of "F" would thermally be formed as formaldehyde.

It is conceivable that the "F" measured by GLC measured "F" metabolites or the "F" generated by formaldehyde dehydrogenase.

The amount of "F" measured by GLC measured "F" metabolites or the "F" generated by formaldehyde dehydrogenase.

More specific measures only metabolite A. The results show that metabolite A is the metabolite generated by formaldehyde dehydrogenase.

The findings and the results provide further indication for the possibility that the "F" measured by GLC measured "F" metabolites or the "F" generated by formaldehyde dehydrogenase.

Table 4.4.1

The release of [¹⁴C]-labelled HCHO from "F" isolated from urine samples from four BALB/c mice which had received a single injection of D[¹⁴C]MF (7.0mmol/kg), and comparison with the amount of "F" measured by GLC.
4.4.3 DISCUSSION

Previous reports suggest on the basis of indirect evidence that the metabolite measured as "F" may be derived from HMF (see Section 1.4). The first study reported here gives an indication of the number of radiolabelled methyl moieties derived from the parent compound which are contained in the metabolite. The observation that more than half of the "F" measured contains radioactivity raises the possibility that some of the "F" could exist as bis-HMF. Bis-HMF could be generated by the enzymatic C-hydroxylation of HMMF, and would thermally degrade to F in the same manner as HMF. It is conceivable that both bis-HMF and HMF could be formed as metabolites of DMF.

The amount of HCHO generated from the "F" metabolite in the second part of this investigation was found to be half of what would have been generated should the metabolite be bis-HMF. This HCHO-release experiment is more specific than those previously reported in that it measures only radioactive HCHO and uses isolated metabolite as the only source. The potential interference from other potential HCHO-precursors is thus minimised. The results from this experiment clearly suggest that HMF is the metabolite.

The findings of these studies, although briefly raising the possibility of bis-HMF being a metabolite of DMF, provide more evidence which favours HMF as the
Nevertheless, the evidence is still indirect and confirmatory work is still required.

1.1 INTRODUCTION

Previous studies described experiments confirming the existence of an activation pathway and a metabolic route presumably leading to the urinary excretion of a metabolite which is thought to be responsible for the hepatotoxicity of the compound.

The nature of this metabolite was tentatively suggested by several authors in 1987: Pearson et al., Baillie et al., and others. It was eventually identified as N-methylcarbamoyl acetamide, a metabolic precursor of the compound metabolized to N-acetyl-N-methylcarbamoyl acetamide, shown below.

These studies were conducted in isolated hepatocytes from the respective species.
4.5 IN VITRO METABOLISM OF N-ALKYLFORMAMIDES BY BALB/C MOUSE HEPATIC MICROSOMES

4.5.1 INTRODUCTION

previous studies described in Section 1.4 and 1.5, and in vivo experiments conducted here (see Section 4.1), have confirmed the existence of what is considered to be a bioactivation pathway for N-alkylformamides. This metabolic route proceeds via GSH conjugation and leads to the urinary excretion of AMCC. The biotransformation pathway is thought to involve the formation of a reactive metabolite which is ultimately responsible for the hepatotoxicity of these compounds.

The nature of this reactive species has so far avoided identification. Methyl isocyanate (MIC) has been tentatively suggested as a possibility (Kestell et al, 1987; Pearson et al, 1987a; Threadgill et al, 1987; Baillie et al, 1989). This subject is discussed at greater length in Sections 4.8 and 4.9.

S-(N-Methylcarbamoyl)glutathione (SMG) has been identified as a biliary metabolite of NMF in BALB/c mice (Threadgill et al, 1987) and is likely to be the metabolic precursor of AMCC. NMF and NEF were shown to be metabolised to N-alkylcarbamoylating species in vitro in isolated hepatocytes (Shaw et al, 1988). The identity of these species was considered to be SMG and S-(N-ethylcarbamoyl)glutathione (SEG) respectively.
However, the metabolic precursor or precursors of the GSH-conjugates remain unidentified, as do other details of this bioactivation pathway. The experiments reported in this part of the study were conducted with the objective of establishing the details of metabolic events which lead to the formation of N-(alkylcarbamoyl) glutathione conjugates of N-alkylformamides. By using hepatic microsomes as an in vitro model for metabolism, we investigated:

(a) whether mouse hepatic microsomes in the presence of GSH metabolise N-alkylformamides to their corresponding N-(alkylcarbamoyl)glutathione conjugates;
(b) the cofactor requirements of this metabolic step;
(c) the effect of time of incubation and substrate concentration on the formation of the GSH-conjugate.

4.5.2 RESULTS
The in vitro experiments were initially conducted using NMF as the substrate, since NMF is known to be metabolised to AMCC in vivo (Kestell et al, 1987) and to SMG in isolated hepatocytes (Shaw et al, 1988; Baillie et al, 1989).

Preliminary experiments, conducted in the Department of Medicinal Chemistry, University of Washington, Seattle, were unsuccessful in finding any microsomal metabolism of NMF to SMG. Hepatic microsomes prepared from BALB/c mice, incubated with NMF in the presence of GSH, NADPH and air
for periods up to 45 min, did not result in the formation of measurable amounts of SMG.

Subsequent experiments conducted in the laboratories at Aston University, using conditions identical to those used previously, demonstrated substantial metabolism of NMF to SMG. Measurement of SMG was achieved by GLC following its conversion to EMC (see Section 3.5.3 and 3.9.3). Typical chromatograms are shown in Figure 4.5.1.

Following the demonstration that NMF is metabolised to SMG in microsomal incubations, the conditions employed were modified as described in Section 3.10 with the objective of establishing which cofactors were necessary for this biotransformation pathway. The effect of these modifications on the formation of SMG is illustrated in Figures 4.5.2 and 4.5.3.

The GLC peak corresponding to SMG was absent when fresh microsomes were substituted with heat-inactivated microsomes. The formation of SMG was also inhibited when either GSH or NADPH were omitted from the incubations. When the incubations were conducted in an atmosphere of nitrogen instead of air, no measurable generation of SMG was observed. These experiments illustrate the requirement for GSH, NADPH and air in order to obtain in vitro microsomal metabolism of NMF to SMG. Therefore subsequent incubations were conducted in the presence of these cofactors.
Figure 4.5.1
GLC chromatograms of derivatised microsomal samples following incubation with NMF (10mM) for 0, 20 and 60min
Figure 4.5.2
GLC chromatograms of derivatised microsomal samples following incubation with NMF (10mM):
(A) control; (B) using heat-inactivated microsomes;
(C) conducted in an atmosphere of N₂
Figure 4.5.3

GLC chromatograms of derivatised microsomal samples following incubation with NMF (10mM): (A) control; (B) omitting GSH; (C) omitting NADPH
The generation of SMG from NMF in microsomal incubations was quantitated during time periods of up to 60min. Substrate concentrations of 1, 5 or 10mM were used. The results are shown in Figure 4.5.4. The dependency of SMG formation on substrate concentration was clearly demonstrated. The amount of metabolite measured increased with time up to 60min.

Further incubations were conducted using either DMF as the substrate or its metabolite, HMMF. Using identical conditions as those used for NMF, there was no evidence of SMG formation from DMF. Even when the derivatisation procedure was modified to include extraction into Pr-1-OH instead of EtOH, which facilitated better GLC separation of the carbamate from DMF, the conjugate was not detected.

