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AN INVESTIGATION OF THE MECHANISM OF HEPATOTOXICITY OF THE ANTITUMOUR AGENT *N*-METHYLFORMAMIDE IN MICE

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Abstract—*N*-Methylformamide (NMF) has been reported to cause liver damage in animals and man. This hepatotoxicty was characterized in BALB/c mice by the release of liver enzymes into the plasma and by histopathological examination of livers after single and repeated administration of NMF. Whereas plasma levels of sorbitol dehydrogenase were elevated dramatically 24 hr after 400 mg/kg given as a single dose, the glutathione content of the livers was not different from controls even after repeated administration. Liver damage was apparent on gross inspection and was defined as periacinar necrosis on histopathology. A dose of 100 mg/kg did not cause damage even after repeated injections on five consecutive days. The hypothesis that NMF is metabolized to a chemically reactive species was tested. Incubation of mouse hepatocytes with 7 mM NMF for 80 min produced a decrease in intracellular glutathione. Exposure of hepatocytes to NMF for 240 min led to the production of breakdown products of lipid peroxides at levels significantly above controls. However, incubation of microsomes or mitochondria with NMF and NADPH did not lead to raised levels of lipid peroxides. The effects described were specific to NMF as incubation of *N,N*-dimethylformamide, *N*-hydroxymethylformamide or formamide with hepatocytes did not result in glutathione depletion or increased lipid peroxidation. NMF undergoes extensive metabolism *in vivo* and the results indicate that NMF forms a chemically reactive metabolite, even though incubation of the drug with liver fractions or hepatocytes did not lead to metabolites at levels which were analytically identifiable.

N-Methylformamide (NMF, Fig. 1) is an industrial solvent and also an investigational antitumour agent currently under clinical evaluation. It is active against a wide spectrum of murine tumours [1–3] and human tumour xenografts in mice [4]. Unlike many other antitumour agents, NMF does not induce myelotoxicity in experimental animals [5, 6].

In the first clinical trial of this drug, symptoms of hepatotoxicity occurred in all five patients treated with NMF [7], and in more recent toxicity studies the hepatotoxic potential of NMF was confirmed in mice [5, 6], rats [8, 9] and dogs [6]. The methyl analogue of NMF, the industrial solvent *N,N*-dimethylformamide (DMF, Fig. 1) is a well-known hepatotoxin in experimental animals [10–13] and man [14–16]. The mechanisms by which NMF and DMF exert toxicity are unclear. The toxicity of DMF has been attributed to NMF, a metabolite of DMF [17–19]. However, what was identified as NMF in the studies by Kimmerle and Eben [18, 19], by gas chromatography in the plasma and urine of animals exposed to DMF, was probably *N*-hydroxymethyl-*N*-methylformamide, the immediate product of *C*-hydroxylation of DMF. This carbinolamine is stable under physiological conditions, but decomposes readily on heating on the gas-chromatography column [20]. Lundberg *et al.* have suggested that NMF itself is the metabolic precursor of another toxicant which is responsible for the hepatotoxicity associated with DMF [9].

In this study possible biochemical mechanisms of the hepatotoxicity of NMF have been investigated. If the biochemical basis for NMF-induced lesions was better understood, liver damage in patients treated with this drug could probably be avoided or minimized. Examples of hepatotoxins of a molecular size comparable to that of NMF are *N,N*-dimethylnitrosamine and the alkyl halides methyl chloride, chloroform and carbon tetrachloride. The hepato-

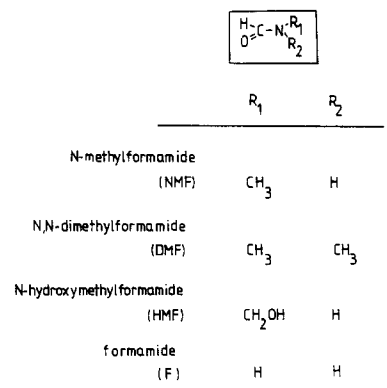

Fig. 1. Chemical structures of the formamide derivatives used in this study.

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toxicity of these compounds appears to be dependent on metabolic activation [21–24]. In general, compounds which form chemically reactive species following hepatic metabolism deplete livers of the non-protein thiol glutathione [25]. Some hepatotoxins such as carbon tetrachloride are considered to produce and propagate free radicals and consequently cause toxicity by initiating the peroxidative breakdown of polyunsaturated fatty acids in membranes [26]. This investigation of the mechanism of NMF induced hepatotoxicity had a 2-fold rationale:

(1) To characterize the nature of the damage by investigating the dose dependency and the time of onset after NMF administration. Toxicity was evaluated in terms of release of liver enzymes, such as sorbitol dehydrogenase (SDH), into plasma, and by gross autopsy and histopathological examination of the livers. SDH plasma levels have been used frequently in the assessment of the severity of drug-induced liver damage [27, 28] and also in the case of NMF [5, 6] and DMF [8, 9].
(2) To test the hypothesis that chemically reactive metabolites of NMF formed in the liver cause necrosis either by directly reacting with bionucleophiles or by initiating lipid peroxidation, both *in vitro* and *in vivo*. In the isolated mouse hepatocyte preparation, the effects of NMF were compared with those of three structurally related formamide derivatives, DMF, *N*-hydroxymethylformamide (HMF, Fig. 1) and formamide (F, Fig. 1). F has been found to be a metabolite of NMF [3], and there is evidence that HMF is a reasonably stable intermediate in the metabolic transformation of NMF to F [29].

