Environmental and Molecular Mutagenesis 27:202–210 (1996)

Unexpected Genetic Toxicity to Rodents of the N′,N′-Dimethyl Analogues of MNU and ENU

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Lijinsky and his colleagues have reported that the N′,N′-dimethyl analogues of ENU and MNU [N′,N′-dimethyl-N-ethyl-N-nitrosourea (DMENU) and trimethylnitrosourea (TMNU), respectively] are carcinogenic to rats despite their extreme hydrolytic stability which would reduce or preclude generation of alkylating species analogous to those formed upon hydrolysis of ENU and MNU. Lijinsky and his colleagues were unable to rationalize those activities of DMENU and TMNU despite extensive experimentation. We therefore decided to study this problem further.

Whichever mode is accepted for the generation of electrophilic/mutagenic/carcinogenic reactive species from ENU and MNU, blocking of the free–NH_2 group with methyl groups (–NMe_2) should ablate or abolish activity. Consistent with this, DMENU and TMNU gave negative results in the NBP alkylation test while the parent compounds gave an instantaneous deep blue coloration. Studies of the rate of hydrolysis of these four compounds revealed ENU and MNU to have half-lives of 8 min, while the alkylated analogues (DMENU and TMNU) had half-lives of 25 and 41 days, respectively. Hydrolysis of ENU and MNU, to yield the alkylating species, proceeds either via proton abstraction from the –NH_2 group or by attack by water on the carbon of the carbonyl group. Methylation will inhibit both of these pathways, the first absolutely (no –NH_2 protons) and the second partially, via steric inhibition. The slow hydrolysis observed for DMENU and TMNU suggests that the latter route of hydrolysis is applicable. Studies with strain TA1535 of *Salmonella typhimurium* (without S9 mix) confirmed the potent mutagenic activity for ENU and MNU (~300-fold increase in revertants at 2,000 μg/plate and ~180-fold increase in revertants at 150 μg/plate respectively). In contrast, the methylated analogues showed only weak mutagenic activity (~3-fold) at ~100-fold higher dose-levels. Addition of S9 mix did not affect the mutagenicity of DMENU or TMNU. To this point, hypothesis and data coincide.

ENU and MNU are potent micronucleus-inducing agents to the mouse bone marrow, and given the above data, it was expected that DMENU and TMNU would show weak or no activity in that assay. In fact, the methylated analogues were as effective as ENU and MNU as clastogens to the mouse bone marrow. Four possible reasons for this conflict of theory and data are explored. The speculative explanation we favour for these effects is that the net alkylation of bone marrow DNA is the same for all four chemicals. With ENU and MNU, most of the alkylating activity is dissipated by rapid hydrolysis. Thus, only a small fraction of the administered dose survives to alkylate the bone marrow. Due to the enhanced stability of the methyl analogues most of the delivered dose will reach the bone marrow. However, because of their lower intrinsic reactivity, only a small fraction of the target dose will alkylate the bone marrow DNA during the time window of the experiment. If these opposing influences happen to balance out, the essentially identical bone marrow genetic toxicity for the four chemicals could be explained.

Key words: ENU, MNU, hydrolysis, –NH_2, methylation

INTRODUCTION

Over the past 20 years Lijinsky and his colleagues have made detailed studies of the mutagenicity and carcinogenicity of a wide range of nitrosamines and nitrosamides. In 1973, Elespuru and Lijinsky described the preparation of the N′,N′-dimethyl derivatives (TMNU and DMENU respectively) of N-methyl-N-nitrosourea (MNU) and N-ethyl-N-nitrosourea (ENU) (for chemical structures and hydrolysis of ENU/MNU, see Figure 1). Subsequent studies [Lijinsky and Taylor, 1975; Andrews et al., 1978; Lijinsky and Andrews, 1983] revealed that these two trialkylnitrosoureas were remarkably stable to hydrolysis, and were essentially non-mutagenic to Salmonella, albeit some activity was observed for DMENU when tested using hamster S9 mix. Nonetheless, both of the chemicals induced tumours of the central nervous system when administered in drinking water to rats. It was not clear what had led to the dramatic increase in hydrolytic stability and the loss of mutagenicity of TMNU and DMENU. Equally, it was noted as curious and inexplicable that

Received May 22, 1995; revised and accepted December 13, 1995.