When HMMF was included as the substrate, using conditions identical to those used in the case of NMF, a small peak corresponding to derivatised SMG was observed (Figure 4.5.5A). In comparison to NMF, HMMF was metabolised to SMG to a much smaller extent (Figure 4.5.5B). Whereas 16.0 ± 1.2nmol SMG/mg protein was formed from NMF (10mM) in 60min, the same concentration of HMMF generated only 1.7 ± 0.04nmol/mg protein. The maximum amount of SMG was formed from HMMF after 20min, and this was unaffected by the concentration of substrate used.
Figure 4.5.4
The metabolism of NMF to SMG by BALB/c mouse hepatic microsomes, incubated in the presence of GSH and NADPH. Values are the mean ± SD of 6 incubations.
(A) GLC chromatogram of derivatised microsomal sample

Incubation period - 0min

Incubation period - 60min

"NMF"

EMC

IS

(B) Measurement of SMG

![Graph showing SMG formation over time with 5mM and 10mM HMMF](image)

**Figure 4.5.5**
The metabolism of HMMF to SMG by BALB/c mouse hepatic microsomes, incubated in the presence of GSH and NADPH. The values in (B) are the mean ± SD of 3 incubations.
4.5.3 **DISCUSSION**

These experiments have demonstrated that NMF and HMMF are oxidised by mouse hepatic microsomes in the presence of GSH to form SMG. The extent of the metabolism of NMF to SMG was far greater than in the case of HMMF, and was linear with time.

The *in vitro* biotransformation of NMF to SMG requires the presence of microsomal enzymes, NADPH and air. This suggests that an enzymatic oxidative step is involved which is perhaps catalysed by cytochrome P-450. This is a significant finding as previously the role of metabolising enzymes in the metabolic activation of formamides was unknown. More detailed and specific investigations of the role of cytochrome P-450 in this metabolic pathway are reported in Section 4.8.

In addition to these cofactors, the *in vitro* metabolism of NMF to SMG requires the inclusion of GSH. This prompts two possibilities for the role of GSH. Firstly, NMF is oxidised to an unstable intermediate which is only detectable when GSH is present to trap it. This is consistent with the role of GSH in the detoxification of electrophilic intermediates (Kosower, 1976; Reed and Beatty, 1980). It has been suggested that methyl isocyanate (MIC) is the reactive species in \(N\)-alkylformamide toxicity (Baillie et al, 1989; Kestell et al, 1987; Pearson et al, 1987a; Threadgill et al, 1987). It is conceivable that should NMF be metabolised to MIC
by microsomes in the absence of GSH, this highly reactive metabolite would be hydrolysed by water in the following manner, and thus escape detection:

\[
\begin{align*}
\text{NMF} & \xrightarrow{\text{MIC}} \left[ \text{O} - \text{C} - \text{N} - \text{CH}_3 \right] + \text{H}_2\text{O} \\
\text{N-methyl carbamic acid} & \rightarrow \left[ \text{O} - \text{C} - \text{N} - \text{H} \right] + \text{CO}_2
\end{align*}
\]

The second proposal for the role of GSH is that the microsomal oxidation of NMF is actually dependent on the presence of GSH. That is, oxidation of NMF and conjugation take place simultaneously and no reactive intermediate is involved. In this reaction GSH would function as a cofactor for the oxidative enzymes. This novel mechanism has previously been considered by Threadgill et al (1987).

Conjugation of electrophilic intermediates with GSH may occur either enzymatically, involving GSH-s-transferases, or non-enzymatically (Ketterer, 1982). The role of GSH-s-transferases in the in vitro generation of SMG from NMF has not been investigated in these studies. However, as GSH-s-transferases are largely present in the cytosol, the substantial metabolism of NMF to SMG by microsomes in the absence of the cytosolic fraction suggests that these enzymes are unimportant in this metabolic pathway.
The derivatisation technique employed in these experiments (see Section 3.5.3 and 3.9.3) converts all N-alkylcarbamoylating species to ethyl N-alkyl carbamate, in this case EMC. It is possible that some EMC measured originates from other N-alkylcarbamoylating metabolites formed in the microsomal incubations in addition to SMG. Glucuronide conjugate might be such a species. Nevertheless, incubations conducted in the absence of GSH demonstrated no metabolism of NMF to any other detectable carbamoylating compounds. Therefore the assumption is made here that any EMC measured is entirely derived from SMG.

The lack of measurable microsomal metabolism of NMF to SMG when identical experiments were conducted in the University of Washington laboratories is interesting. One explanation is that the metabolising ability of the microsomes used differed, even though BALB/c mice were used in both laboratories. If cytochrome P-450 is involved in the metabolism of NMF to SMG, it is conceivable that the isozyme(s) concerned is lacking in the American mice.

Although DMF is known to be metabolised to AMCC in vivo in mice (see Section 4.1), in these experiments there was no measurable generation of SMG, its metabolic precursor. This is consistent with the lack of in vitro DMF metabolism to SMG in isolated hepatocytes (Shaw et al, 1988). There are several possible explanations for this
observation. The extent of the biotransformation of DMF to SMG in vitro could be so small as to escape detection. The minor extent of the metabolism of DMF to AMCC in vivo supports this suggestion. Nevertheless, HMMF generated SMG in vitro in these experiments in detectable amounts. As the in vivo generation of AMCC from either DMF or HMMF was quantitatively similar (see Section 4.3.2), it might be envisaged that these two substrates would behave similarly in vitro. DMF is presumably biotransformed to HMMF before forming SMG (see Section 4.3.3). Indeed DMF was metabolised to "NMF", presumably HMMF, by microsomes in these experiments, although the amount was not quantified. Scailteur et al (1984) reported that the metabolism of DMF to HMMF by rat hepatic microsomes was far less than expected from the in vivo generation of HMMF. In their studies the in vitro formation of HMMF from DMF was slow and increased in a linear fashion for approximately 2hr. The slow biotransformation of DMF in vitro is in accordance with the lack of generation of SMG observed here.

The microsomal generation of SMG from NMF was substantially more than from HMMF. These in vitro results correlate with the extent of metabolism of these two compounds to AMCC in vivo. The proportion of the NMF dose metabolised to AMCC in mice was 16% (Kestell et al, 1985) compared to 1.2 ± 0.2% in the case of HMMF (see Section 4.3.2). As HMMF is largely excreted unchanged in vivo (Section 4.3.2) and only metabolised to SMG to a small
extent in vitro, it appears to be relatively resistant to metabolism by microsomal enzymes. The observations that SMG generation from HMMF was unaffected by substrate concentration, and that after 20 min no further metabolism was apparent, are consistent with this suggestion.

In summary, NMF is oxidised by hepatic microsomes in the presence of GSH and NADPH to form SMG. HMMF, the major metabolite of DMF, but not DMF itself, is also metabolised by microsomes to SMG albeit to a far lesser extent. Mouse hepatic microsomes seem to provide a useful model for reproducing the bioactivation pathway of N-alkylformamides in vitro. This metabolic model system is used in further exploratory studies which are reported in the next chapter.
4.6 IN VITRO METABOLISM OF N-ALKYLFORMAMIDES BY HUMAN HEPATIC MICROSONES

4.6.1 INTRODUCTION
Extrapolation of data on the metabolism of chemicals in animals to humans is often complicated due to marked species differences in the metabolic fate of the compounds. Indeed, comparative studies of the in vivo metabolism of DMF reported in Section 4.2 demonstrated a marked quantitative difference between humans and rodents in the metabolic pathway which is linked to DMF toxicity. Access to human metabolic data is therefore useful, although generally in vivo studies are impossible due to ethical considerations. In vitro metabolic experiments using human tissue can provide important information which may then be correlated with in vitro animal data and results from in vivo studies.