It has been shown previously that a single intraperitoneal injection of NMF to mice caused an acute depletion of liver non-protein thiols in mice [3]. This effect was dose-related and specific for NMF when compared with other formamide derivatives. Here we report the effect of NMF on liver glutathione levels *in vivo* after repeated administration of the drug and also the glutathione status of hepatocytes exposed either to NMF, DMF, HMF or F. The formation of breakdown products of lipid peroxides was investigated in liver fractions prepared 24 hr after NMF administration and also in hepatocytes incubated with either NMF or other formamide derivatives.

MATERIALS AND METHODS

Male BALB/c mice (19–25 g) were used in all experiments except those described in Table 1 where CBA/CA and BDF_1 mice were also used. Details of tumour passages and maintenance are given elsewhere [3]. Treatment with NMF or saline was initiated on day 3 (TLX5-bearing mice) or day 1 (M5076-bearing mice) after passage.

Animals were purchased from Bantin and Kingman Ltd. (Hull, U.K.) and were fed on Heygate 41B breeding diet and allowed tap water *ad libitum*.

Chemicals. Formamide derivatives were commercially available and were purified by distillation. HMF was synthesized by E. N. Gate in our laboratories according to ref. [30]. All formamides were mixed with saline for administration to mice.

Reduced and oxidized glutathione (GSH and GSSG, respectively) were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). Malonaldehyde-bis-dimethylacetal (MDA), used as a standard for the lipid peroxide assay, and 2-vinyl pyridine for the GSSG determination were purchased from Aldrich Ltd. (Poole, Dorset, U.K.). Collagenase was obtained from Boehringer Ltd. (U.K.), horse serum from Gibco (Europe) and all other chemicals, substrates and cofactors were from Sigma.

Gross autopsy and histopathology. Mice were injected with NMF in 0.1 ml sterile saline i.p. at 100, 200 and 400 mg/kg as single doses or daily for 5 days. Twenty-four hours after the last dose, animals were killed by cervical dislocation. Total body weight, appearance and liver weight were recorded and livers were excised, blotted and examined for gross pathological changes and, in some cases, fixed in Bouin's fluid [31]. Representative specimens of liver were embedded in paraffin wax and stained with haemalum and eosin. Damage was also examined at various times up to 24 hr after a single dose of

Table 1. Hepatic glutathione levels (mean ± S.D.) 24 hr after the fifth of five daily i.p. injections of 400 mg/kg NMF (number of animals in parentheses)

Mouse strain	Glutathione content (GSH + GSSG, μmoles/g liver)	GSSG content (μmoles/g liver)
BALB/c (male)		
control (3)	6.9 ± 0.5	0.65 ± 0.18
NMF treated (3)	7.0 ± 2.9	0.46 ± 0.19
CBA/CA (female)		
control (5)	6.3 ± 1.4	0.42 ± 0.06
NMF treated (6)	7.8 ± 1.0	0.26 ± 0.06
CBA/CA (female, with TLX5 lymphoma)		
control (5)	5.5 ± 1.0	0.53 ± 0.06
NMF treated (6)	7.2 ± 1.5	0.50 ± 0.22
BDF_1 (female, with M5076 sarcoma)		
control (5)	4.3 ± 0.9	0.97 ± 0.11
NMF treated (5)	6.6 ± 1.2	0.67 ± 0.27

Table 2. TARM content of liver fractions (mean ± S.D.) 24 hr after administration of 400 mg/kg NMF in BALB/c mice (number of experiments in parentheses)

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

400 mg/kg NMF. Control animals were injected with saline.

Assays of liver enzymes in plasma. Mice were anaesthetized with sodium pentobarbitone (200 mg/kg) i.p. and blood samples (1 ml) were collected in heparinized syringes after cardiac puncture. Plasma was obtained by centrifugation in a Beckman Microfuge. Plasma SDH levels were determined according to Rose and Henderson [32] 24 hr after either a single dose of 100, 200 or 400 mg/kg NMF or five daily doses of 100 or 200 mg/kg NMF. After the repeated NMF administration, plasma levels of L-alanine and L-aspartate aminotransferases (ALT and AST, respectively) were also determined according to Kachmar and Moss [33].

Determination of glutathione. Glutathione levels were determined in hepatocytes exposed to NMF and in liver homogenates 24 hr after the fifth of five daily doses of 400 mg/kg NMF i.p., by the spectrophotometric method originally described by Tietze [34] and modified by Griffiths [35]. The method was employed with the following changes: livers were homogenized in 10% (w/v) metaphosphoric acid (40 ml/g liver). The cell pellet obtained from hepatocyte suspensions, or aliquots of the supernatant were also deproteinized with this acid. Addition of trisodium phosphate solution to neutralize the acid was titrated so that the final solution had a pH of 7.0–7.5. This had been determined to be the optimum pH range for glutathione reductase activity and also for the maximum electrophilic reactivity of 2-vinylpyridine in the GSSG assay.