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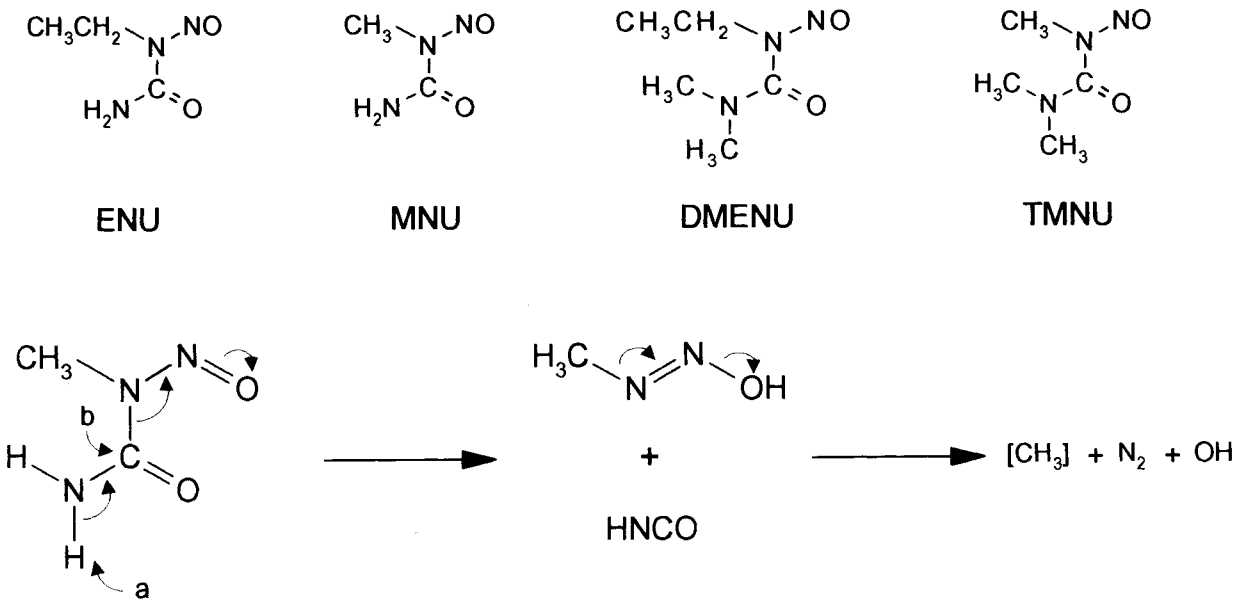


Fig. 1. Chemical structures of ENU, MNU, DMENU and TMNU. Schematic showing how either hydrolytic proton abstraction (**a**) or attack at the carbonyl (**b**) could yield alkylating species from ENU or MNU. Substitution of the $-NH_2$ group with methyl groups would abolish step (a) and attenuate step (b) by steric inhibition of the carbon atom.

each compound should have produced tumours in rats given their failure to decompose to the alkyldiazonium alkylating species formed by the parent compounds MNU and ENU. A weakness of the studies on TMNU and DMENU by Lijinsky and colleagues was that few direct comparisons with ENU and MNU were made. We therefore decided to evaluate these four compounds in parallel to determine their rates of hydrolysis, their relative alkylating potential, their relative mutagenicity to *Salmonella typhimurium* (TA1535) and their relative activity in the mouse bone marrow micronucleus (MN) assay.

MATERIALS AND METHODS

Chemicals

Corn oil was obtained from Kraft-Wesson. ENU, MNU, sodium azide and 2-aminoanthracene were purchased from Sigma Chemical Company, Poole, Dorset. DMENU was prepared by reaction of dimethylamine with ethyl isocyanate, followed by nitrosation, as described by Elespuru and Lijinsky [1973]. The N',N'-dimethyl-N-ethylurea had a melting point of 54–55°C and was found to be pure by elemental analysis, NMR and mass spectroscopy. Nitrosation, as described by Elespuru and Lijinsky [1973], gave DMENU as an oil. HPLC (water/methanol 15:85, Spherisorb 5ODS2) showed the product to be 97.3% pure (λ_{max} 239 nm). C, H and N analysis suggested retention of a trace (1/20 mol) of methylene chloride, confirmed by ^1H NMR.

