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HEPATOTOXICITY OF N-METHYLFORMAMIDE IN MICE—II

COVALENT BINDING OF METABOLITES OF [^{14}C]-LABELLED *N*-METHYLFORMAMIDE TO HEPATIC PROTEINS

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Abstract—Incubation of the hepatotoxin *N*-methylformamide (NMF) labelled either in the methyl group ($OHCNH^{14}CH_3$) or the formyl group ($OH^{14}CNHCH_3$) with mouse hepatic microsomes in the presence of NADPH, but not in its absence, led to covalent binding of metabolites to microsomal proteins. When [^{14}C]NMF was injected into BALB/c mice radioactivity was found to be associated with liver and, to a much lesser extent, with kidney proteins. Association of radioactivity derived from $OHCNH^{14}CH_3$ with hepatic proteins was higher in BALB/c mice than in CBA/CA mice and in these it was higher than in BDF_1 mice. Association of label derived from either isotopomer was significantly reduced but not abolished by pretreatment of mice with cycloheximide suggesting both covalent binding and metabolic incorporation of NMF metabolites. Depletion of hepatic glutathione by pretreatment of mice with buthionine sulfoximine or diethyl maleate prior to administration of $OH^{14}CNHCH_3$ enhanced the association of label with hepatic proteins measured 1 hr after drug injection. Covalent binding of [^{14}C]NMF to hepatic microsomes *in vitro* was abolished in the presence of glutathione. It is argued that the generation of the toxic lesion and the association of NMF metabolites with hepatic proteins may be causally related even though certain mechanistic and enzymatic details of this link remain obscure.

The biotransformation of relatively inert chemicals to reactive metabolites, commonly referred to as "metabolic activation", is now considered to be an obligatory initial event in a number of toxicities induced by chemicals [1]. The experimental antitumour drug *N*-methylformamide (NMF) [2, 3] is known to be hepatotoxic [4, 5]. In an accompanying paper [6], compelling evidence is presented for the contention that NMF undergoes metabolic activation to a hepatotoxic metabolite. Reactive metabolites generated from such chemicals can bind covalently to cellular macromolecules, thus modifying their biological properties, possibly in a detrimental way [7]. The objectives of the work described in this paper were to provide a better understanding of the mechanism by which NMF causes hepatotoxicity, and, more specifically, to test the hypothesis that like other hepatotoxic drugs, NMF is metabolised to species which can bind covalently to hepatic macromolecules.

MATERIALS AND METHODS

Chemicals and animals. Materials were purchased in the purest commercially available forms: Glutathione, NADP, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, phenobarbital and cycloheximide (Sigma Chemical Co., Poole, U.K.); diethyl maleate and NMF (Aldrich Chemical Co., Gillingham, U.K.). NMF was distilled before use. (±)-Buthionine sulfoximine was prepared as described previously [8] and [^{14}C]-labelled NMF with the label either in the methyl group, $OHCNH^{14}CH_3$, or in the formyl group, $OH^{14}CNHCH_3$, was also synthesized according to a published method [9].

Experiments were conducted using male BALB/c, CBA/CA or BDF_1 mice (18–25 g, supplier: Bantin and Kingman, Hull, U.K.). Animals were housed on wire mesh and allowed free access to tap water and food (Heygate 13 breeding diet, Pilsbury's Ltd., Birmingham, U.K.) in rooms with a daily light cycle from 6 a.m. to 6 p.m. All chemicals were injected i.p. after dissolution in isotonic saline (injection volume: 0.2 ml) except diethyl maleate which was injected as a solution in arachis oil (0.2 ml). Chemicals were administered at the following doses (unless otherwise stated) which were based on the references quoted: NMF, 400 mg/kg [3], cycloheximide, 2 mg/kg 45 min before NMF or leucine, and [^{3}H] leucine, 20 mg/kg with 2 μCi/mouse [10]; (±)-buthionine sulfoximine, 1600 mg/kg 4 hr before NMF [11]; diethyl maleate, 0.3 ml/kg 1 hr before NMF [12]. In the *in vivo* binding studies 5 μCi [^{14}C] NMF was administered together with the amount of NMF to make up the desired dose.