Determination of lipid peroxidation. Aldehydic compounds which reacted with thiobarbituric acid (TARM) were assayed in hepatocytes exposed to NMF and in liver fractions obtained 24 hr after NMF administration. TARM was measured spectrophotometrically according to the method of Fong *et al.* [36] with the following modifications: suspension of hepatocytes, liver microsomes, postmicrosomal supernatant or mitochondria were deproteinized by addition of 70% (w/v) trichloroacetic acid (final concentration in solution was 7% w/v). After centrifugation the acidic supernatant was adjusted to pH 3.5 by addition of 12 M sodium dihydrogen phosphate solution. This pH has been reported to be optimum for the reaction between aldehydes and thiobarbituric acid [37]. It also prevented trichloroacetic acid from hydrolysing NMF to products which gave yellow-coloured agents with thiobarbituric acid. MDA was used as a standard. Values in Table 2 and Figs. 5 and 6 are expressed as nmole of MDA equivalents per liver fraction equivalent to 1 g liver, or 10^6 cells, or mg protein.

Preparation and incubation of liver fractions. For determinations of TARM in liver fractions, haemoglobin was removed from livers prior to homogenization by perfusion with 10 mM Tris buffer, pH 8.0. Livers were homogenized in ice-cold 0.25 M sucrose (10% w/v). After centrifugation of the homogenates at 700 *g* for 10 min, supernatants were centrifuged at 10,000 *g* for 10 min at 2°. After washing, the mitochondrial pellet was resuspended in 0.15 M KCl. Postmitochrondrial supernatants were centrifuged at 100,000 *g* for 90 min at 2° to give the postmicrosomal supernatant and the microsomal pellet, which was washed and suspended in 0.15 M KCl. In experiments with NMF-treated mice, the liver fractions thus obtained were investigated for TARM contents immediately. In the *in vitro* studies, microsomal or mitochondrial fractions were pooled to give solutions 1 ml of which contained subcellular fractions equivalent to 1 g original liver weight. A NADPH generating solution in Tris buffer was prepared according to Slater and Sawyer [38] and added to microsomal or mitochondrial suspensions. Aliquots (2.5 ml) of this mixture were incubated for up to 60 min at 37° in 25 ml beakers after addition of either 20 ml saline or saline containing NMF to give 7 mM in the final solution. Microsomal and mitochondrial protein contents were determined according to Lowry *et al.* [39].

Preparation and incubation of hepatocytes. Mouse hepatocytes were prepared according to the method of Renton *et al.* [40] and suspended in Krebs–Henseleit buffer containing 0.1% (w/v) bovine serum albumin and 10% (w/v) horse serum. Initial cell viability measured by trypan blue exclusion was over 80%. Viability was also checked by determining lactate dehydrogenase leakage [41] which gave results similar to those obtained with trypan blue. Incubations of 2×10^6 hepatocytes per ml were carried out in 2.5 ml buffer at 37° with continuous gassing with a 95% O_2–5% CO_2 mixture, in 25 ml silanized conical flasks with rubber stoppers, under gentle shaking. Contents of the flasks were

transferred to centrifuge tubes and cells were separated from supernatant by standing on ice for 10 min.

The concentration of NMF, DMF, F or HMF in the hepatocyte incubations was 7 mM, which has been shown to be the peak plasma concentration of NMF in mice after i.p. injection of 400 mg/kg [29]. Formamides were added to incubates in 0.1 ml saline and incubation periods were up to 80 min in glutathione experiments and up to 240 min in lipid peroxidation experiments.

Unlike rat hepatocytes, mouse liver cells have not been used extensively in studies of this kind. Therefore we carefully investigated the influence of different incubation conditions on cell viability and the extent to which glutathione levels and the generation of TARM in mouse hepatocytes responded to the challenge of agents known to disturb the homeostasis of these endogenous compounds in rat hepatocytes. We found levels of GSH immediately after isolation to vary considerably, unrelated to slight differences in cell viability, but apparently dependent on the batch of collagenase used. The mean ±S.D. of the intracellular glutathione concentration was 19.41 ± 7.93 nmole/10^6 cells in 19 experiments. Incubation of the hepatocytes for a long time period unavoidably led to a decrease in viability, e.g. after 80 min to 61.1 ± 14.6% (n = 7) in the cells exposed to NMF and to 71.6 ± 10.1% (n = 9) in suspensions without NMF. Incubations of hepatocytes with NMF at concentrations even as high as 0.1 M did not lead to a marked decrease in cell viability compared with control incubations. In experiments with the oxidizing agent menadione, a pattern of intracellular GSH depletion and extracellular GSSG increase was observed (not shown here), which was almost identical to that reported by Eklöw *et al.* [42] for rat hepatocytes. Likewise, incubation of mouse hepatocytes with the hepatotoxin carbon tetrachloride led to raised levels of TARM.