The in vitro metabolism of N-alkylformamides using mouse hepatic microsomes has been reported in Section 4.5. In this part of the study experiments were conducted using microsomes prepared from human liver samples. The objectives of these experiments were twofold:
(a) to elucidate whether NMF, DMF or HMMF are metabolised to SMG by human hepatic microsomes;
(b) to study whether the quantitative difference between mice and humans in the metabolism of DMF to AMCC in vivo is reflected in the extent of DMF biotransformation to SMG in vitro.
4.6.2 RESULTS

Incubations of microsomes prepared from human liver samples were conducted using the same conditions used in the case of mouse hepatic microsomes (Section 3.9.2). The formation of SMG from the substrates NMF (10mM), DMF (5 or 10mM) and HMMF (10mM) was measured over a period of 60min.

NMF, in the presence of GSH and NADPH, was metabolised by human microsomes to SMG (Figure 4.6.1). The amount of conjugate measured in the microsomes from the male donor (40.3nmol/mg protein) was much greater than in the second human, a female (10.8nmol/mg protein). SMG formation in the microsomes from the man was also markedly greater than in mouse microsomes over the same period of time (16.0 ± 1.2nmol/mg protein). In both cases SMG generation was linear with time.

When DMF was used as substrate, at a concentration of either 5 or 10mM, SMG was not detected in the case of either donor. However, DMF was shown to be metabolised to "NMF", presumably HMMF, by both samples of microsomes. The amount of "NMF" generated was not quantified.

Incubation of human microsomes with HMMF and the necessary cofactors demonstrated the formation of SMG. With microsomes from the male donor (Figure 4.6.2), the amount of SMG formed from HMMF was substantially less than when NMF was used as the substrate. After incubation
Figure 4.6.1
The metabolism of NMF (10mM) to SMG by microsomes from two human liver samples, incubated in the presence of GSH and NADPH. The donors were both healthy young adults, one male and one female. Incubations were conducted in duplicate.
Figure 4.6.2
The metabolism of HMMF (10mM) to SMG by microsomes from two human liver samples, incubated in the presence of GSH and NADPH. Incubations were conducted in duplicate.
for 60min, 5.6nmol SMG/mg protein was formed from HMMF, compared to 40.3nmol/mg protein in the case of NMF. In the second experiment, using microsomes from the female donor, there was some evidence of formation of the conjugate from HMMF. However the amount was small and amounted to less than 1.0nmol/mg protein.

4.6.3 DISCUSSION

Human hepatic microsomes, in the presence of GSH, NADPH and air, were found to metabolise NMF to SMG. HMMF was also metabolised to SMG, albeit to a lesser degree.

The two samples of liver tissue obtained, both from healthy humans of similar age, one male and one female, demonstrated a marked quantitative difference in their ability to metabolise NMF and HMMF. The capacity of the microsomes from the male donor to metabolise NMF to SMG was nearly four times that of the microsomes from the female donor.

Several in vitro studies have reported wide variations in the activities of various drug metabolising enzymes using human liver microsomes (Kapitalnik et al, 1977; Lorenz et al, 1984; von Bahr et al, 1980). There is similarly a marked variability between individuals in the metabolism of drugs in vivo, which may result from both genetic and environmental factors (Vessell, 1983). Therefore it is unsurprising that the apparent ability of hepatic
microsomes from these two human donors to metabolise NMF or HMMF to SMG show clear quantitative differences.

The metabolising capacity of the two liver samples could have been usefully quantified by either measuring the cytochrome P-450 content or the extent of aminopyrine N-demethylation (see Sections 3.9.6 and 3.9.7). These standard tests were employed in studies reported in Section 4.8. The limited amount of human liver tissue available prevented such control experiments.

Quantitative comparison of results from this experiment with the in vitro animal data (see Section 4.5.2) showed that hepatic microsomes from the male donor metabolised NMF to SMG at a rate which was more than twice that observed in mouse microsomes. Hepatic microsomes from the second donor demonstrated a reduced ability to metabolise NMF compared to mouse microsomes. The amount of SMG measured within 60 min in the case of the female donor was approximately 60% of that formed when incubations were conducted with mouse liver microsomes. The greater extent of metabolism of NMF to SMG in the microsomes of the male donor is surprising in view of the generally lower drug metabolising enzyme activities observed in human liver compared to animal liver (Lorenz et al, 1984).

The rate of formation of SMG from NMF in the case of both human and mouse hepatic microsomes was paralleled by the generation of SMG from HMMF, although in significantly
smaller amounts. Therefore the differential metabolising capacity of the different samples of microsomes appeared to effect both substrates, NMF and HMMF, to a similar extent.

As in the case of mouse hepatic microsomes, when DMF was used as the substrate there was no detectable metabolism to SMG by human liver microsomes. However, DMF was metabolised to "NMF". The explanations presented in Section 4.5.3 may therefore also apply here. However, in view of the greater in vivo metabolism of DMF to AMCC in humans compared to rodents (see Section 4.2), it might have been anticipated that this quantitative difference would be reflected in these in vitro studies. That is, DMF would be metabolised to SMG by human hepatic microsomes to a greater extent than by mouse liver microsomes. As there was no detectable formation of SMG from DMF with mouse microsomes, one might have expected some SMG to be formed with human microsomes.

The quantitative species difference in the in vivo metabolism of DMF to AMCC is not reflected in the in vitro microsomal formation of SMG, the metabolic precursor of AMCC. Therefore it could be argued that the difference between rodents and humans in DMF metabolism along this pathway does not originate from the differential ability of the hepatic microsomes to metabolise DMF.
In conclusion, hepatic microsomes from both humans and mice, have been shown to perform \textit{in vitro} part of what is considered to be the bioactivation pathway for N-alkylformamides \textit{in vivo}. Hepatic microsomes can thus provide a useful \textit{in vitro} system for studying the metabolic activation of these compounds.
4.7 INVESTIGATION OF SPECIES AND STRAIN DIFFERENCES IN
THE MICROSOMAL METABOLISM OF NMF

4.7.1 INTRODUCTION

In vivo studies of the hepatotoxicity of N-alkylformamides in animals, previously described in Section 1.3 and 1.5, had demonstrated both strain and species variation.

BALB/c mice have been shown to be more vulnerable to NMF-induced liver damage than either CBA/CA or BDF1 mice, and correspondingly demonstrated the most marked depletion in hepatic GSH levels (Langdon et al, 1985; Pearson et al, 1987a). A comparative study of NMF toxicity in mice and rats demonstrated marked strain variations (Tulip and Timbrell, 1988). The hepatotoxic dose of NMF in BALB/c mice was approximately one fifth of that which induced marginal liver damage in Sprague Dawley rats. Similarly, NMF depleted hepatic non-protein sulphydryls in mice but not in rats.

These investigations suggest that the differing sensitivities of experimental animals to NMF-induced hepatotoxicity are a consequence of differences in their metabolism of NMF. The following experiments were conducted with the objective of establishing whether these strain and species variations have an identifiable metabolic origin. The questions addressed in this part of the study were:
(a) is NMF metabolised to SMG in vitro using hepatic microsomes from either BALB/c mice, CBA/CA mice and Sprague Dawley rats?
(b) does the extent to which NMF is biotransformed to SMG in vitro reflect the species and strain differences observed in NMF-induced hepatotoxicity in vivo?