TMNU was described as an oil by Elespuru and Lijinsky [1973]. The present sample was synthesized by treatment of trimethylurea (1.1 g; Alfred Bader Chemicals) in 5 N sulphuric acid (2.5 ml) with a small excess of sodium nitrite (0.875 g; added in portions over 10 min) at <5°C. After 20 min, the yellow oil which had formed was extracted into ethyl acetate (2 × 20 ml). The extract was dried (magnesium sulphate), filtered and evaporated (rotary evaporator, 15°C) to produce a yellow oil (0.945 g). This product remained as an oil when stored at 4°C. Mass spectroscopy failed to demonstrate the presence of a mass ion (m/e = 131), the highest ion observed being m/e 102 (= trimethylurea). Elemental analysis produced inconsistent results for C, H and N content, perhaps indicating an intrinsic instability in this material. Proton, ^{13}C and ^{15}N NMR ($DMSOd_6$) were, however, consistent with the required structure. ^1H: 2.45 ppm (d) NCH_3; 2.74 (s) $N(CH_3)2$; 6.18 (b) NH_2; 258.2 (s) N-NO; 557.5 (s) NO. Assignments of the ^{15}N chemical shifts are by reference to Levy and Lichter [1979].

Alkylating Potential of the Nitrosamines

The 4-(4'-nitrobenzyl)pyridine (NBP) assay was performed as described by Epstein et al. [1955].

Hydrolytic Stability of the Nitrosamines

A solution of triethylamine in methanol (5 equiv.) was added to a solution of the nitrosourea in methanol and shaken gently at 20.5°C. At suitable time intervals, samples were examined by HPLC on Spherisorb 50DS eluting with water/methanol (15/85) at 1 ml/min. The concentration of the nitrosamine was estimated by integration of the chromatogram and the rate of reaction was calculated from a plot of log [area] against time. Small variations in the hydrolysis conditions used, e.g. the nature of the solvent or the concentration of the reagents, can affect the half-life values determined. Consequently, results generated using this method are not absolute.

Salmonella Assays

Assays were conducted using a plate incorporation assay, as described previously [Ashby et al., 1993] using strain TA1535 in the absence of

S9. The positive control chemicals, sodium azide and 2-aminoanthracene, have been described earlier [Ashby et al., 1985; Lefevre et al., 1994 respectively]. DMENU and TMNU were also tested in the presence of β-naphthoflavone/phenobarbital induced S9 mix [Lefevre et al., 1994].

Animals and Dosing

Male CBA mice aged 8–12 weeks were used in all experiments (Tables II–IV) with the exception of Expt. 2, Table IV (male CD-1 mice of the same age were used) and Table V (male AP mice of the same age were used). All animals were housed and maintained as described previously [Tinwell and Ashby, 1989]. Each animal received a single administration of the appropriate compound 24 or 48 hr prior to sacrifice. A dosing volume of 10 ml/kg bodyweight was used in all experiments and all compounds were homogenised in corn oil immediately prior to dosing [Ashby, 1987]. The route of exposure is shown in Tables II–V.

The dose-levels employed in the main experiments (Tables III–V) were based on the preliminary data shown in Table II. The initial dose-levels of ENU were derived from previously published data [CSGMT, 1988]. Higher doses of DMENU were used due to its predicted reduced toxicity. The preliminary dose-levels of MNU and TMNU were based on those of ENU. In all dose-ranging studies, bone marrow toxicity, not morbidity or clinical toxicity, was the determining factor. Thus, maximum tolerated doses were estimated to be 350 mg/kg for ENU, 750 mg/kg for DMENU and 100 mg/kg for MNU and TMNU.