RESULTS

Studies in vivo

Plasma SDH levels and gross autopsy and histopathological examination of the livers of mice 24 hr after single doses of NMF showed that the NMF-induced damage was related to the dose. In Fig. 2A plasma SDH levels are plotted against the dose. Plasma ALT and AST values showed similar trends when compared with the SDH levels. Gross inspection and histopathology of the livers revealed damage invariably after a dose of 400 mg/kg, three out of eight mice exhibited damage after 200 mg/kg NMF, and no damage was observed after administration of 100 mg/kg. In damaged livers there was evidence of periacinar necrosis, often confluent in nature. Figure 3 shows a typical liver of a BALB/c mouse after administration of 400 mg/kg NMF compared with a liver of a mouse treated with saline. The time period between the administration of NMF and the appearance of liver damage was at least 10 hr, as the SDH levels were similar to control levels up to this time (Fig. 2B). Likewise, gross inspection of the livers did not reveal obvious changes before 12 hr after injection of NMF. The repeated administration of either 100, 200 or 400 mg/kg NMF led to a pattern of toxicity similar to that described for the single doses. Whereas five daily doses of 100 mg/kg did not cause any symptoms of general or hepatic toxicity 24 hr after the fifth dose, three out of six animals exhibited a loss of body weight during the course of five daily injections of 200 mg/kg with no elevation of liver enzyme levels in the plasma or abnormal appearance of the liver on inspection of any of the mice. The administration of 400 kg/kg was too toxic in most mice to be repeated more than once. The abnormal appearance of the livers in the mice which survived the repeated administration of 400 mg/kg resembled those seen in animals after one injection of 400 mg/kg NMF.

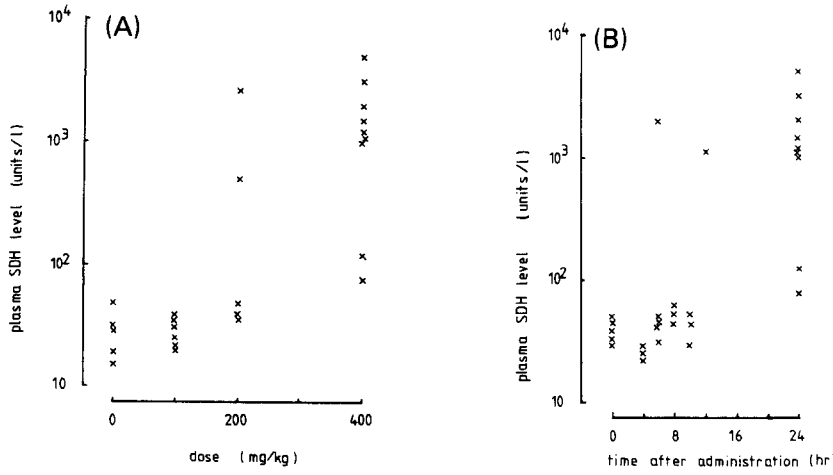

Fig. 2. Plasma sorbitol dehydrogenase (SDH) levels after i.p. administration of NMF; (A) 24 hr after injection of saline (zero dose values), 100, 200 or 400 mg/kg NMF; (B) at different time intervals after administration of 400 mg/kg NMF.