Determination of association of label with liver and kidney macromolecules in vivo. Tissues were excised, weighed and frozen until analysis. After thawing a 25% (w/v) homogenate in water was prepared. Proteins were precipitated with 3 vol. of acetone. The precipitate was exhaustively washed with water

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Table 1. Covalent binding of metabolites of [^{14}C]NMF to hepatic microsomal proteins

Experimental conditions*	Covalent binding (ng NMF equivalents/mg protein, mean ± SD) OHCNH^{14}CH$_3$	OH^{14}CNHCH$_3$
Microsomes	14.6 ± 2.0 (7)†	14.4 ± 6.1 (4)
Heat-inactivated microsomes with NADPH	32.4 ± 5.1 (3)	13.8 ± 2.6 (3)
Microsomes with NADPH	123.6 ± 13.2 (9)‡	60.7 ± 9.8 (5)‡
Microsomes, NADPH and glutathione (10 mM)	10.0 ± 3.5 (3)§	13.9 ± 6.2 (3)‖

* [^{14}C]NMF (7 mM) was incubated with microsomes obtained from BALB/c mice for 2 hr as described under Materials and Methods.
† Number of experiments in brackets.
‡ Significantly different from microsomes alone ($P < 0.001$).
§‖ Significantly different from microsomes with NADPH (§$P < 0.001$, ‖$P < 0.01$).

(10 times) and water : methanol (1 : 1) (5 times), after which no further non-covalently bound radioactivity could be removed. The tissue pellet was solubilized in 2 ml of soluene 350 (Packard United Technologies, Reading, U.K.). The amount of protein which had been precipitated was determined according to Lowry *et al.* [13], protein loss during the washing procedure was less than 5%. The results were calculated as ng NMF equivalents bound to 1 mg protein, unless described otherwise, and are either presented as such or as percent of control binding. Radioactivity bound to DNA and RNA was determined as summarized by Pohl and Branchflower [14].

Determination of covalent binding to metabolites of [^{14}C]*NMF to liver microsomes* in vitro. Livers were excised from BALB/c mice at 9 a.m. and a homogenate (20% w/v) was prepared in phosphate buffer (50 mM, pH 7.4). Microsomes were prepared in the standard way involving centrifugation of the homogenate at 9000 *g* for 20 min and centrifugation of the 9000 *g* supernatant at 100,000 *g* for 60 min. The microsomal pellet was resuspended in phosphate buffer and aliquots (equivalent to 200 mg liver) were incubated in open beakers with [^{14}C]NMF (7 mM, specific activity 71 μCi/mol). This concentration equals the peak concentration of NMF in the blood of mice which received 400 mg/kg NMF [15]. The final incubation volume measured 2 ml and, when appropriate, contained also NADP (1 mM), glucose-6-phosphate (5 mM), glucose-6-phosphate dehydrogenase (2 units/ml) as NADPH generating system and $MgCl_2$ (5 mM). The metabolic viability of the microsomes was assessed in control incubations with aminopyrine. Aminopyrine metabolism to formaldehyde was determined according to Nash [16]. Under the conditions of the covalent binding assay aminopyrine (5 mM) was metabolized to 5.5 ± 0.8 nmoles HCHO/mg protein/min (mean ± SD, N = 3) in the presence of NADPH and to 0.9 ± 0.2 nmoles HCHO/mg protein/min without NADPH. Covalent binding experiments were carried out in duplicate. Incubations were performed with shaking at 37° for 2 hr. Microsomal proteins were precipitated with acetone (4 ml). The precipitate was washed as described for the *in vivo* experiments and the pellet obtained was dissolved in 1 M NaOH (2 ml). Aliquots of the solute were used for protein determination (50 μl) and for scintillation counting (1 ml).

Measurement of radioactivity Aqueous samples (1 ml) were mixed with Fisofluor mpc scintillant (10 ml, Fisons Ltd., Loughborough, U.K.), tissue protein samples dissolved in soluene 350 (1 ml) were mixed with Beckman Readysolve (10 ml; Beckman Instruments Ltd., High Wycombe, U.K.) and the dissolved microsomal proteins in aqueous NaOH (1 ml) were mixed with Dimilume 30 (10 ml; Packard United Technologies). Radioactivity was counted in a Packard Tricarb 2660 scintillation counter using the external standardization mode. For all samples the counting efficiency was > 80%.