Slide Preparation and Scoring

Bone marrow smears were prepared and stained with acridine orange as described previously [Tinwell and Ashby, 1989]. The frequency of micronucleated polychromatic erythrocytes (MPE) was determined among 2,000 polychromatic erythrocytes (PE) and the erythropoietic ratio (PE/NE) was assessed among 1,000 PE and normochromatic erythrocytes (NE).

RESULTS

Alkylating Potential

TMNU and DMENU gave a negative response in the NBP test. Under similar conditions of test, MNU and ENU gave the expected blue coloration within 1 min. The results for the parent compounds are similar to previously reported data [Bartsch et al., 1983]. Conducting the experiment at pH 3 prior to addition of base failed to produce coloration from DMENU despite activity for ENU.

Stability of the Nitrosoureas

The half-lifes for ENU and MNU were calculated to be 7.5 and 8 min respectively whereas those for TMNU and DMENU were 25 and 41 days respectively. The results for the two analogues suggest that the addition of methyl groups to ENU/MNU greatly diminishes the rate of hydrolysis, but does not completely abolish it. Similar and more extensive aqueous hydrolysis data over a range of pH values have been described by Lijinsky and Taylor [1975].

TABLE I. Mutagenicity of ENU, MNU, DMENU and TMNU, to Strain TA1535 of *Salmonella typhimurium*

	Mean no. revertants/plate ± SD		
Compound dose (μg/plate)	Experiment 1 (−S9 mix)	Experiment 2 (−S9 mix)	Experiment 3 (+S9 mix)
ENU			
5,000	1740 ± 219.5**	Lawn absent	
3,750	1448 ± 212**	Lawn absent	
2,500	875 ± 168**		
2,000		3276 ± 435**	
1,250	76 ± 53*	1972 ± 164**	
875	43 ± 31*	619 ± 200	
500	15 ± 6.4*	56 ± 12.8**	
MNU			
250	1003 ± 134**	Lawn absent	
200	413 ± 239**	Lawn absent	
150	37 ± 8**	1832 ± 471**	
100	15 ± 2**	62 ± 6**	
50	9 ± 4	56 ± 16**	
20	11 ± 6	21 ± 1.5**	
DMENU			
30,000	39 ± 6**	25 ± 1.5**	
20,000	33.3 ± 2.5**	22 ± 4**	
10,000	19**[a]	19 ± 3**	
7,500	17 ± 4**	16 ± 2.5**	25 ± 3**
5,000	17 ± 4**	16 ± 1.5**	23 ± 4**
3,750	14 ± 2**	9 ± 1	18 ± 3**
2,500			16 ± 2.5**
1,250			15 ± 4
875			12 ± 1
TMNU			
7,500	16 ± 10	15 ± 11	31 ± 1.5**
5,000	17 ± 7*	18 ± 2	23 ± 5**
3,750	21 ± 2**	12 ± 1.5	20 ± 2**
2,500	19 ± 1**	12 ± 1	16 ± 8.5
1,250	15 ± 5*	12 ± 3	12 ± 1.5
875	11 ± 2	10 ± 1.5	10.3 ± 2.5
DMSO (100 μl)	8 ± 3	10 ± 2	12 ± 2
Positive control	Sodium azide	Sodium azide	2-Amino-anthracene
2	903.5**	1248**	108.5**
1	595**	768**	39**
0.5	253**	268.5**	24.5**

In Experiments 1 and 2 tests were conducted in the absence of S9 and in Experiment 3 they were conducted in the presence of S9 mix. Three plates per dose-level were analyzed for the four test compounds, two plates per dose-level of sodium azide and 2-aminoanthracene were used and five DMSO control plates were used. Untransformed data were assessed for statistical significance using a one-sided Student's *t*-test, $*P < 0.05$; $**P < 0.01$.

[a]One plate in this group was contaminated.