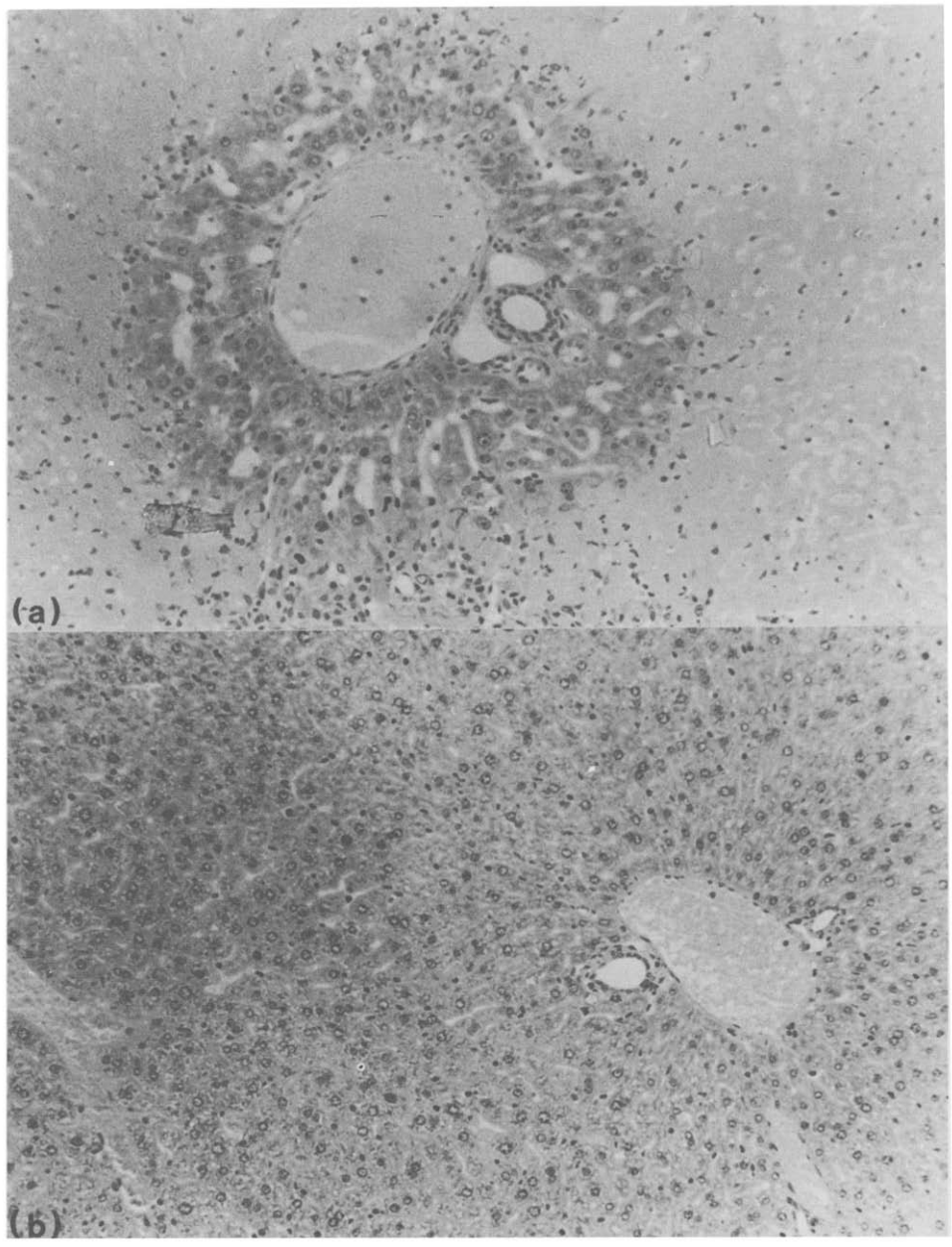

Fig. 3. Sections from livers of BALB/c mice obtained 24 hr after treatment with NMF, 400 mg/kg (A), or saline (B) i.p. for 5 days. Magnification ×160, haemalum and eosin stains. Note severe necrosis in (A) in periacinar region.

Comparing the appearance and well-being of the mice observed in this study with mice of different strains given similar or higher doses of NMF in the evaluation of the drug's antineoplastic activity [3], we noted indications of a marked difference between strains in their susceptibility to NMF-induced toxicity. For example, the LD_{10} for a single dose of NMF determined in BDF_1 mice was about three times the value obtained in BALB/c mice [5]. However, in the antitumour studies liver appearance was investigated only sporadically and further investigation of this strain-dependent hepatotoxicity is warranted. It has been shown previously that hepatic glutathione levels were significantly reduced 1 hr after a single injection of NMF in a dose-related fashion [3]. At 100 mg/kg this acute effect of NMF on GSH levels was reversed within 15 hr after NMF administration (A. Gescher, unpublished results). Table 1 shows hepatic GSH levels 24 hr after the fifth of five daily doses of 400 mg/kg in BALB/c mice, in which this dose causes serious toxicity, and in two other strains of mice. In no strain did this schedule lead to a long-lasting disturbance of the hepatic GSH status.

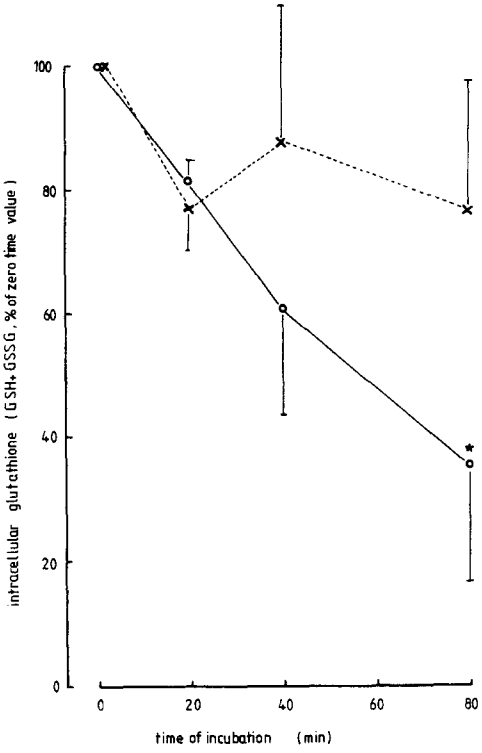

Fig. 4. Intracellular concentrations of glutathione in mouse hepatocytes incubated with 7 mM NMF (○——○) or saline (×---×). Values are the mean ± S.D. of between 4 (20 min time point) and 11 experiments (80 min time point). Star indicates difference is statistically significant ($P < 0.001$).

TARM values, an indicator of lipid peroxidation, obtained after injection of a single dose of 100, 200 or 400 mg/kg NMF to BALB/c mice were only markedly elevated in mitochondria and cytosol after the high dose of NMF (Table 2). Values were so variable that statistical comparison of the results was not possible.

Studies in vitro

Figure 4 shows that incubation of hepatocytes with 7 mM NMF for up to 80 min produced a significant decrease in intracellular glutathione. The extracellular concentration of glutathione did not change significantly within the incubation period (data not shown). Extracellular glutathione consisted almost exclusively of GSH at the beginning and almost entirely of GSSG at the end of the experiments. Hardly any GSSG was detectable within the cells at the beginning of the incubation and intracellular GSSG levels increased to 2.05 ± 1.42 nmol/10^6 cells (8.6% of total intracellular GSH + GSSG, $n = 5$) after 80 min in control incubations and to 1.85 ± 0.24 nmole GSSG/10^6 cells (19.5% of total GSH + GSSG, $n = 7$) after 80 min incubation with NMF.