4.7.2 RESULTS

NMF, in the presence of GSH, was metabolised to SMG by hepatic microsomes from BALB/c and CBA/CA mice, and from Sprague Dawley rats. The amount of SMG generated in these in vitro experiments is illustrated in Figure 4.7.1. In each case the quantity of SMG formed increased with time in a linear fashion.

Comparison of the amount of SMG measured does not indicate a marked difference between the two murine strains. After a 60 min incubation period, the amount of SMG formed with BALB/c and CBA/CA mouse microsomes was 30.9 ± 5.6 and 38.2 ± 8.8nmol/mg protein respectively.

NMF metabolism to SMG in the case of Sprague Dawley rat microsomes was significantly less than with either mouse strain. The amount of SMG generated in 60min was 18.0 ± 3.2nmol/mg protein, which amounts to about half of that formed by mouse microsomes.
**Figure 4.7.1**

The metabolism of NMF to SMG by hepatic microsomes from BALB/c mice, CBA/CA mice or Sprague Dawley rats, incubated in the presence of GSH and NADPH. Values are the mean ± SD of 4 incubations.
4.7.3 DISCUSSION

The hepatotoxic effects of NMF in experimental animals has been shown to be dependent on both strain and species (Langdon et al, 1985; Pearson et al, 1987a; Tulip and Timbrell, 1988). This differential hepatotoxicity of NMF could be a result of the capacity of the microsomal enzymes to activate the compound in vivo. Therefore it might be anticipated that the strain and species differences would be reflected by the extent to which NMF was metabolised to SMG in vitro.

There was no marked strain difference in the extent to which NMF was metabolised to SMG by hepatic microsomes from either BALB/c or CBA/CA mice. This suggests that the greater susceptibility of BALB/c mice to NMF-induced hepatotoxicity is not a consequence of the increased ability of the microsomal enzymes to bioactivate NMF. The strain difference is likely to be a result of some other factor which is not demonstrable in this in vitro model.

The extent of NMF metabolism to SMG by rat microsomes was much less than in the case of both murine strains. This result correlates with in vivo observations which show rats to be relatively insensitive to NMF-induced hepatotoxicity (Tulip and Timbrell, 1988). The findings presented here suggest that the reduced hepatotoxicity of NMF in rats compared to mice is a consequence of the reduced ability of rat microsomal enzymes to metabolise NMF.
Extending these in vitro findings to the in vivo situation, it might be anticipated that rats would excrete a smaller proportion of NMF as AMCC than mice. Tulip and Timbrell (1988) found this not to be the case, both species excreting similar amounts of AMCC. However, mice excreted more methylamine than rats. Methylamine is a metabolite of NMF which is considered to be formed as a result of the same oxidative step which leads to SMG and AMCC (Threadgill et al., 1987). Methylamine may be formed from an unstable toxic intermediate, or as a degradation product of either AMCC or SMG. The greater extent of the oxidative metabolism of NMF in mice compared to rats in vivo (Tulip and Timbrell, 1988), resulting in methylamine and AMCC, may originate from the increased metabolising capacity of microsomal enzymes.

This latter point might prompt a comparative examination of the extent of methylamine and AMCC excretion in BALB/c and CBA/CA mice, which could be correlated with these in vitro results.

In conclusion, in vitro studies of NMF metabolism in rats and mice indicate that species differences in NMF-induced liver damage may originate from differential ability of microsomal enzymes to metabolise NMF. Conversely, strain differences in NMF toxicity between the two murine strains do not appear to arise from the activity of microsomal enzymes, but from some other cause which is not apparent in these in vitro experiments.
4.8 STUDIES OF THE INVOLVEMENT OF CYTOCHROME P-450 IN THE METABOLIC ACTIVATION OF N-ALKYLFORMAMIDES

4.8.1 INTRODUCTION

Experiments described in Sections 4.5, 4.6 and 4.7 have shown that NMF is oxidised by mouse, rat and human hepatic microsomes, in the presence of GSH, to form SMG. The oxidative metabolism of NMF is thought to occur in the formyl moiety and to lead to the generation of a reactive species which is ultimately responsible for hepatotoxicity (Kestell et al, 1987; Threadgill et al, 1987). The identity of the toxic species, or any other precursors of SMG, are unknown. Similarly the enzymatic nature of the oxidation of NMF is unconfirmed.

Many drugs and industrial chemicals are now recognised as being metabolically activated by cytochrome P-450. Examples of such compounds include bromobenzene, paracetamol, carbon tetrachloride, benzene, and butylated hydroxytoluene (Monks and Lau, 1988; Nelson and Harvison, 1987).

Preliminary evidence suggests that cytochrome P-450 is involved in the microsomal oxidation of NMF (see Section 4.5). The requirements for NADPH, air and microsomal enzymes for the biotransformation of NMF to SMG are all characteristic of cytochrome P-450 involvement. However, they are not specific for cytochrome P-450 and further confirmatory evidence is required. A range of experimental methods can be employed to establish
cytochrome P-450 involvement in metabolic pathways (for review see Burke, 1981). Additional evidence for cytochrome P-450 involvement may be obtained by monitoring the incorporation of molecular oxygen, as the $^{18}$O form, into metabolically generated SMG.

The following experiments were conducted with the objective of establishing whether cytochrome P-450 is involved in the bioactivation of N-alkylformamides. The following specific questions were asked:

(a) is the microsomal metabolism of NMF to SMG inhibited by carbon monoxide?

(b) what is the effect of the known inhibitor of cytochrome P-450, SKF 525-A, on the microsomal biotransformation of NMF and HMMF?

(c) do phenobarbitone (PB) and beta-naphthoflavone (BNF), inducers of P-450 and P-448 respectively, effect the formation of SMG from NMF or HMMF?

(d) is atmospheric $^{18}$O incorporated into SMG generated from NMF by microsomes?

4.8.2 RESULTS

Experiments in this section were all conducted using BALB/c mouse hepatic microsomes with NMF (10mM) as the substrate. As HMMF is also metabolised to SMG by hepatic microsomes (see Section 4.5), the effect of SKF 525-A, PB and BNF on its biotransformation was also examined.
Aminopyrine N-demethylation was inhibited by an atmosphere of CO/air (1:1) to 60% of the control value (Figure 4.8.1A). The same atmosphere completely inhibited the microsomal metabolism of NMF to SMG (Figure 4.8.1B). Control values were determined from incubations conducted in an atmosphere of nitrogen/air (1:1). This allowed for any reduction in metabolism which might result from lowered partial pressure of air. Metabolism of NMF and aminopyrine were only 28% and 11% higher than controls, respectively, when incubations had unrestricted access to air.

The extent of the inhibition of aminopyrine N-demethylation was shown to be dependant on SKF 525-A concentration (Figure 4.8.2). Addition of SKF 525-A (0.5mM) to microsomal incubations reduced aminopyrine metabolism only to 84% of the control value. This concentration of SKF 525-A had no inhibitory effect on NMF metabolism (Figure 4.8.3). Subsequent experiments were performed using SKF 525-A at a final concentration of 3.0mM. At this concentration SKF 525-A reduced aminopyrine N-demethylation to 42% of the control value and resulted in a substantial decrease in the metabolism of NMF to SMF (Figure 4.8.4). After 60min SMG generation was reduced by SKF 525-A to 36% of the control value. In all cases SKF 525-A was added to microsomal incubations 5min prior to the addition of substrate.
Figure 4.8.1
The effect of carbon monoxide on the activity of microsomal enzymes:
(A) aminopyrine (5mM) N-demethylation
(B) the metabolism of NMF (10mM) to SMG.
Hepatic microsomes were prepared from BALB/c mice.
Values in (A) are the mean ± SD of 4 incubation.

