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TOXICITY OF METHYLATING AGENTS IN ISOLATED HEPATOCYTES

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Abstract—To investigate the pathogenesis of hepatotoxicity by methylating agents, we exposed isolated hepatocytes to *N*-nitrosodimethylamine (NDMA), *N*-methyl-*N'*-nitro-*N* nitrosoguanidine (MNNG), *N*-methyl-*N*-nitrosourea (MNU), or methyl methanesulfonate (MMS). Although NDMA is a potent *in vivo* hepatotoxicant in rats, no evidence of hepatocyte injury, measured by the leakage of lactate dehydrogenase (LDH) activity into the medium, was observed following exposure to a 1–100 mM concentration of either NDMA or MNU. In contrast, exposure of hepatocytes to MMS or MNNG resulted in ≥90% LDH release. These differences in toxicity were not related to the extent of covalent binding to hepatocytes. Following MMS or MNNG, but not MNU or NDMA exposure, a significant rise in the generation of thiobarbiturate (TBA)-reactive species was observed. When hepatocytes were exposed to the antioxidant promethazine prior to the addition of MMS or MNNG, the formation of TBA-reactive species was inhibited completely. Although promethazine blocked MNNG-mediated cell injury, the antioxidant had no effect on MMS intoxication. These data suggest that methylating agents can cause hepatotoxicity by more than a single mechanism. For MNNG, lipid peroxidation may be involved in the pathogenesis of acute hepatotoxicity.

Exposure to some alkylating agents results in acute cytotoxic hepatic injury [1]. This hepatotoxicity is thought to result from the covalent binding of alkylating moieties to subcellular target sites critical for normal cell function, culminating in irreversible cell injury [2, 3].

Methylating agents represent perhaps the simplest form of alkylating agents. Numerous studies have indicated that the methylating agent *N*-nitrosodimethylamine (NDMA)§ is a potent hepatotoxicant *in vivo* [4, 5]. However, isolated hepatocytes were not killed by exposure to NDMA [6]. This lack of toxicity may have been due to an inadequate amount of tissue methylation, since generation of methylating moieties from NDMA requires cytochrome P-450 activation [7], and cytochrome P-450 levels can become decreased in isolated hepatocytes [8]. Alternatively, NDMA and/or other methylating agents may not be toxicants in this model system. Therefore, the purpose of the present study was to characterize the toxicity of methylating agents in freshly isolated hepatocytes. To this end, hepatocytes were exposed to either NDMA or to the direct-acting methylating agents methyl methanesulfonate (MMS), *N*-methyl-*N*-nitrosourea (MNU) or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG). At various time points, hepatocyte suspensions were examined for viability and biochemical markers indicative of toxic mechanisms. The results of this study suggest that methylating agents can induce acute hepatocellular necrosis by more than a single mechanism.

MATERIALS AND METHODS

Materials. NDMA and MMS were obtained from the Aldrich Chemical Co. (Milwaukee, WI) and the Eastman Kodak Co. (Rochester, NY), respectively. MNU and MNNG were obtained from the Sigma Chemical Co. (St. Louis, MO). *N*-[^{3}H]Methyl-*N'*-nitro-*N*-nitrosoguanidine (1 Ci/mmol), [^{14}C]methyl methanesulfonate (50 mCi/mmol), *N*-[^{14}C]methyl-*N*-nitrosourea (61 mCi/mmol), and nitroso-*N*,*N*-di[^{14}C]methylamine (8 mCi/mmol) were obtained from the Amersham Corp. (Arlington Heights, IL). Collagenase was obtained from Worthington Biochemicals (Freehold, NJ). Other chemicals were either of reagent grade quality or better.

Hepatocyte preparation. Hepatocytes were prepared from male Sprague–Dawley rats (250–300 g) by the method of Zahlten and Stratman [9], and were suspended in Krebs–Henseleit buffer supplemented with 10 mM glucose, 5 mM lactose, 5 mM glutamate, 10 mM fumarate, 1 mg/ml bovine serum albumin (BSA), and 2.5 mM $CaCl_2$, at a concentration of 25 mg wet cell weight/ml. Following isolation, 85–95% of the hepatocytes excluded trypan blue. Cells were incubated in a rotary shaker (80 cycles/min) at 37° under a constant flow of humidified carbogen (95% oxygen, 5% carbon dioxide).

Treatment of hepatocytes with methylating agents.