Levels of TARM were raised markedly above control levels in hepatocytes after 240 min exposure to 7 mM NMF (Fig. 5A). The pro-oxidant effect of NMF in hepatocytes led to the examination of TARM levels in different compartments within the cell, the endoplasmic reticulum and mitochondria. However, incubation of isolated microsomes and mitochondria with 7 mM NMF and NADPH did not lead to the formation of TARM in amounts exceeding the control levels (Figs. 5B and 5C). Both effects of NMF in hepatocytes, the slow depletion of GSH and the pro-oxidant effect were found to be specific for NMF, as neither DMF, HMF or F at equimolar concentrations were able to elicit similar lesions (Figs. 6A and 6B).

DISCUSSION

The liver necrosis caused by NMF has been shown previously to be of the centrilobular (or periacinar) type in rats [9] and Swiss mice [4]. Similar necrogenic activity of NMF was observed in this study in BALB/c mice (Fig. 3). Using plasma levels of the cytoplasmic enzyme SDH as an indicator of hepatotoxicity, the damage was further characterized by its manifestation not before 10 hr after NMF administration (Fig. 2B) and by its dependency on the dose

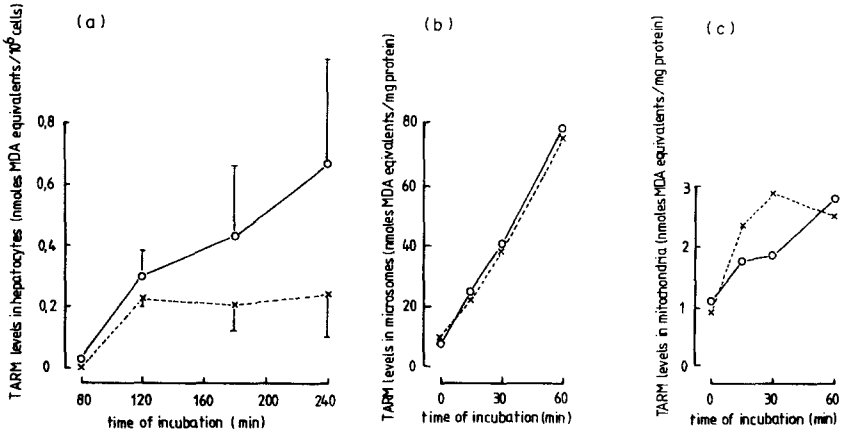

Fig. 5. Lipid peroxidation (A) in hepatocytes, (B) in microsomes and (C) in mitochondria incubated with (○——○) and without (×---×) 7 mM NMF. Values in (A) are the mean ± S.D. of 4–8 experiments; values in (B) and (C) are the mean of 2 experiments.

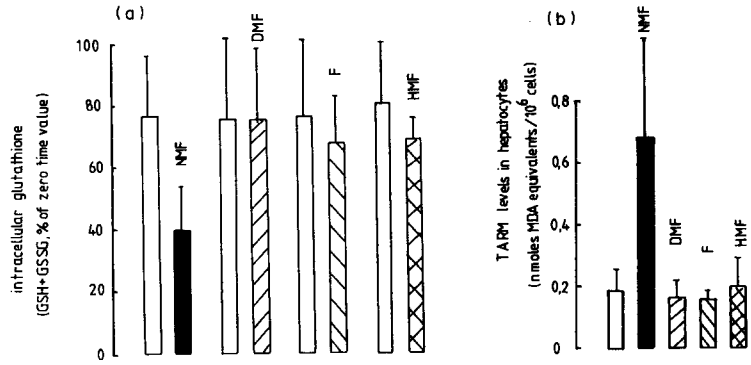

Fig. 6. Influence of either NMF, DMF, F or HMF on (A) intracellular concentrations of glutathione and (B) TARM levels in hepatocytes. Hepatocytes were incubated with 7 mM drug for 80 min (A) or 240 min (B) and values are the mean ± S.D. of 5–11 experiments in (A) and 3–8 experiments in (B). Open bars represent control values; in the case of (A) the experimental design required control incubations for each derivative.

(Fig. 2A). In this particular mouse strain the dose threshold above which a single dose was likely to cause necrosis appeared to be 200 mg/kg. After five daily doses of 100 mg/kg, NMF did not cause damage, indicating that accumulation of the toxic species responsible for the lesion did not occur. The delayed onset of the hepatotoxicity (Fig. 2B) suggests that NMF is not directly toxic but requires metabolism to the necrogenic species. The NMF-induced necrosis appeared to be restricted to the periacinar region of the liver acinus. As the monooxygenase enzymes which catalyse the oxidation of xenobiotics are concentrated in this area of the liver [43], the tissue distribution of the NMF-induced injury seems to mirror the distribution of cytochrome P-450. A similar distribution in the case of liver damage caused by carbon tetrachloride has been suggested to reflect a major role for this enzyme in either the activation of the toxicant or the propagation of lipid peroxidation [44]. By analogy, the involvement of cytochrome P-450 and/or lipid peroxides in the genesis of the damage induced by NMF is suggested. It has been demonstrated in the mouse that NMF undergoes extensive biotransformation to unknown metabolites which are rapidly excreted in the urine [29]. However, we have no chemical analytical evidence that NMF is metabolized by hepatic monooxygenases or dehydrogenases in experiments using liver microsomes, 9000 *g* supernatant or isolated hepatocytes [3, 29]. But the observation that NMF depleted hepatic non-protein thiol stores *in vivo* by 59.8% 1 hr after administration [3] is consistent with the hypothesis that an electrophilic metabolite is formed. This depletion did not persist and 24 hr after the fifth dose of NMF, glutathione levels were not significantly different from controls (Table 1).