Statistical analysis. Results were compared using Student's *t*-test.

RESULTS

On incubation with hepatic microsomes and an NADPH generating system, [^{14}C]NMF was metabolised to compounds which bound covalently to microsomal protein (Table 1). Approximately twice as much radioactivity derived from OHCNH^{14}CH$_3$ was bound than activity derived from OH^{14}CNHCH$_3$. When [^{14}C]NMF was administered to BALB/c mice it bound to hepatic proteins in a dose dependent manner (Fig. 1). Injection of 20 mg/kg led to only negligible binding. At the dose of 400 mg/kg approximately twice as much radioactivity was associated with proteins when OHCNH^{14}CH$_3$ (Fig. 1A) was injected as compared to OH^{14}CNHCH$_3$ (Fig. 1B) which parallels the results obtained in the *in vitro* experiments (Table 1). When calculated as percentage of the 400 mg/kg dose, 2.4 ± 0.2% in the case of OHCNH^{14}CH$_3$ and 0.8 ± 0.3% in the case of OH^{14}CNHCH$_3$ were found bound to liver macromolecules 8 hr after drug administration. Activity was not associated with nucleic acids. Therefore, we assume that metabolites were associated mainly, if not exclusively, with proteins.

In the kidneys, an organ to which NMF does not appear to be toxic, label derived from OHCNH^{14}CH$_3$ was also bound to a much greater extent than label

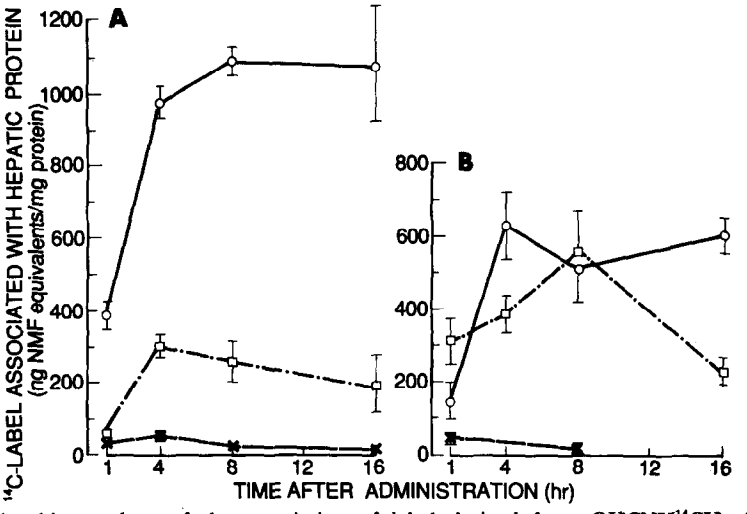

Fig. 1. Relationship to dose of the association of label derived from $OHCNH^{14}CH_3$ (A) and $OH^{14}CNHCH_3$ (B) with hepatic proteins in BALB/c mice; ×– – –× 20 mg/kg, □–·–·–□ 100 mg/kg, ○——○ 400 mg/kg. Association of activity with proteins was measured as described under Material and Methods. Values are the mean ± SD of 3 mice.

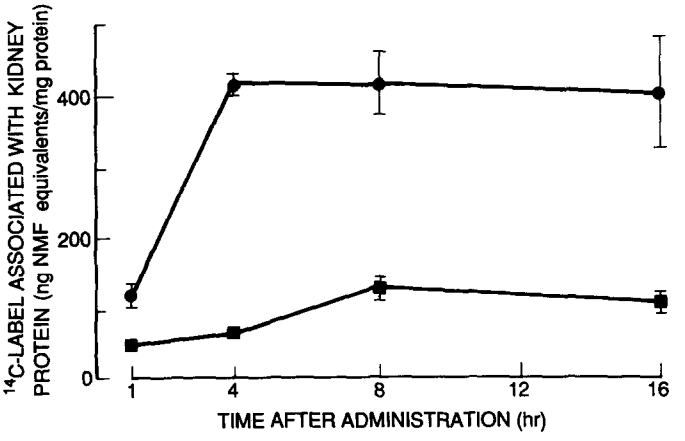

Fig. 2. Association of label derived from 400 mg/kg $OHCNH^{14}CH_3$ (●) or $OH^{14}CNHCH_3$ (■) with kidney proteins in BALB/c mice. Activity associated with proteins was determined as described under Material and Methods. Values are the mean ± SD of 3 mice.