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Figure 4.8.2
The effect of varying concentrations of SKF 525-A on the metabolism of aminopyrine (5mM) by BALB/c mouse hepatic microsomes. SKF 525-A was added to incubations 5min prior to adding the substrate. Values are the mean ± SD of 3 incubations.
Figure 4.8.3

The effect of SKF 525-A (0.5mM) on the metabolism of NMF (10mM) to SMG by BALB/c mouse hepatic microsomes, incubated in the presence of GSH and NADPH. Values are the mean ± SD of 4 incubations.
Figure 4.8.4
The effect of SKF 525-A (3.0mM) on the metabolism of (A) aminopyrine (5mM), and (B) NMF (10mM) by BALB/c mouse hepatic microsomes. SKF 525-A was added to incubations 5min prior to the substrate. Values are the mean ± SD of 4 incubations.
The effect of PB on microsomal enzyme activity was investigated using two dosing regimes, either 50mg/kg for 4 days or 80mg/kg for 4 days. The former treatment resulted in only slightly increased cytochrome P-450 content and elevated aminopyrine N-demethylase activity, amounting to 135% and 112% of the control value respectively (Figure 4.8.5). Microsomal generation of SMG from NMF was not increased in this case. The latter, higher dose of PB raised cytochrome P-450 content of microsomes and increased N-demethylation of aminopyrine to 167% and 160% of control values respectively (Figure 4.8.6). Again, there was no observed increase in the yield of SMG compared to controls.

In one experiment of four, conducted in an identical manner to those described previously, PB-induced microsomes (80mg/kg for 4 days) metabolised NMF to a greater extent than did control microsomes (Figure 4.8.7). The amount of SMG generated in 60min was 159% of that formed by control microsomes. Subsequent experiments were unable to reproduce this increased metabolism. These results are thus presented separately.

Microsomes from mice treated with 50mg/kg BNF for 4 days had a cytochrome P-450 content that was 153% of the control value (Figure 4.8.8). However, the metabolism of NMF to SMG by these microsomes was no higher than in the case of control microsomes.
Figure 4.8.5

The effect of phenobarbitone (50mg/kg x 4 days) on:
(A) cyt. P-450 content of BALB/c mouse hepatic microsomes
(B) aminopyrine N-demethylation (5mM)
(C) the metabolism of NMF (10mM) to SMG by microsomes.
Incubations were conducted in the presence of GSH and NADPH.
Values are the mean ± SD of 6 incubations.
Figure 4.8.6
The effect of phenobarbitone (80mg/kg x 4 days) on:
(A) cyt. P-450 content of BALB/c mouse hepatic microsomes
(B) aminopyrine N-demethylation (5mM)
(C) the metabolism of NMF (10mM) to SMG by microsomes.
Incubations were conducted in the presence of GSH and NADPH.
Values are the mean ± SD of 4 incubations.
Figure 4.8.7

The effect of phenobarbitone (80mg/kg x 4 days) on the metabolism of NMF (10mM) to SMG by BALB/c mouse hepatic microsomes. The results are from a single experiment.
(A) Cytochrome P-450 content

(B) NMF metabolism

Figure 4.8.8
The effect of BNF on (A) the cytochrome P-450 content of microsomes, and (B) the microsomal metabolism of NMF (10mM) to SMG. BALB/c mice were treated with BNF (50mg/kg) for 4 days. Incubations were conducted in the presence of GSH and NADPH.
Values are the mean ± SD of 3 incubations.
The effect of enzyme inhibitors and inducers on the microsomal metabolism of HMMF to SMG are shown in Figures 4.8.9 and 4.8.10. As with NMF metabolism, SKF 525-A resulted in decreased SMG formation when a 3.0mM concentration was used. The absence of induction with either PB or BNF was also similar to the lack of effect on NMF metabolism.

Derivatised samples, which were obtained from incubations conducted in an atmosphere of either $^{16}$O$_2$ or $^{18}$O$_2$, were analysed by Dr Paul Pearson of the University of Washington, Seattle. Samples were analysed by GC-MS, with selective ion monitoring between m/z 103 and 107. Ion chromatograms for authentic EMC, EMC derived from $^{16}$O incubations and EMC derived from $^{18}$O incubations are shown in Figure 4.8.11. The MH$^+$ ion for authentic EMC, that is containing $^{16}$O, would be detectable at m/z 104. The MH$^+$ ion of EMC which contained one atom of $^{18}$O would be detectable at 106.

The relative intensities of the ions at m/z 104 and 106 were established for each sample (Table 4.8.1). Authentic EMC had a very low 106/104 ratio, demonstrating a predominance of the MH$^+$ ion at 104. EMC derived from $^{18}$O incubations demonstrated a low intensity of the ion at m/z 106, suggesting that there was no appreciable incorporation of $^{18}$O into the compound. However, EMC derived from both incubation atmospheres exhibited low ion intensities at both m/z 104 and 106. This indicates
Figure 4.8.9

The effect of SKF 525-A (3.0mM) on the metabolism of HMMF (10mM) to SMG by BALB/c mouse hepatic microsomes. Values are the mean ± SD of 4 incubations.
Figure 4.8.10
The effect of various inducers of microsomal enzymes on the metabolism of HMMF (10mM) to SMG by BALB/c mouse hepatic microsomes. Values are the mean ± SD of 4 incubations (A & B) or 3 incubations (C).
Figure 4.8.11
MH+ ion chromatograms following GC-MS analysis of
(A) authentic EMC; (B) EMC derived from $^{16}O$ incubations;
and (C) EMC derived from $^{18}O$ incubations, using selective
ion monitoring between m/z 103 and m/z 107
### Table 4.8.1

Intensities of MH⁺ ions following GC-MS analysis of either authentic EMC or derivatised microsomal samples incubated in $^{16}O_2$ or $^{18}O_2$.

<table>
<thead>
<tr>
<th>Sample</th>
<th>INTENSITY OF MH⁺ ION</th>
<th>RELATIVE INTENSITY</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>m/z 104</td>
<td>m/z 106</td>
</tr>
<tr>
<td>Authentic EMC</td>
<td>20272</td>
<td>86.6</td>
</tr>
<tr>
<td>EMC derived from $^{16}O_2$</td>
<td>545.7</td>
<td>6.6</td>
</tr>
<tr>
<td>EMC derived from $^{18}O_2$</td>
<td>229.1</td>
<td>2.7</td>
</tr>
</tbody>
</table>
that a low concentration of EMC was present in both of the derivatised samples.

4.8.3 DISCUSSION
In these experiments known inhibitors and inducers of microsomal enzymes were used to clarify enzymatic involvement in the metabolic activation of N-alkylformamides.

Inhibitors of microsomal monooxygenases can act in one of several ways (for reviews see Netter, 1980; Netter, 1987; Testa and Jenner, 1981). Two types of inhibitor were used in these studies, CO and SKF 525-A.

CO binds to the heme iron of cytochrome P-450. By competing with oxygen for the same site, CO prevents the transfer of activated oxygen to the substrate. Inhibition of a reaction by CO is a classic method for establishing cytochrome P-450 involvement (Netter, 1987; Testa and Jenner, 1981). Complete inhibition of the microsomal metabolism of NMF to SMG reported in this study is thus evidence of cytochrome P-450 involvement in this reaction.