Nevertheless, the depletion of glutathione by NMF could be demonstrated *in vitro* in suspensions of isolated hepatocytes (Fig. 4). It is noteworthy that this depletion was apparent only in suspensions incubated with NMF for more than 1 hr. Glutathione levels in isolated hepatocytes are rapidly reduced by electrophilic agents such as diethylmaleate which avidly undergo chemical and enzyme-catalysed reactions with glutathione, whereas compounds which are metabolized to electrophilic species, such as paracetamol, deplete hepatocytes more gradually [45]. Therefore the gradual depletion of glutathione in hepatocytes by NMF suggests the formation of an electrophilic metabolite. Metabolites of NMF could also deplete hepatic stores of glutathione by other mechanisms. The biosynthesis of this tripeptide can be inhibited by xenobiotics [46]. Alternatively drug-induced rapid conversion of GSH to its oxidized form, GSSG, would also lead to depletion of the reduced tripeptide. Finally, agents like aspirin appear to deplete hepatic glutathione as a result of an increased leakage of GSH from hepatocytes [47]. It is most unlikely that a redox process is involved in the NMF-induced glutathione depletion as hepatic GSSG levels were not markedly increased *in vivo* (Table 1) or in hepatocyte suspensions. Furthermore, it is unlikely that NMF increases the efflux of GSH out of the hepatocytes as extracellular GSH levels did not differ significantly between NMF and control incubations.

Probably NMF causes hepatotoxicity by forming a metabolite which initiates the production or propagation of free radicals leading to the peroxidative breakdown of membrane polyunsaturated fatty acids. Indeed, a raised level of breakdown products of lipid peroxides was found in liver mitochondria 24 hr after administration of a hepatotoxic dose (Table 2). This finding has to be interpreted with caution as the necrosis was already well-established at the time TARM was determined, so that the increased levels of TARM in the livers may have been the consequence of the damage rather than its cause. On the other hand, incubation of hepatocytes with NMF did lead to marked lipid peroxidation, even though it took a long incubation period for this effect to become apparent (Fig. 5A). The long time span required for NMF to induce lipid peroxidation in hepatocytes may reflect the fact that the pro-oxidant metabolite of NMF took a long time to be generated in sufficient quantities. Alternatively, this phenomenon may not be due to the pro-oxidant activity of a NMF metabolite but may be a consequence of the NMF-induced depletion of hepatic glutathione, which in turn makes the hepatocyte

more susceptible to endogenous oxidative stress [48]. In order to be able to interpret the metabolic basis of the NMF-induced glutathione depletion and lipid peroxidation, we are currently investigating its ability to bind covalently to, or to be incorporated into, liver macromolecules. In addition, the chemical nature of two very polar radioactively labelled metabolites found in the urine of mice after administration of [^{14}C]NMF is under scrutiny. Also, in order to define early cellular lesions associated with the necrogenic action of NMF, we are currently investigating the ability of NMF to interfere with the homeostasis of Ca^{2+} in different cell organelles.

It is remarkable that the biochemical lesions which might be involved in the genesis of hepatotoxicity, glutathione depletion and lipid peroxidation were found to be specific to NMF when compared with the other formamide derivatives studied (Fig. 6). DMF possesses a well-documented hepatotoxic potential [10–16], but it may well be much less potent in its toxicity than NMF. This contention seems to be supported by the almost 4-fold difference in LD_{50} values in mice between DMF and NMF [49]. In view of this hepatotoxic specificity of NMF it is worthwhile to recall that the antineoplastic activity of this drug in mouse tumour models was far superior to that of its metabolites or a large number of other formamide derivatives [1–3].