Salmonella Mutagenicity

All four compounds were tested in TA1535 in the absence of S9 and those data are shown in Table I and Figure 2. Both MNU and ENU were potent mutagens to Salmonella as previously reported [Serebryani et al., 1990; Kier et al., 1986]. DMENU and TMNU showed

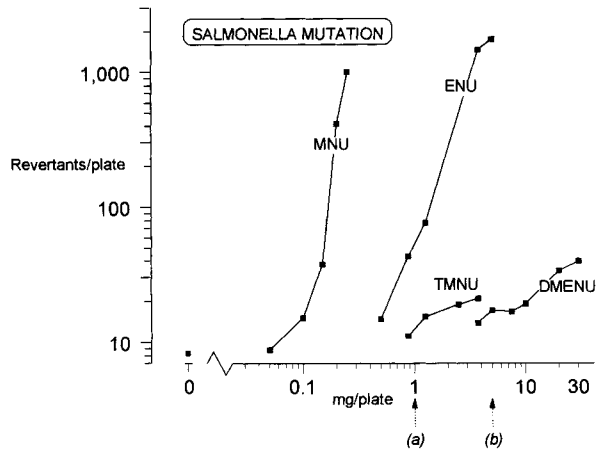


Fig. 2. Mutagenicity of ENU, MNU, DMENU and TMNU to strain TA1535 of *S. typhimurium* tested in the absence of S9 mix. Data for higher dose levels and SDs are shown in Table I. A log/log scale has been used. (*a*) Top dose level employed by Lijinsky and Andrews [1983] (+S9 mix only); (*b*) Top dose-level usually employed with this assay.

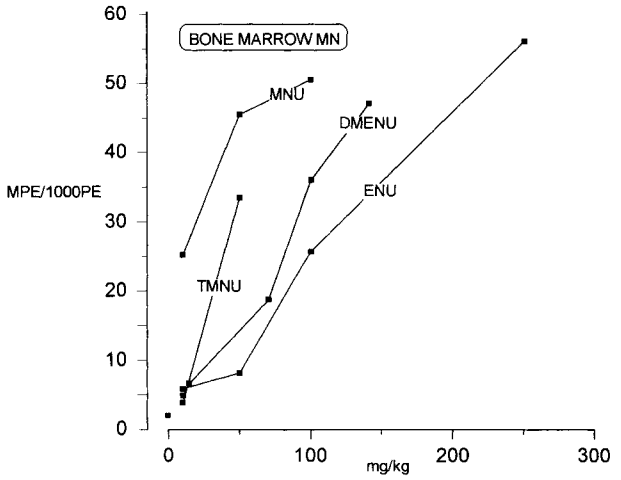


Fig. 3. Activity of ENU, MNU, DMENU and TMNU in mouse bone marrow micronucleus (MN) assays 24 hr after dosing. Higher dose levels and SDs are shown in Table III.

TABLE II. Data Generated From Dose-Ranging Studies Performed on All Four Compounds

Compound	Dose (mg/kg)	No. of animals	MPE/1000 PE based on 2000 PE assessed per animal		PE/NE ± SD
			Individual animal data	Group mean ± SD	
ENU	25	3	15, 17, 14	15.3 ± 1.5	1.1 ± 0.1
	100	3	49, 57.5, 30	45.5 ± 14.1	0.7 ± 0.1
	250	3	83.5, 95, 79	85.8 ± 8.3	0.45 ± 0.1
	350	3	52.5, 87, 84	74.5 ± 19.1	0.5 ± 0.1
DMENU	250	3	41.5, 50.5, 31	41 ± 9.8	0.9 ± 0.1
	700	3	71.5, 49, 39.5	53.3 ± 16.4	0.35 ± 0.03
	750	3	34.5, 42, 48	41.5 ± 6.8	0.36 ± 0.1
MNU	100	3	27.5, 20.5, 20.5	22.8 ± 4	0.5 ± 0.1
TMNU	100	3	19.5, 31, 48.5	33 ± 14.6	0.64 ± 0.2

Each animal received a single oral dose of the appropriate compound 24 hr prior to sacrifice. Based on the depression of erythropoiesis (PE/NE ratio) the MTD values were estimated to be 350 mg/kg for ENU, 750 mg/kg for DMENU, 100 mg/kg for MNU and 100 mg/kg for TMNU.