SKF 525-A may be used to alter microsomal enzyme activity in one of several ways. The procedure for using SKF 525-A in these experiments, that is adding it to microsomal incubations 5min prior to adding the substrate, results in its own metabolism. The formation of a stable complex
between a SKF 525-A metabolite, reduced cytochrome P-450 and oxygen causes non-competitive inhibition of cytochrome P-450 activity (Buening and Franklin, 1976; Schenkman et al, 1972; Testa and Jenner, 1981).

In this study, when SKF 525-A was used at a concentration which decreased aminopyrine N-demethylation by more than half, the microsomal generation of SMG from NMF was substantially diminished. This inhibitory effect, in conjunction with that of CO, provides clear support for the hypothesis that the oxidative biotransformation of NMF is catalysed by cytochrome P-450. The corresponding inhibition of HMMF metabolism to SMG by SKF 525-A suggests the same role for cytochrome P-450 in the biotransformation of this compound.

Inducers of cytochrome P-450, which can be used to discriminate between the various isozymes, fall into several categories (Conney, 1967; Okey et al, 1986). The two most well-known of these groups are those containing compounds which act like PB and ones which act like 3-methylcholanthrene (3-MC). The latter group are known inducers of cytochrome P-448. In these investigations PB was used as an example from the former group, and BNF from the latter group.

Pre-treatment of mice with either PB or BNF resulted in elevated cytochrome P-450 content of microsomes and increased aminopyrine N-demethylation. However, these
increases were never more than twice the control values which is below that reported in other studies. Investigations of DMF metabolism using rat liver microsomes, for instance, reported increased aminopyrine N-demethylation following PB treatment that was three times the control value (Scailteur et al, 1984).

The increased cytochrome P-450 activity in these experiments, whether induced by either PB or BNF, caused no corresponding increase in the metabolism of NMF to SMG. The oxidative metabolism of NMF which, in the presence of GSH, leads to the formation of SMG thus appears to be catalysed by a cytochrome P-450 isozyme which is inducible neither by PB or BNF.

HMMF biotransformation to SMG was similarly unaffected by PB or BNF induction of cytochrome P-450. The degree to which HMMF is metabolised to SMG by microsomes is substantially less than in the case of NMF (see Section 4.5). However, the similar extent to which inhibitors and inducers effect this pathway in the case of both substrates suggests that HMMF is metabolised to SMG via NMF. As the metabolic activation of DMF is considered to occur following its metabolism to HMMF (see Section 4.3.3), it follows that the bioactivation pathway for DMF also proceeds via NMF. The subsequent metabolic events resulting in toxicity are common to both N-alkylformamides
The single experiment where NMF metabolism appeared to be induced by PB, but was not reproducible, is difficult to explain in the light of all conditions being identical to previous experiments. Nevertheless, it suggests that the possible involvement of a PB-inducible isozyme cannot be discarded completely.

The effect of enzyme inducers and inhibitors on the metabolic activation of NMF has previously been investigated by Pearson (1985). Pre-treatment of mice with PB (50mg/kg for 4 days) or SKF 525-A (60mg/kg, 1hr before dosing with NMF) did not alter NMF-induced hepatotoxicity or hepatic GSH-depletion. It was concluded that the bioactivation of NMF did not involve cytochrome P-450.

The absence of any PB-inductive effect on NMF metabolic activation reported by Pearson (1985) is in agreement with the results presented here. However, the lack of SKF 525-A inhibition reported in the same investigation, and the subsequent conclusions on the involvement of cytochrome P-450, are in contrast to the findings and conclusions of this study. The differing effect of SKF 525-A may have resulted from it being administered in vivo to mice (Pearson, 1985) compared to its in vitro use in this study. The ability of SKF 525-A to alter microsomal enzyme activity varies considerably according to the method of treatment used and it may act as either an inhibitor or an inducer (Bornheim et al, 1983; Buening

A positive result in the $^{18}$O experiment would have provided unequivocal evidence of the oxidation of NMF in the formyl moiety. Further, it would have supported the proposal that N-methylcarbamic acid is an intermediate in the bioactivation of NMF (Baillie et al, 1989). The proposed pathway, illustrating $^{18}$O incorporation, is shown in Figure 4.8.12.

The results of the $^{18}$O study presented here are inconclusive in that there was no demonstration of $^{18}$O incorporation into metabolically-generated SMG. However, the low concentration of EMC measured in both of the derivatised samples suggests that appreciable loss of the compound had occurred. It is possible that under the alkaline conditions required for derivatisation, any $^{18}$O incorporated into SMG may have been washed out (see Figure 4.8.12). These two factors may have contributed to the lack of detectable $^{18}$O incorporation in this experiment. The absence of any evidence of $^{18}$O incorporation in this experiment does not repudiate the proposed bioactivation pathway for NMF, particularly in the light of the role of cytochrome P-450 in SMG formation (see earlier in this Section).
Figure 4.8.12
Proposed metabolic pathway for NMF leading to the formation of SMG showing (A) the incorporation of $^{18}$O and (B) a possible means by which the incorporated $^{18}$O could be lost during derivatisation to EMC.
It can be concluded from these studies that the metabolic activation of NMF and DMF is catalysed by cytochrome P-450. The P-450 isozyme(s) involved appear to be neither inducible by PB nor by BNF. Although demonstration of the role of cytochrome P-450 in NMF metabolism is consistent with a bioactivation pathway which proceeds via N-methylcarbamic acid (Baillie et al., 1989), it is not unequivocal evidence. Proof of $^{18}$O incorporation into microsomal-generated SMG would provide substantial evidence in support of this proposed pathway. It is suggested that this experiment requires further attention.
4.9 INVESTIGATIONS OF THE IDENTITY OF THE REACTIVE METABOLITE OF N-ALKYLFORMAMIDES

4.9.1 INTRODUCTION

Evidence from previous studies suggests that oxidation of the formyl moiety is a necessary step in the metabolic activation of N-alkylformamides (Baillie et al, 1989; Kestell et al, 1987; Threadgill et al, 1987). In the case of NMF, this biotransformation pathway is considered to proceed subsequently to the generation of SMG and AMCC. 

In vitro metabolic studies conducted here using hepatic microsomes are consistent with this suggestion (Sections 4.5 and 4.8). In the preceding section, the role of cytochrome P-450 in this oxidative step was clearly established.

So far, SMG (Baillie et al, 1989; Threadgill et al, 1987) and AMCC (Kestell et al, 1986; Tulip et al, 1986) are the only metabolites involved in this bioactivation pathway which have been identified. The precise details of the metabolic events which stem from NMF oxidation have not yet been established. A suggested bioactivation pathway for NMF was summarised in Section 4.8.3, involving the formation of N-methylcarbamic acid and methyl isocyanate (MIC) as intermediates (Baillie et al, 1989).

The implications of MIC being a metabolite of N-alkylformamides are important. Isocyanates are highly reactive compounds with the ability to react with various functional groups on biological macromolecules (Brown et
al, 1987). The severity of the toxic effects resulting from MIC exposure was clearly demonstrated following the leak of MIC gas at the Union Carbide factory in Bhopal in 1984. MIC has been shown to be a potent irritant to the eyes and respiratory tract (Fowler et al, 1987; Misra et al, 1988), to adversely effect reproduction (Varma, 1987; Varma et al, 1987) and to damage the bone marrow (Conner et al, 1987). Additionally MIC is able to carbamylate haemoglobin resulting in tissue hypoxia (Ramachandran et al, 1988), and several isocyanates have been shown to inhibitor cholinesterase activity (Brown et al, 1982; Brown et al, 1987).