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REFERENCES

1. D. A. Clarke, F. S. Philips, S. S. Sternberg, R. K. Barclay and C. C. Stock, *Proc. Soc. exp. Biol. Med.* **84**, 203 (1953).
2. A. Furst, W. C. Cutting and H. Gross, *Cancer Res.* **15**, 294 (1955).
3. A. Gescher, N. W. Gibson, J. A. Hickman, S. P. Langdon, D. Ross and G. Atassi, *Br. J. Cancer* **45**, 843 (1982).
4. D. L. Dexter, E. N. Spremulli, G. M. Matook, I. Diamond and P. Calabresi, *Cancer Res.* **42**, 5018 (1982).
5. S. P. Langdon, D. Chubb, A. Gescher, J. A. Hickman and M. F. G. Stevens. (submitted for publication).
6. K. N. Newman, R. G. Meeks and S. Frick, Phase 1 Report of Task 1 Preclinical Intravenous Toxicity Study of *N*-Methylformamide (NSC 3051) in CDF_1 Mice and Beagle Dogs. Birmingham Al, Southern Research Institute (1981).
7. W. P. L. Myers, D. Karnofsky and J. H. Burchenal, *Cancer* **9**, 949 (1956).
8. V. Scaiiteur, J. P. Buchet and R. Lauwerys, in *Organ-Directed Toxicity, Chemical Indices and Mechanisms* (Eds. S. S. Brown and D. S. Davies), p. 169. Pergamon Press, Oxford (1981).
9. I. Lundberg, S. Lundberg and T. Kronevi, *Toxicology* **22**, 1 (1981).
10. J. W. Clayton, J. R. Barnes, D. B. Hood and G. W. H. Schepers, *Am. ind. Hyg. Ass. J.* **24**, 144 (1963).
11. W. Massmann, *Br. J. ind. Med.* **13**, 51 (1956).
12. W. T. Matthew, R. Karunanithy, M. H. Yee and P. N. Natarajan, *Lab. Invest.* **42**, 257 (1980).
13. H. Unger, S. F. Sullman and A. J. Zuckerman, *Br. J. exp. Path.* **57**, 157 (1976).
14. H. P. Potter, *Archs Environ. Hlth* **27**, 340 (1973).
15. W. Reinl and H. S. Urban, *Int. Archs Gewebepath.* **21**, 333 (1965).
16. F. Tolot, M. Droin and M. Genevois, *Archs Malad. Prof.* **19**, 602 (1958).
17. J. R. Barnes and K. E. Ranta, *Toxic. appl. Pharmac.* **23**, 271 (1972).
18. G. Kimmerle and A. Eben, *Int. Archs Arbeitsmed.* **34**, 109 (1975).
19. G. Kimmerle and A. Eben, *Int. Archs Arbeitsmed.* **34**, 127 (1975).
20. C. Brindley, A. Gescher and D. Ross, *Chem.-Biol. Interact.* **45**, 387 (1983).
21. D. F. Heath, *Biochem. J.* **85**, 72 (1962).
22. F. Sperling, F. J. Macri and W. F. von Oettingen, *Archs ind. Hyg.* **1**, 215 (1950).
23. H. Uehlecke and T. Werner, *Archs Toxic.* **34**, 289 (1975).
24. R. O. Recknagel and E. A. Glende, *CRC Crit. Rev. Toxic.* **2**, 263 (1973).
25. J. L. Plummer, B. R. Smith, H. Sies and J. R. Bend, in *Methods in Enzymology* (Ed. W. B. Jacoby), Vol. 77, p. 50. Academic Press, London (1981).
26. T. F. Slater, *Free Radical Mechanisms in Tissue Injury*. Pion, London (1972).
27. G. O. Korsrud, H. G. Grice and J. M. McLaughlan, *Toxic. appl. Pharmac.* **22**, 474 (1972).
28. G. O. Korsrud, H. G. Grice, T. K. Goodman, J. E. Knipfel and J. M. McLaughlan, *Toxic. appl. Pharmac.* **26**, 299 (1973).
29. C. Brindley, A. Gescher, E. S. Harpur, D. Ross, J. A. Slack, M. D. Threadgill and H. Whitby, *Cancer Treat. Rep.* **66**, 1957 (1982).
30. D. Grady and R. R. Stott, U.K. Patent 1.092.632 (1965).
31. R. A. B. Drury and E. A. Wallington, *Carleton's Histological Technique*, 5th edn, p. 52. Oxford University Press, Oxford (1980).
32. C. I. Rose and A. R. Henderson, *Clin. Chem.* **21**, 1619 (1975).
33. J. F. Kachmar and D. W. Moss, in *Fundamentals of Clinical Chemistry* (Ed. N. W. Tietz), p. 672. W. B. Saunders, Philadelphia (1976).
34. F. Tietze, *Analyt. Biochem.* **27**, 502 (1969).
35. O. W. Griffiths, *Analyt. Biochem.* **106**, 207 (1980).
36. K. L. Fong, P. B. McCay, J. L. Poyer, B. B. Keele and H. Misra, *J. biol. Chem.* **248**, 7792 (1973).
37. H. Ohkawa, N. Ohishi and K. Yagi, *Analyt. Biochem.* **95**, 351 (1979).
38. T. F. Slater and B. C. Sawyer, *Biochem. J.* **123**, 805 (1971).
39. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
40. K. W. Renton, L. B. Deloria and G. J. Mannering, *Molec. Pharmac.* **14**, 672 (1978).
41. P. L. Leathwood and D. T. Plumer, *Enzymologia* **37**, 240 (1969).
42. L. Eklöw, H. Thor and S. Orrenius, *FEBS Lett.* **127**, 125 (1981).
43. P. E. Gooding, J. Chayen, B. Swayer and T. F. Slater, *Chem.-Biol. Interact.* **20**, 299 (1978).
44. T. F. Slater, in *Free Radicals, Lipid Peroxidation and Cancer* (Eds. D. C. H. McBrien and T. F. Slater), p. 242. Academic Press, London (1982).
45. J. Hogberg and A. Kristoferson, *Eur. J. Biochem.* **74**, 77 (1977).
46. O. W. Griffiths, in *Methods in Enzymology* (Ed. W. B. Jacoby), Vol. 77, p. 59. Academic Press, London (1981).
47. N. Kaplowitz, J. Kuhlenkamp, L. Goldstein and J. Reeve, *J. Pharmac. exp. Ther.* **212**, 240 (1980).
48. M. T. Smith, H. Thor and S. Orrenius, *Biochem. Pharmac.* **32**, 763 (1983).
49. P. G. Cooksey, E. N. Gate, A. Gescher, J. A. Hickman, S. P. Langdon and M. F. G. Stevens, *Biochem. Pharmac.* **32**, 3037 (1983).