derived from $OH^{14}CNHCH_3$ (Fig. 2). The radioactivity found in the kidneys 8 hr after administration of 400 mg/kg [^{14}C]NMF was only 36% of that associated with liver proteins in the case of $OHCNH^{14}CH_3$ and 25% after injection of $OH^{14}CNHCH_3$. Radioactivity associated with other tissues (e.g. lungs, spleen, heart) was less than a quarter of that measured in the liver (results not shown). In these tissues NMF does not appear to cause toxicity [17].

There was a marked difference between strains of mouse in the extent to which metabolites of [^{14}C]NMF were bound to or incorporated into proteins. Figure 3 shows that association of radioactivity was highest in BALB/c mice, both in the liver and the kidneys. In the case of $OHCNH^{14}CH_3$, the amount of label associated with hepatic proteins in CBA/CA mice was significantly lower than that measured in BALB/c mice but significantly higher than that measured in BDF_1 mice (Fig. 3).

In order to distinguish between covalent binding and incorporation of [^{14}C]NMF metabolites, bound radioactivity was determined in livers of mice which had been pretreated with cycloheximide, an inhibitor of protein synthesis, prior to drug administration. The decrease in association of label from [^{14}C]NMF with liver proteins caused by cycloheximide was greater than (1 hr after NMF injection) or similar to (8 hr after NMF administration) the effect which cycloheximide exerted on the incorporation of [^{14}C] leucine (Fig. 4), indicating that metabolites of NMF were incorporated to some extent into proteins via precursors of endogenous substrates. However, this result has to be interpreted with caution as the possibility cannot be excluded that cycloheximide affects the enzymes involved in the metabolism of NMF to the reactive species.

With the view of studying the role of glutathione in the processes leading to the association of NMF

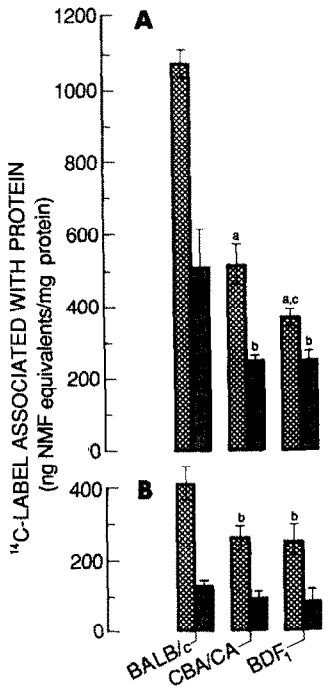

Fig. 3. Association of label derived from 400 mg/kg $OHCNH^{14}CH_3$ (hatched bars) or $OH^{14}CNHCH_3$ (stippled bars) with proteins in the liver (A) and the kidneys (B) of BALB/c, CBA/CA and BDF_1 mice. Values are the mean ± SD of 3 mice; [a,b]significant difference compared to BALB/c mice ($^aP < 0.001$, $^bP < 0.01$), [c] significant difference compared to CBA/CA mice ($P < 0.05$).