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§ Abbreviations: NDMA, *N*-nitrosodimethylamine; MMS, methyl methanesulfonate; MNU, *N*-methyl-*N*-nitrosourea; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; GSH, reduced glutathione; LDH, lactate dehydrogenase; MDA, malondialdehyde; CCl_4, carbon tetrachloride; ESR, electron spin resonance; and TBA, thiobarbituric acid.

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NDMA (10 or 100 mM) was added directly to cell suspensions. MMS (2.0 mM) was diluted in water and then added immediately to cell suspensions. MNNG (0.5 mM) was dissolved in ethanol (1% v/v) vehicle, and MNU (10 mM) was dissolved in dimethyl sulfoxide (DMSO, 0.5% v/v) vehicle, to assure adequate dispersion in the cell suspensions. The doses of the toxicants chosen for examination were based on results from preliminary studies. For MNNG (0.5 mM) and MMS (2.0 mM), the lowest consistently lethal concentrations were chosen. For NDMA, 10 mM and 100 mM saturate the high-affinity and low-affinity NDMA demethylases respectively [10]. Because similar effects were observed at both doses, a single symbol is employed in the figures for clarity. No effects were observed at lower doses. For MNU, the limits of its solubility in DMSO and the tolerance of hepatocytes to this vehicle dictated a maximal MNU concentration of 10 mM. Since exposure of hepatocytes to either vehicle alone did not affect significantly any parameter examined, these data were omitted from the figures for clarity.

Cell thiol assays. Hepatocyte suspensions were acidified by treatment with 5% trichloroacetic acid, and the precipitates were pelleted by centrifugation at 3000 rpm for 1 min. Reduced glutathione (GSH), reflected by acid soluble thiol concentration, was then determined in the resulting supernatant solutions [11].

Lipid peroxidation. Hepatocyte suspensions were assayed for products of lipid peroxidation by the method of Burk *et al.* [12]. Briefly, cell suspensions precipitated with 5% trichloroacetic acid were centrifuged for 1 min at 3000 rpm. Aliquots of the resulting supernatant solutions were assayed for thiobarbituric acid (TBA)-reactive species as previously described [13], using malonaldehyde bis-dimethylacetal (MDA) as the standard.

Covalent binding. Calculations from preliminary studies, based upon the specific activities of the radiolabeled compounds, and the percentage of the compounds that become covalently bound, indicated that the amount of radioactive materials required to detect binding utilizing the same concentrations employed in the toxicity experiments would have been prohibitively expensive. Therefore, tracer quantities (1.0 μCi) of NDMA, MNNG, MNU or MMS were added to 4 ml of cell suspensions. Cell suspension aliquots of 0.4 ml were removed after 5, 15 and 60 min and were added to 0.8 ml of ice-cold ethanol. These mixtures were incubated for 5 min at 0°, then centrifuged at 3000 rpm for 2 min, and the supernatant solutions were decanted. The pellets were washed with ethanol four additional times by repeated sonication and centrifugation. This procedure recovered 98% of the protein and 100% of the DNA. The final pellets were solubilized in 0.25 ml of 1 N NaOH. Following the addition of 0.2 ml of this preparation to 1 ml water and 10 ml Aquasol-2 (New England Nuclear), the radioactivity present in each sample was determined by liquid scintillation spectrophotometry, using a Packard Tri-Carb 460 CD Liquid Scintillation System. The data obtained were expressed as a percentage of the total radioactivity added to the hepatocyte suspensions that bound to cell macromolecules. In addition, estimates for total binding occurring at the doses employed in the toxicity studies were calculated, and expressed as nanomoles of CH_3 bound per milligram of cell wet weight.

Assessment of hepatocyte viability. Hepatocellular viabilities subsequent to chemical addition were routinely assessed by measuring lactate dehydrogenase (LDH) activity associated with the hepatocytes and the suspension medium [14], and expressed as a fraction of the LDH activity that had leaked from the hepatocytes into the suspension medium.