The metabolic generation of MIC from NMF would clearly account for any toxic effects at the site of its formation. The objective of this part of the study was to test the hypothesis that MIC is a metabolic intermediate in the biotransformation of NMF. Employing a technique that has successfully demonstrated the generation of MIC from the antitumour agent caracemide, preliminary experiments were conducted which addressed the following questions:

(a) under the conditions which enable the release and trapping of MIC from caracemide, is there any evidence of MIC release from synthetic SMG?

(b) is there evidence of MIC generation when microsomes are incubated with NMF in the absence of GSH?
4.9.2 RESULTS

A preliminary experiment was conducted to confirm the release of MIC from caracemide, using a modified trapping procedure (see Section 3.14.1). GLC analysis demonstrated a substantial peak at the same retention time as derivatised MIC, that is PMC. This confirmed that MIC was released from caracemide during the 30min incubation period.

The efficiency of recovering MIC by this trapping method was established using known amounts of MIC, either alone or mixed with 0.2M phosphate buffer (Table 4.9.1). Addition of MIC only to the reaction flask enabled virtual complete recovery. When MIC was mixed with buffer, recovery was reduced by nearly 50%.

When synthetic SMG was substituted for caracemide in the reaction flask, there was no measurable release of MIC over a period of 30min. Even when the reaction was continued for 90min, no peak corresponding to PMC could be detected.

Using the same trapping procedure, mouse hepatic microsomes were incubated with NMF in the presence of a NADPH-generating system. GSH was omitted from the incubations. The basis for this experiment was that any MIC generated by the microsomal metabolism of NMF would, in the absence of GSH, be removed and derivatised to PMC.
4.9.3 Discussion

The table below shows the results of the experiment where MIC was added to the reaction flask with and without buffer. The table includes the volume of buffer added, the MIC recovered, and the percentage recovery of MIC.

<table>
<thead>
<tr>
<th>MIC added to reaction flask (mMol)</th>
<th>Volume of buffer (ml)</th>
<th>MIC recovered (mMol)</th>
<th>% Recovery of MIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.21</td>
<td>2.0</td>
<td>0.12</td>
<td>58</td>
</tr>
<tr>
<td>0.21</td>
<td>0</td>
<td>0.22</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 4.9.1

Estimation of the efficiency of recovery of MIC with or without buffer, using a trapping solution containing Pr-1-OH and KOH. Duplicate samples were used.
GLC analysis demonstrated no detectable amounts of PMC in this experiment.

4.9.3 DISCUSSION
The method used for detecting MIC released from the antitumour agent caracemide (Newman and Farquhar, 1987) was successfully repeated in this study, with the modified trapping procedure that enables MIC to be measured as PMC. This technique provides a useful means of obtaining direct evidence of the generation of MIC as a metabolite of NMF or of its release from one or more of the conjugates.

The release of MIC from synthetic SMG was investigated on the basis of the known reversibility of the reaction of isocyanates with some biological macromolecules (Brown et al, 1987). The reaction of isocyanates with sulphhydryl groups results in a product which is stable under physiological pH. However, under slightly alkaline conditions this reaction becomes reversible yielding the isocyanate. This has led to the suggestion that proteins or peptides containing a sulphhydryl group could act as a transport mechanism for isocyanates. The isocyanate subsequently released could then react with other macromolecules.

Studies of the distribution of radiolabelled MIC have supported this suggestion (Bhattacharya et al, 1988; Ferguson et al, 1988). When MIC was absorbed through the
lungs, radioactivity was found in the blood and numerous other tissues. It was proposed that the radiolabelled MIC could have been transported in the blood in the form of a conjugate, for example a GSH-conjugate (Ferguson et al, 1988). The reversibility of this conjugation reaction could result in the release of MIC in tissues away from the original site of absorption.

The reversibility of conjugation reactions of GSH-conjugates related to SMG, and the resultant toxicity, has been demonstrated in vitro (Bruggeman et al, 1986). Allyl and benzyl isothiocyanate were found to be transported by conjugation with GSH and cysteine. Toxicity occurred on the release of the isothiocyanates from the conjugates.

Extending these findings to an explanation for the toxicity of N-alkylformamides, it could be suggested from the structure of SMG that under appropriate conditions this conjugate could yield MIC. According to the proposals outlined above, GSH conjugation could act as a transport mechanism for the reactive intermediate formed from NMF. The formation of SMG would thus not be a detoxification step as was previously considered. Indeed studies that are currently being conducted at Aston University and at the University of Washington, Seattle suggest that a number of N-alkylcarbamic acid thioesters derived from formamides, including SMG, possess cytotoxic properties (Han et al, 1989).
This suggestion that GSH-conjugation acts as a transport mechanism for a potentially toxic species would thus be supported by evidence of the release of MIC from synthetic SMG. The experiments conducted here provided no such evidence. The lack of any measurable release of MIC from synthetic SMG may be explained in one of several ways. Firstly, it is possible that under the conditions used in this investigation, although not necessarily under in vivo conditions, SMG does not yield MIC readily. Secondly, the amount of MIC released from SMG in these experiments may be so small as to avoid detection. Indeed, determination of the recovery of MIC in this study demonstrated that only approximately half of the original amount was recovered from a buffered solution.

Similarly there was no evidence of the generation of MIC from NMF in microsomal incubations. Previous experiments conducted in this study (see Section 4.5) showed that the NMF is only metabolised by microsomes to SMG when GSH is present. Two possible explanations were proposed (see Section 4.5.3), both of which are consistent with the absence of detectable MIC observed here. The most probable explanation is that any MIC generated in the incubations would immediately be hydrolysed via N-methylcarbamic acid to methylamine and carbon dioxide. The second, novel, explanation suggested that the oxidation step is dependent on the presence of GSH in the form of a cofactor.
In conclusion, the investigations reported here provide no evidence which lends support to the proposal that MIC is a reactive metabolite of NMF. Therefore it is improbable, but not impossible, that MIC is generated during the metabolic activation of NMF.
SECTION 5

GENERAL DISCUSSION
5 GENERAL DISCUSSION

The studies reported in this thesis were conducted with the objective of clarifying the means by which DMF causes hepatotoxicity. Using both in vivo and in vitro methods, the involvement of metabolism in the toxicity of DMF was examined.

The role of metabolism in the toxicity of other N-alkylformamides has previously been demonstrated (see Section 1.5). The mechanism of hepatotoxicity is considered to be linked specifically to the metabolic pathway which leads to the excretion of N-alkylcarbamic acid thioesters (Kestell et al, 1987). Prior to this study there has been no evidence that DMF is metabolised in this manner in animals.

The in vivo experiments reported in Section 4.1 demonstrate that DMF is rapidly and extensively metabolised in rodents. In addition, these investigations show that DMF is metabolised, not only to HMMF, "F" and alkylamines as had previously been reported, but also to AMCC. This is the first time that the metabolism of DMF to AMCC has been demonstrated in rodents. By modifying a previously described analytical method (see Section 3.5.3) and making it more sensitive, low urinary concentrations of AMCC which had previously avoided detection were measured. DMF-induced hepatotoxicity in rodents is marginal in comparison to that of NMF and NEF (see Section 1.3). In this investigation AMCC was found
to be excreted by rodents in very small amounts. This finding is in accordance with the proposal of Kestell et al (1987) that the hepatotoxicity of N-alkylformamides is linked to the extent to which they are metabolised to their corresponding N-alkylcarbamic acid thioesters.