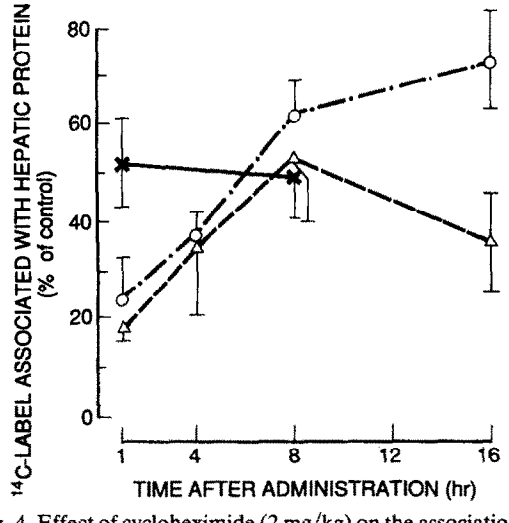

Fig. 4. Effect of cycloheximide (2 mg/kg) on the association of label derived from $OHCNH^{14}CH_3$ (○-·-·-○) or $OH^{14}CNHCH_3$ (△- - -△) with hepatic proteins, or on the incorporation of [^{3}H] leucine (×——×) into hepatic proteins in BALB/c mice. Cycloheximide (2 mg/kg) was administered 45 min prior to either 400 mg/kg [14C]NMF or 2 μCi [^{3}H]leucine. Results are expressed as percentage of values measured in mice which were not pretreated with cycloheximide (controls), on the basis of ng NMF equivalents per mg protein. Values are the mean ± SD of 3 mice.

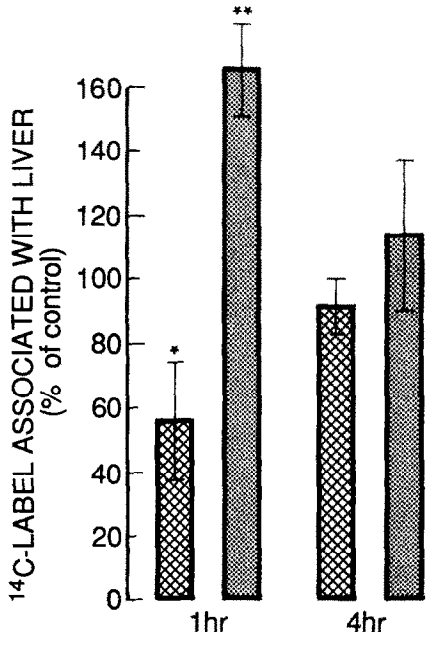

Fig. 5. Influence of pretreatment with buthionine sulfoximine (1600 mg/kg) on the association of label derived from $OHCNH^{14}CH_3$ (hatched bars) or $OH^{14}CNHCH_3$ (stippled bars) with hepatic proteins in BALB/c mice. Buthionine sulfoximine (1600 mg/kg) was injected 4 hr prior to [^{14}C]NMF (400 mg/kg) and label was measured 1 hr and 4 hr after NMF administration. Association is expressed as percent of binding observed in animals which were not pretreated with buthionine sulfoximine (controls), calculated on the basis of the amount of metabolites associated with the whole liver rather than with a mg of protein. As in these animals necrosis occurred and blood proteins accumulated in the livers within 4 hr of NMF administration, this representation was considered to reflect changes in association appropriately [20]. Values are the mean ± SD of 3 mice; stars indicate significant difference with controls, $^*P < 0.05$, $^{**}P < 0.005$.

metabolites with hepatic proteins, hepatic glutathione was depleted by administration of buthionine sulfoximine, an inhibitor of glutathione synthesis, prior to administration of [^{14}C]NMF. This pretreatment led to a decrease in hepatic glutathione to 19% of control levels at the time of [^{14}C]NMF injection [6]. Whereas pretreatment with buthionine sulfoximine inhibited the association of label derived from $OHCNH^{14}CH_3$ to a moderate extent, it markedly enhanced the association in the case of $OH^{14}CNHCH_3$ when covalently bound label was determined 1 hr after NMF administration (Fig. 5). However, 4 hr after administration, radioactivity derived from either radioisotopomer was associated with liver proteins to an extent similar to that observed in control mice. At this time livers of mice which had received NMF after buthionine sulfoximine exhibited severe haemorrhagic lesions and some animals were clearly moribund. Therefore, the interpretation of the amount of radioactivity associated with hepatic proteins at this stage may not be meaningful. Nevertheless, such hepatic damage was not apparent on macroscopic inspection of livers

1 hr after administration of NMF. Pretreatment of mice with diethyl maleate, a compound which depletes hepatic glutathione by avid electrophilic attack, also raised the level of activity associated with proteins 1 hr after administration of $OH^{14}CNHCH_3$ to 218 ± 20% (mean ± SD, N = 3) of control, whereas the association of label derived from $OHCNH^{14}CH_3$ was only 33 ± 9% (N = 3) of that observed in control mice. Thus, removal of hepatic glutathione appears to increase the propensity of liver proteins towards association with metabolites of the formyl moiety of the NMF molecule, a finding congruent with the observation that the covalent binding from both isotopomers of [^{14}C]NMF to hepatic microsomes *in vitro* was completely abolished in the presence of 10 mM glutathione (Table 1).