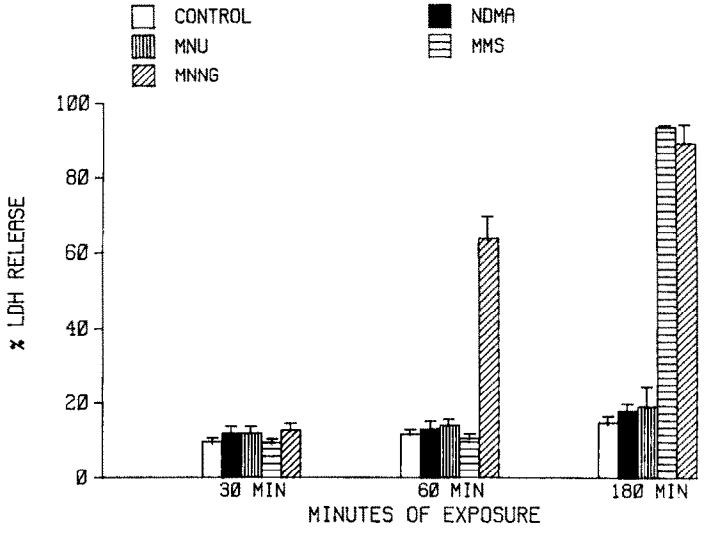

Fig. 1. Effects of methylating agents on hepatocyte viabilities. Hepatocytes were exposed to the four methylating agents and, at the indicated times, viabilities were assessed by measuring LDH leakages as described in Materials and Methods. Values represent mean ± SEM from four to six separate hepatocyte preparations.

Statistics. Statistical evaluations were conducted using Student's *t*-test; $P < 0.05$ was considered significant.

RESULTS

Effects of methylating agents on hepatocellular viability. Hepatocyte suspensions were exposed to either NDMA, MNNG, MMS or MNU at time 0, and hepatocyte viabilities were assessed at 30, 60 and 180 min by measuring the extent of LDH leakage into the culture medium (Fig. 1). At the concentrations employed, MMS and MNNG were acutely cytotoxic, as indicated by the nearly complete leakage of LDH activity into the culture medium within 180 min. In contrast, neither MNU or NDMA were hepatotoxic, indicated by a lack of LDH leakage when compared to controls over the same period of time. When followed to 300 min, no evidence of diminished cell viability induced by these latter two compounds was observed (data not shown).

Covalent binding. To determine whether the variable cytotoxicity of these methylating agents was due to differences in either their uptake by hepatocytes or the subsequent extent of alkylation of cell macromolecules, the covalent binding of tracer quantities of each compound was measured. Calculated as a percentage of total radiolabeled material added to the cell suspensions, significant and comparable levels of covalent binding were observed for all four compounds (Table 1). When expressed as the estimated total amount of material bound to cell macromolecules, however, NDMA, MNU and MMS demonstrated comparable and significantly greater binding than MNNG over 60 min.

Cell thiol status. The lack of a clear relationship between covalent binding and toxicity led to the examination of other effects on hepatocytes. Since the decomposition or metabolism of methylating agents results in the formation of reactive electrophiles, the effects of compound exposure on the concentrations of the major cellular nucleophile, GSH, were examined. Hepatocellular GSH content fell to less than 25% of control values afer 30 min of exposure to NDMA (100 mM), or the other three methylating agents (Fig. 2). For hepatocytes exposed to MMS and MNNG, this GSH depletion was sustained for an additional 150 min. In contrast, following this initial decline, GSH levels rose significantly ($P < 0.05$) after an additional 150 min in hepatocytes exposed to MNU or NDMA. Thus, loss of cell viability was related to profound and sustained depletion of cellular GSH.

Lipid peroxidation. GSH depletion is known to predispose hepatocytes to oxidative injury [15]. Therefore, a marker of lipid peroxidation was measured following exposure to these methylating agents. The quantity of TBA-reactive species increased in suspensions of hepatocytes exposed to MMS or MNNG (Fig. 3). In contrast, there was no production of TBA-reactive species following exposure to either MNU or NDMA.

Effects of promethazine. Since we had shown previously that radical-mediated, CCl_4-induced injury in isolated hepatocytes is characterized by GSH depletion and MDA formation, and that pretreatment with the antioxidant promethazine diminishes both injury and MDA formation [13], the effect of promethazine on injury induced by MMS and MNNG was examined. Promethazine completely prevented MDA formation following MNNG or MMS exposure (Fig. 4A). Moreover, promethazine prevented MNNG-induced cytotoxicity; however, it did not protect against MMS-induced injury (Fig. 4B).