weak mutagenic activity to Salmonella only at dose-levels at which MNU and ENU were toxic. These data therefore support the hydrolysis data described above, i.e. hydrolysis could not have been completely abolished as low levels of carbonium ions must have been generated from the two analogues to account for their marginal activity in the Salmonella assay. Addition of S9 mix marginally increased the mutagenicity of the two methylated analogues (Table I). The negative results reported by Lijinsky and Andrews [1983] for DMENU and TMNU, when tested in the absence of S9 mix, can probably be explained by those tests being conducted to a top dose-level of only 1 mg/plate (see Fig. 2).

Mouse Bone Marrow Micronucleus Assay

The MN assay data for ENU, MNU and their respective methylated analogues, are presented in Tables II–V and Figure 3. ENU and DMENU were assessed in the MN assay 24 and 48 hr following their oral administration (Table III). ENU induced a potent dose-related increase in MPE 24 hr after dosing, with activity detected at doses as low as 10 mg/kg. In addition, this dose-related increase in MPE was coupled to a concomitant depression of erythropoiesis. These data confirm previous reports of the activity of ENU in the MN assay [Hayashi et al., 1982; CSGMT, 1988]. ENU was also clearly active in the bone marrow 48 hr after dosing.

Contrary to expectations, DMENU also induced a dose-related increase in MPE of similar magnitude to that induced by ENU. Further, adjustment of the dose of DMENU so that it was equivalent in molarity to ENU, led to the induction of a stronger response by the dimethyl analogue (>2-fold; Expt. 2, Table III). As with the parent compound, DMENU was also active 48 hr after dosing. However, DMENU was not as toxic to the bone marrow

TABLE III. Results From Two Mouse Bone Marrow Micronucleus Assays Testing ENU and DMENU

Compound	Dose (mg/kg)	No. of animals	Sample time (hr)	MPE/1000 PE based on 2000 PE assessed per animal		PE/NE ± SD
				Individual animal data	Group mean ± SD	
Experiment 1						
Corn oil	10 ml	5	24	1, 3, 0.5, 2, 3.5	2 ± 1.3	1.1 ± 0.1
		5	48	3, 3, 3.5, 3, 3.5	3.2 ± 0.3	1.1 ± 0.1
ENU	10	5	24	6, 6, 8, 4, 7	6.2 ± 1.5**	1.0 ± 0.1
	100	5	24	36.5, 28.5, 19.5, 42.5, 36.5	32.7 ± 8.9**	1.0 ± 0.1
		5	48	27, 29.5, 25.5, 19, 14.5	23.1 ± 6.2**	0.6 ± 0.1*
	350	5	24	60.5, 63, 89.5, 69, 76.5	71.7 ± 11.7**	0.45 ± 0.1**
DMENU	10	5	24	5, 6, 6, 3	5.2 ± 1.3**	1.1 ± 0.1
	100	5	24	42, 39.5, 44.5, 29, 25	36 ± 8.5**	1.0 ± 0.1
		5	48	16, 26.5, 14.5, 30, 28.3	23.1 ± 7.3**	0.7 ± 0.15**
	350	5	24	21, 14, 23.5, 29.5, 25	22.6 ± 5.7**	0.9 ± 0.1**
		5	48	12.5, 12, 16.5, 19, 18	15.6 ± 3.2**	1.0 ± 0.1
	700	5	24	27, 40.5, 54.5, 58, 75	51 ± 18.2**	0.45 ± 0.1**
Experiment 2						
Corn oil	10 ml	5	24	0.5, 2.5, 3, 2, 1	1.8 ± 1.0	1.1 ± 0.05
		4	48	2, 1.5, 2.5, 2	2.0 ± 0.4	1.1 ± 0.1
ENU	10	7	24	4.5, 4, 7.5, 6.5, 6.5, 3, 8	5.7 ± 1.9**	1.1 ± 0.1
	50	6	24	10.5, 7.5, 8.5, 9, 5.5, 8	8.2 ± 1.7**	1.0 ± 0.2
	100	6	24	30, 7.5, 23.5, 21, 23.5, 14	19.9 ± 8.0**	0.85 ± 0.1**
		6	48	15.5, 33.5, 17.5, 14.5, 15, 23.5	19.9 ± 7.4**	0.7 ± 0.2**
	250	6	24	66, 48.5, 61.5, 28, 56, 76.5	56 ± 16.7**	0.6 ± 0.2**
DMENU	14.1	7	24	5.5, 6, 10.5, 11, 3.5, 4.5, 6	6.7 ± 2.9**	1.1 ± 0.1
	70.4	6	24	26, 6.5, 23.5, 27.5, 13, 16	18.8 ± 8.3**	0.9 ± 0.2*
	141	6	24	16.5, 47.5, 66, 48, 49, 55.5	47.1 ± 16.5**	0.7 ± 0.1**
		6	48	22, 34, 70, 32, 60, 42	43.3 ± 18.2**	0.7 ± 0.15**
	700	6	24	19, 16, 58, 33.5, 35.5, 58	36.7 ± 18.2**	0.3 ± 0.1**