DISCUSSION

It is shown here for the first time that metabolites of NMF bind covalently to hepatic macromolecules, and there are three pieces of evidence which suggest that the generation of the toxic lesion caused by NMF and the covalent binding of it metabolites are causally related.

(i) The binding observed *in vivo* was appreciably higher in the organ in which toxicity is expressed than in other tissues.

(ii) The rank order of association of metabolites of [^{14}C]NMF with liver macromolecules in three strains of mouse *in vivo* (Fig. 3) paralleled differences between the sensitivity of these strains towards NMF-induced hepatotoxicity and hepatic glutathione depletion [6].

(iii) Modulation of the hepatic glutathione status prior to NMF administration affected both the severity of the hepatic damage and the binding in a fashion which suggests the involvement of a reactive, toxic intermediate. Depletion of hepatic glutathione exacerbated toxicity and increased binding of metabolites of the NMF formyl moiety *in vivo* (Fig. 5). Conversely, pretreatment of mice with thiol compounds protected the livers against toxicity and, likewise, the presence of glutathione in the incubation medium totally abolished covalent binding measured *in vitro* (Table 1).

The radioactivity found to be associated with the hepatic proteins *in vivo* was at least partially due to incorporation into proteins of metabolites which were precursors of endogenous substrates. This conclusion is based on the finding that inhibition of protein synthesis markedly decreased the association. What percentage of the overall association was due to covalent adduct formation is difficult to establish. However, since covalent binding occurred *in vitro*, it is likely that it also contributed to overall association with tissue macromolecules *in vivo*.

There was a difference between the two [^{14}C]NMF isotopomers with regard to both the extent of binding *in vitro* and *in vivo* and the relationship between dose and their binding *in vivo*. This suggests that metabolic rupture of the amide bond in the NMF molecule precedes binding or incorporation of at least a portion of the dose of [^{14}C]NMF injected. The end products of NMF metabolism have recently been identified [18]. Carbon dioxide and methylamine are the major metabolites of NMF and both, or their immediate precursors, could conceivably contribute to incorporation of label into protein, as they are substrates of endogenous metabolic pathways. On the basis of the recent finding that *S*-(*N*-methylcarbamoyl)-*N*-acetylcysteine is a urinary metabolite of NMF in rodents and man [19], a number of chemically plausible electrophilic metabolic intermediates of NMF can be proposed. The formation of these intermediates could explain both the hepatic necrosis [4, 5] and the hepatic covalent binding which NMF causes in mice. Which enzyme system is responsible for the activation of NMF is still unclear.

Pretreatment of mice with buthionine sulfoximine exacerbated NMF toxicity [6] and it increased the association of label derived from $OH^{14}CNHCH_3$ with liver proteins *in vivo*. Assuming that the binding characterised in this study is indeed linked to the genesis of the toxic lesion one could conclude that the metabolic fate of the NMF formyl moiety is more crucial for the development of toxicity than that of the methyl moiety. It is, however, difficult to reconcile this conclusion with the difference between the two isotopomers in the relationship of the binding to dose. The amount of radioactivity derived from $OH^{14}CNHCH_3$ which was found in the liver after administration of the toxic dose of 400 mg/kg was only approximately twice the amount bound after the innocuous dose of 100 mg/kg, whereas in the case of $OHCNH^{14}CH_3$ the level of association after 400 mg/kg was approximately four times that observed after 100 mg/kg (Fig. 1). This would indicate that the extent of binding of the $^{14}CH_3$-labelled isotopomer reflects more convincingly the dose threshold for toxicity than does the binding of the ^{14}CHO-labelled isotopomer. This interpretation would be in accordance with the postulate that events leading to the association of metabolites of the NMF formyl moiety are less important for toxicity than those causing the binding of metabolites of the methyl group.

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