DISCUSSION

The present study was designed to examine the mechanism of hepatic injury induced by a series of methylating agents. Since it is believed that sufficient alkylation of tissue macromolecules results in acute cellular injury [2, 3], it was expected that freshly isolated hepatocytes exposed to any of these methylating agents at sufficient concentrations would lose viability rapidly. Indeed, previous studies had shown that MMS is lethal to cultured hepatocytes [16] and that NDMA is a potent *in vivo* hepatotoxicant [4].

Table 1. Covalent binding of radiolabel to cell macromolecules

Compound concentration	Covalent binding					
	5 min		15 min		60 min	
	Percent*	Total†	Percent	Total	Percent	Total
NDMA (10 mM)	0.08 ± 0.02	0.32 ± 0.06	0.15 ± 0.02	0.58 ± 0.09	0.57 ± 0.07	2.30 ± 0.29
MNU (10 mM)	0.38	1.54	0.33	1.32	0.32	1.26
MMS (2 mM)	0.67 ± 0.10	0.53 ± 0.08	0.83 ± 0.11	0.67 ± 0.09	1.21 ± 0.09	0.97 ± 0.07
MNNG (0.5 mM)	0.46 ± 0.04	0.09 ± 0.01	0.43 ± 0.04	0.09 ± 0.01	0.43 ± 0.04	0.09 ± 0.01

Hepatocytes were exposed to radiolabeled methylating agents. At the indicated times, the extent of covalent binding of methylating moieties to cell macromolecules was determined as described in Materials and Methods. Values represent means ± SEM from four (MMS, NDMA, MNNG) or the average of two (MNU) separate hepatocyte preparations.

* Represents the percentage of radioactivity added to the hepatocyte suspensions that had become covalently bound.

† Represents binding expressed as nmoles CH_3 bound/mg cell weight, which was calculated from the percent binding data and the indicated concentrations.

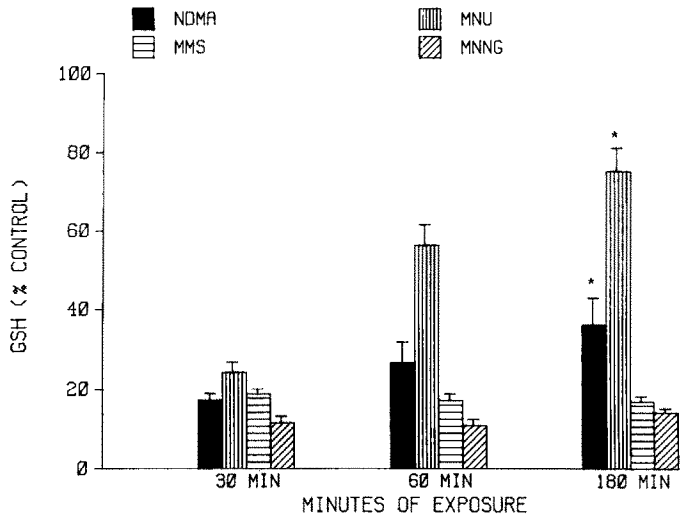

Fig. 2. Effects of methylating agents on hepatocyte GSH concentrations. Hepatocytes were exposed to the four methylating agents and, at the indicated times, GSH concentrations were assessed as described in Materials and Methods. Values are based on the means ± SEM from four to six separate hepatocyte preparations, and a GSH concentration for untreated hepatocytes of 3.60 nmol/mg cell weight. An asterisk denotes significantly higher than corresponding 30-min value, $P < 0.05$.

However, our results indicate that the pathogenesis of liver injury by this group of compounds may be far more complex.

The compounds employed in the current study were chosen for comparison because they all result in tissue alkylation by the transfer of a methyl group. However, significant differences were known to be inolved in their methylation of cellular macromolecules which potentially could influence resultant toxicity. MMS methylates cell substituents by an SN2 mechanism [17]. The three *N*-nitroso compounds (NDMA, MNU, and MNNG) are thought to alkylate cell substituents by an SN1 mechanism, with $CH_3N_2^+$ as the postulated methylating intermediate [18, 19]. Moreover, within the nitroso group of compounds, the method of generation of the methylating intermediate is known to vary. For NDMA, the intermediate is generated enzymatically by cytochrome P-450 [7]. For MNU, the intermediate is generated by base-catalyzed hydrolysis [18, 20]. For MNNG, intermediate formation requires the interaction of the electron-deficient carbon atom of the parent compound with cellular sulfhydryl groups [21, 22]. Because much of this information was generated by studies concerned with mutagenicity or carcinogenicity of these compounds, few data were