Each animal received a single oral dose of the appropriate compound 24 or 48 hr prior to sacrifice. In the second experiment the dose-levels of DMENU were adjusted so that they were the molar equivalent of those for ENU. Data were assessed for statistical significance using a one-sided Student's *t*-test using untransformed data: $*P < 0.05$; $**P < 0.01$.

TABLE IV. Results From Two Mouse Bone Marrow Micronucleus Assays Testing MNU and TMNU

Compound	Dose (mg/kg)	No. of animals	Sample time (hr)	MPE/1000 PE based on 2000 PE assessed per animal		PE/NE ± SD
				Individual animal data	Group mean ± SD	
Experiment 1 (CBA)						
Corn oil	10 ml	6	24	2, 2.5, 3.5, 0.5, 1, 2	1.9 ± 1.1	1.2 ± 0.1
MNU	10	6	24	29, 35.5, 17.5, 20, 31.5, 17.5	25.2 ± 7.8**	1.0 ± 0.2
	50	6	24	52, 23.5, 33, 30.5, 25.5, 27	32 ± 10.4**	0.8 ± 0.1*
	100	6	24	50.5, 38.5, 73.5, 41.5, 58.5, 40.5	50.5 ± 13.5**	0.5 ± 0.1**
TMNU	10	6	24	3, 5.5, 7.5, 4.5, 1, 2	3.9 ± 2.4*	1.2 ± 0.1
	50	6	24	36.5, 30.5, 34, 23, 32, 33	31.5 ± 4.6**	1.0 ± 0.1**
	100	6	24	29, 14, 22.5, 17, 17.5, 8.5	18.1 ± 7.1**	0.7 ± 0.05**
Experiment 2 (CD-1)						
Corn oil	10 ml	10	24	1, 2, 3, 2.5, 4, 3.5, 3, 1, 2.5, 2.5	2.5 ± 1.0	0.9 ± 0.1
MNU	50	5	24	72, 56, 49.5, 61, 71	61.9 ± 9.7**	0.8 ± 0.1
TMNU	50	5	24	36.5, 41, 24.5, 47, 30.5	35.9 ± 8.8**	0.8 ± 0.1

Each animal received a single oral dose of the appropriate compound 24 hr prior to sacrifice. Animals used in Experiment 1 were CBA mice and those used in Experiment 2 were CD-1 mice. As these latter animals were novel to this series of investigations, a greater number of control animals were employed in the second experiment. Data were assessed for statistical significance using a one-sided Student's *t*-test: $*P < 0.05$; $**P < 0.01$.

as was ENU. This was evidenced by the 2-fold increase in dose-level which could be employed for the analogue without any deleterious effect to the animal and also by the observation of a greater level of cytotoxicity by ENU when both compounds were tested at similar (lower) doses.