The studies of volunteers that were conducted by Dr Mraz (see Section 4.2) show that humans metabolise DMF to AMCC to a far greater extent than rodents. If the extent of metabolism to AMCC correlates with toxicity as has been suggested, DMF may be more toxic to humans than has been assumed on the basis of results obtained in rodents. This conclusion prompts the suggestion that rodents may be unsuitable for assessing the health risks for humans resulting from DMF exposure.

The widespread use of DMF in industrial processes has been described in Section 1.1. An understanding of the role of metabolism in the toxicity of DMF may be usefully applied in this area. Health risks associated with occupational exposure to chemicals may be evaluated by environmental monitoring, health surveillance and/or biological monitoring. Environmental monitoring is routinely used to estimate exposure to chemicals which may be inhaled. In the U.K., exposure limits which are recommended for long-term and short-term exposure to DMF are set at 10ppm (30mg/m³) and 20ppm (60mg/m³) respectively. These limits are designed with the aim of "protecting against the effects of .... exposure or
reducing the risks to an insignificant level" (Health and Safety Executive, 1988).

DMF may be inhaled, absorbed through the skin or ingested. Therefore measurement of airborne levels of DMF may not accurately reflect the risks associated with exposure. Indeed Lauwerys et al (1980) reported that skin absorption of DMF was a more important route of absorption for humans than inhalation. In addition, various factors may influence the intake of DMF including work practices, hygiene habits and individual variation (Bernard and Lauwerys, 1986).

A second means of evaluating health risks, health surveillance, is aimed at detecting early signs of adverse effects. The measurement of plasma enzyme levels, for instance, has been employed in several studies as a means of detecting liver damage in workers exposed to DMF (see Section 1.2). Hepatotoxicity associated with exposure to DMF was demonstrated in a large proportion of the workforce at a fabric coating factory (Redlich et al, 1988). Elevated plasma levels of AST or ALT, indicative of hepatic damage, were reported in 62% of those workers investigated. Symptoms amongst the affected workers were generally absent or minimal and prior to the study liver damage had gone undiagnosed. The authors raised the concern that liver injury associated with routine exposure to DMF, or other solvents, may frequently go undetected. This report highlights the need for an
appropriate means of detecting excessive exposure to DMF before the onset of toxicity.

Biological monitoring provides a means of estimating the dose of a chemical which has been inhaled, absorbed through the skin or ingested by measuring levels of the chemical or its metabolites in body fluids or in exhaled breath. In the case of compounds which undergo metabolic toxification, measurement of the reactive metabolite is considered to be more relevant than measurement of the parent compound or of metabolites which are unrelated to toxicity (Bernard and Lauwerys, 1986). Biological monitoring may thus avoid some of the limitations of other methods which have previously been described.

The measurement of urinary levels of "NMF" by GLC has been used as a means of assessing human exposure to DMF in several volunteer and occupational studies (Catenacci et al., 1980; Kimmerle and Eben, 1975; Krivanek et al., 1978; Lauwerys et al., 1980; Maxfield et al., 1975; Yonemoto and Suzuki, 1980). The results presented in this thesis, together with previous work, clearly suggest that the hepatotoxic properties of DMF are associated with its metabolism to AMCC. As described in Section 4.2, this metabolic route seems to be a major biotransformation pathway in humans. Therefore this work leads logically to the proposal that analysis of urinary levels of AMCC might provide a more accurate indicator of the
hepatotoxic risk to humans of exposure to DMF than measurement of urinary "NMF".

The in vivo studies reported in Sections 4.1 and 4.2 have helped to improve our understanding of the metabolism of DMF and its relationship to the generation of hepatotoxicity in rodents and in humans. The second part of this investigation turned to in vitro methods as a means of focussing on the mechanisms involved in this toxicity.

The metabolism of N-alkylformamides to their corresponding N-alkylcarbamic acid thioesters is thought to involve the generation of a reactive precursor. A necessary step in the metabolic activation of NMF is the oxidation of the formyl moiety (Baillie et al, 1989; Threadgill et al, 1987). So far the only products of this bioactivation pathway to be identified unequivocally are AMCC (Kestell et al, 1986b; Mraz and Turecek, 1987; Tulip et al, 1986) and its metabolic precursor, SMG (Baillie et al, 1989; Threadgill et al, 1987). By using in vitro metabolic systems the details of this bioactivation pathway, specifically its enzymatic nature, may be investigated. These studies may thus provide indirect evidence as to the identity of the toxic intermediate which at the present time remains unconfirmed.

The in vitro experiments conducted here (Sections 4.5, 4.6, 4.7 and 4.8) show that NMF is metabolised by hepatic
microsomes from mice, rats and humans to an N-
methylcarbamoylating metabolite. The requirement for GSH
for the detection of this metabolite and evidence from
similar studies using isolated hepatocytes (Baillie et
al, 1989; Shaw et al, 1988) suggest that this metabolite
is SMG. This is the first time that hepatic microsomes
have been shown to metabolise NMF to SMG. Thus microsomes
seem to be a suitable in vitro metabolic system useful
for the clarification of further details of the metabolic
events leading to SMG formation. This model system should
appropriately complement isolated hepatocytes which have
been used previously in studies of the metabolism of
formamides (Shaw et al, 1988).

Using microsomes, we were able to demonstrate that the in
vitro metabolism of NMF to SMG involves an oxidation step
which is catalysed by cytochrome P-450 (Section 4.8). The
role of cytochrome P-450 in the metabolic activation of
N-alkylformamides has not previously been established,
and further investigations (Section 4.8) have shown that
the isozyme involved is not inducible by PB or by BNF.

The role of HMMF in the bioactivation of its parent
compound DMF was investigated using in vivo and in vitro
methods. HMMF was metabolised to AMCC in vivo by rodents
approximately to the same extent as was DMF (see Section
4.3). Other marked similarities between the metabolic
fate of DMF and HMMF in vivo lead to the conclusion that
the former compound is metabolised largely, perhaps
exclusively, via the latter compound. Unlike DMF, HMMF was shown to be metabolised \textit{in vitro} by microsomes to SMG although to a lesser extent than NMF (Sections 4.5, 4.6 and 4.8). The apparent inability of microsomes to generate SMG from DMF under the same conditions (Sections 4.5 and 4.6) suggests that the amount of HMMF and perhaps NMF formed by the microsomal metabolism of DMF is so low that their further metabolism to SMG proceeds at a rate which is below the detection limit.

The studies which have been reported in this thesis have extended our understanding of the toxicity of not only DMF but of \textit{N}-alkylformamides in general. The \textit{in vivo} metabolic studies clearly suggest that the hepatotoxic properties of DMF are linked to its metabolism, specifically the metabolic pathway leading to AMCC. Therefore the mechanism by which DMF causes toxicity is probably the same as that proposed for \textit{N}-monoalkylformamides (Baillie et al, 1989; Kestell et al, 1987; Threadgill et al, 1987). The \textit{in vitro} studies have demonstrated that microsomes provide a useful metabolic model for investigating the mechanistic details of \textit{N}-alkylformamide bioactivation. Further, these investigations have for the first time provided evidence of the role of cytochrome P-450 in catalysing the oxidation step which is considered to be fundamental to \textit{N}-alkylformamide toxicity. The incorporation of these findings into what was formerly known about DMF
biotransformation leads to the tentative metabolic scheme for DMF which is summarised in Figure 5.1.
Figure 5.1 Proposed metabolic pathways for DMF
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