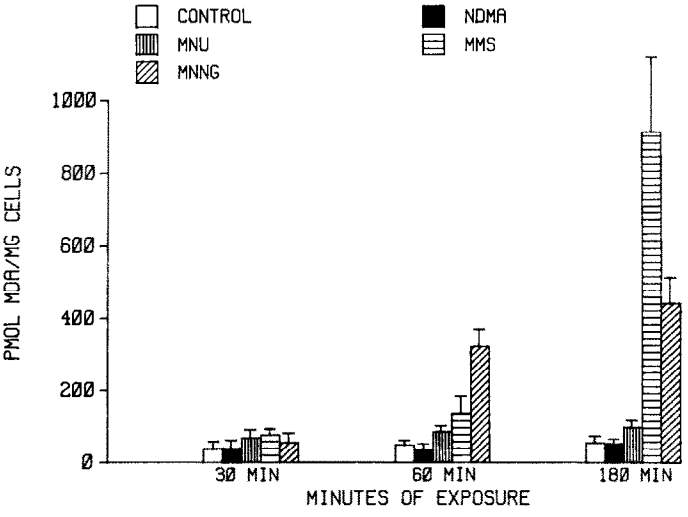

Fig. 3. Evidence of hepatocellular lipid peroxidation. Hepatocyte suspensions were exposed to the four methylating agents and, at the indicated times, the concentration of TBA-reactive species in the suspensions was measured and quantitated by comparison to an MDA standard curve. Values are means ± SEM from four to six separate hepatocyte preparations.

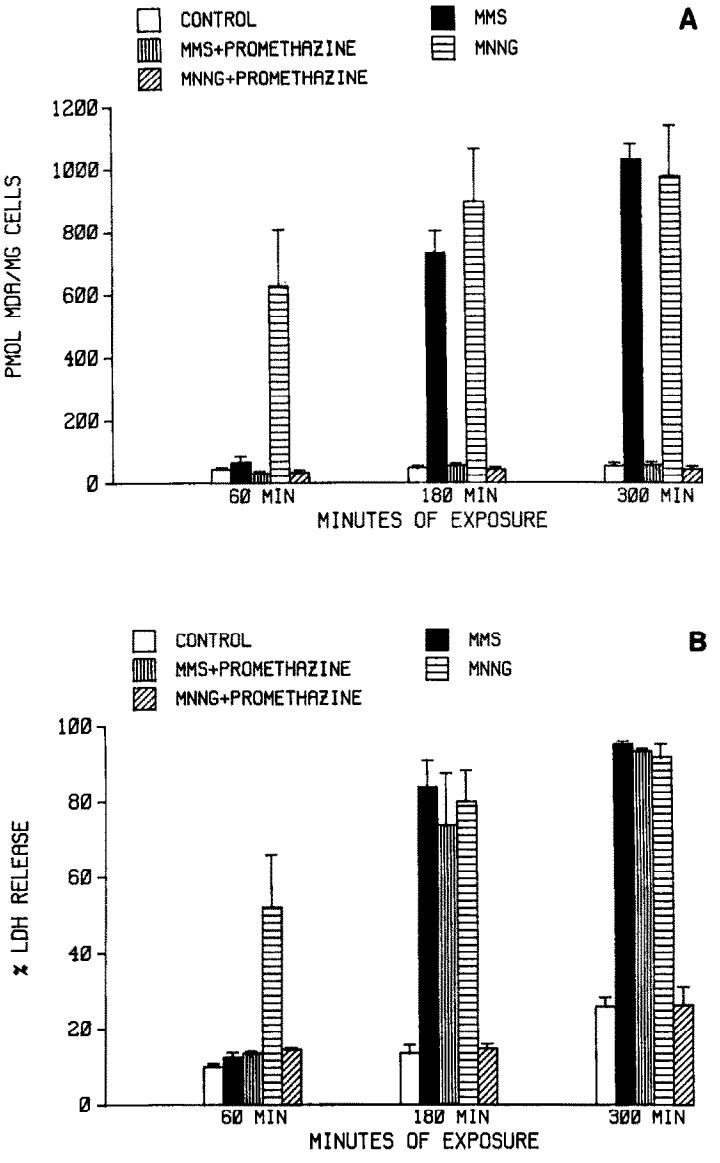

Fig. 4. Effect of promethazine pretreatment on (A) lipid peroxidation or (B) cytotoxicity following MMS or MNNG exposure. At various times, aliquots of cell suspensions were assayed for TBA-reactive species (A) or LDH leakage (B) as described in Materials and Methods. Values are means ± SEM from four to six separate hepatocyte preparations.

available regarding their potential as acute hepatotoxicants.

Since each of the four methylating agents rapidly produced a substantial and comparable depletion of GSH in hepatocytes, we ruled out the possibility of attributing qualitative differences in toxicity to differences in cell permeability or metabolism. To evaluate the possible role of alkylation of cellular macromolecules as a causative factor, we compared the amount of covalent binding calculated either as a percentage of radiolabel bound, or estimated for the amount of binding that would occur at the doses employed in the toxicity studies. The latter analysis assumed that for each compound the relative amount of covalent binding was constant for both the toxicity and binding studies.

The observed lack of correlation of the extent of methylation with subsequent toxicity would seem to be at variance with a large body of evidence that supports a direct role for tissue alkylation or arylation in the mediation of liver toxicity, mutagenesis, and carcinogenesis by a variety of compounds, including NDMA [1–3, 23]. Dose relationships between binding and toxicity have been demonstrated for individual compounds rather than within a group of compounds. This suggests that the course of liver injury may be influenced by other factors including the mechanisms and sites of compound activation, and the nature of the reactive metabolites produced. We hypothesize that the "hardness" of the reactive metabolites generated by these methylating agents is one important factor.

Model hepatotoxicants, such as bromobenzene and acetaminophen, are metabolized into soft electrophiles which tend to bind to soft nucleophilic sites in the cell [24, 25]. Such sites include the thiol groups of cysteinyl residues in protein or GSH, but not the relatively hard electrophilic sites such as the oxygens and certain nitrogens of the nucleic acids [26]. Thus, high doses of these compounds are required to deplete liver GSH before hepatoxicity results. In the current studies, MMS exposure induced a sustained depletion of GSH. This is compatible with the SN2 mechanism by which MMS alkylates cell substituents because it results in the attack of soft nucleophilic sites. This has been inferred from a lower O^6-thymine/N^7-guanine alkylation ratio for MMS when compared to MNU [27, 28], MNNG [18] or NDMA [29], as well as the observations that alkylsulfonates are known to alkylate proteins more extensively than alkylnitrosoureas or alkylnitrosamines [26].

In contrast, the postulated alkylating intermediate of the nitroso compounds is $CH_3N_2^+$ [19], a relatively hard electrophile [26]. This results in the preferential alkylation of hard nucleophilic sites [26, 30], particularly nucleic acids. For NDMA, this reactivity is reflected by high nucleic acid/protein [31] and O^6-thymine/N^7-guanine alkylation ratios [29]. Thus, it might be expected that NDMA alkylation would attack different sites when compared to bromobenzene, acetaminophen, or MMS and require much higher doses to deplete cellular GSH. Indeed, the employment of exceedingly high doses of NDMA in the current studies probably accounts for the decline in cellular GSH; however, this was neither sustained nor accompanied by acute toxicity. Since NDMA hepatoxicity *in vivo* is not preceded by appreciable GSH depletion [5], it seems likely that the observed GSH decline was a pharmacologic effect without toxicologic relevance. Moreover, the hardness of the reactive intermediate of NDMA may also explain why NDMA-induced liver cell injury was not observed, since inadequate levels of binding to toxicologically relevant critical sites may not have occurred. Such a possibility will be difficult to pursue until the mechanistic etiology of NDMA toxicity *in vivo* is better defined. Similar considerations may also pertain to the absence of toxicity observed following MNU exposure.

In contrast to both NDMA and MNU, MNNG exposure produced acute toxicity, accompanied by a sustained depletion of GSH and significant MDA formation. This suggested that one potential effect of MNNG exposure was radical-mediated oxidative stress, resulting in acceleration of the development of cell injury. This hypothesis is supported by a diminution of both toxicity and MDA formation by the antioxidant promethazine. Indeed, the production of a radical metabolite from MNNG has been proposed [21] and an ESR signal indicating free radical formation has been detected [32]. MMS also induced significant MDA formation; this did not appear to be of primary importance to toxicity, since promethazine suppressed MDA formation without affecting the course of injury. Thus, the current data suggest that multiple mechanisms of injury may be induced by a series of methylating agents. We propose that the course of injury may be influenced by the nature of their reactive intermediates.

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REFERENCES

1. H. J. Zimmerman, *Hepatotoxicity, the Adverse Effects of Drugs and Other Chemicals on the Liver.* Appleton-Century-Crofts, New York (1978).
2. J. R. Gillette, J. R. Mitchell and B. B. Brodie, *A. Rev. Pharmac.* **14**, 271 (1974).
3. J. R. Mitchell, H. Hughes, B. H. Lauterberg and C. V. Smith, *Drug Metab. Rev.* **13**, 539 (1982).
4. R. C. Shank, *Toxic. appl. Pharmac.* **31**, 361 (1975).
5. H. G. Shertzer, M. W. Tabor and M. L. Berger, *Expl molec. Path.* **47**, 211 (1987).
6. D. L. Story, S. J. Gee, C. A. Tyson and D. H. Gould, *J. Toxic. environ. Hlth* **11**, 483 (1983).
7. T. Kawanishi, Y. Ohno, A. Takahashi, A. Takanaka, Y. Kasuya and Y. Omori, *Archs Toxic* **56**, 7 (1984).
8. G. L. Englemann, A. G. Richardson and J. A. Fierer, *Archs Biochem. Biophys.* **238**, 359 (1985).
9. R. N. Zahlten and F. W. Stratman, *Archs Biochem. Biophys.* **163**, 600 (1974).
10. J. C. Arcos, D. L. Davies, C. E. L. Brown and M. F. Argus, *Z. Krebsforsch.* **89**, 181 (1977).
11. J. Sedlak and R. L. Lindsay, *Analyt. Biochem.* **25**, 192 (1968).
12. R. F. Burk, K. Patel and J. M. Lane, *Biochem. J.* **215**, 441 (1983).
13. M. L. Berger, H. Bhatt, B. Combes and R. W. Estabrook, *Hepatology* **6**, 36 (1986).
14. H. Bergmeyer and E. Bernt, in *Methods of Enzymatic Analysis* (Ed. H. Bergmeyer), pp. 574–8. Academic Press, New York (1974).
15. G. Bellomo and S. Orrenius, *Hepatology* **5**, 876 (1985).
16. F. A. X. Schanne, A. B. Kane, E. E. Young and J. L. Farber, *Science* **206**, 700 (1979).
17. G. A. Sega, K. W. Wolfe and J. G. Owens, *Chem. Biol. Interact* **33**, 253 (1981).
18. P. D. Lawley, *Mutation Res.* **23**, 283 (1974).
19. W. Lijinsky, J. Loo and A. E. Ross, *Nature, Lond.* **218**, 1174 (1968).
20. E. G. Garrett, S. Gato and J. F. Stubbins, *J. pharm. Sci.* **54**, 119 (1965).
21. P. D. Lawley, in *Topics in Chemical Carcinogenesis* (Eds. E. Nakahara, S. Takayama, T. Sugimura and S. Odashima), pp. 237–58. University Park Press, Baltimore (1971).
22. P. D. Lawley and C. J. Thatcher, *Biochem. J.* **116**, 693 (1970).
23. R. Montesano and H. Bartsch, *Mutation Res.* **32**, 179 (1976).
24. J. R. Mitchell, D. J. Jollow, W. Z. Potter, J. R. Gillette and B. B. Brodie, *J. Pharmac. exp. Ther.* **187**, 211 (1973).
25. D. J. Jollow, J. R. Mitchell, N. Zampaglione and J. R. Gillette, *Pharmacology* **11**, 151 (1974).
26. B. Coles, *Drug Metab. Rev.* **15**, 1307 (1984–1985).
27. A. Loveless, *Nature, Lond.* **223**, 206 (1969).
28. J. Krepinsky, J. P. Carver, S. Rajalakshmi, P. M. Rao and D. S. R. Sarma, *Chem. Biol. Interact.* **27**, 381 (1979).
29. B. Singer, *Prog. nucleic Acid Res. molec. Biol.* **15**, 219 (1975).
30. F. P. Guengerich and D. C. Liebler, *CRC Crit. Rev. Toxic.* **14**, 259 (1985).
31. P. N. Magee and E. Farber, *Biochem. J.* **83**, 114 (1962).
32. C. Nagata, Y. Ioki, M. Kodama and Y. Tagashira, *Ann. N.Y. Acad. Sci.* **222**, 1031 (1973).