Although tested to a lesser extent, it was clear that the activity of MNU and TMNU followed a similar trend

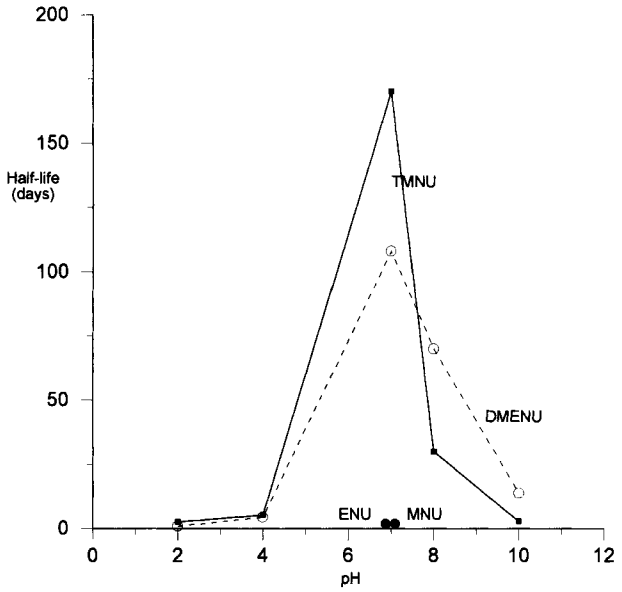


Fig. 4. Hydrolysis half-lives of the four test chemicals over a range of pH values (plotted from data tabulated in Lijinsky and Taylor [1975]).

to that of ENU/DMENU (Table IV). In particular, both compounds induced significant increases in MPE in the bone marrow 24 hr after dosing either CBA or CD-1 mice. However, and in contrast to the observation for ENU/DMENU, the response observed for TMNU was lower than that induced by the parent compound. The data for MNU in the bone marrow of male CBA mice (Expt. 1, Table IV) have previously been reported [Ashby et al., 1994a] and are in agreement with the earlier data of Goncharova et al. [1988]. As with DMENU, it was apparent from the PE/NE ratios that the mice could probably have been exposed to a higher dose of TMNU.

The hydrolysis data presented earlier by Lijinsky and Taylor [1975] are shown in Figure 4. In Figure 4 it is clear that the rate of hydrolysis of the methylated derivatives was increased at low pH. However, a limited experiment using the ip route of administration (to avoid the acidic environment of the stomach) failed to modify the relative activities of ENU and DMENU in the MN assay (Table V). Further, DMENU failed to produce a blue colour in the NBP test when maintained at pH 3 overnight followed by basification. ENU gave a positive response under those conditions of test.

DISCUSSION

The present observations remain to be rationalized. On the one hand the chemical concept that led to the synthesis of the methylated analogues seems to have been validated by the dramatically reduced rates of hydrolysis of the methylated analogues, by their inactivity in the NBP alkylation test, by their minimal mutagenicity to Salmonella and by their reduced acute toxicity to mice. These collective observations would suggest that DMENU and TMNU are of little or no hazard to mammals. In contrast, the mouse bone marrow MN assay data indicate genetic toxicity to mice for DMENU and TMNU equal to that of the parent compounds, and both of these analogues are carcinogenic to the rat [Lijinsky and Taylor, 1975].

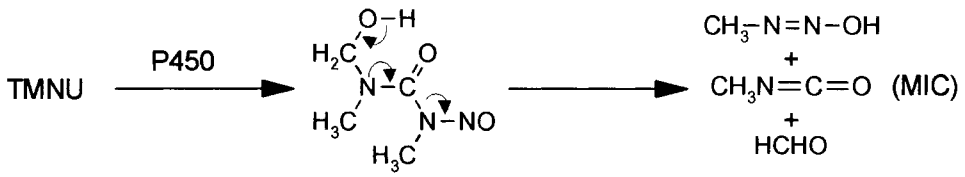
Four possible explanations for this unexpected divergence of theory and data are considered (Fig. 5). First, a novel method of activation such as α-hydroxylation of the new methyl groups (probably catalysed by cytochrome P-450) may be feasible for the two analogues (Fig. 5a). This would lead to three possible alkylating species. However, this seems unlikely given that the mutagenicity to Salmonella of the methylated analogues is not significantly enhanced by S9 mix (Table I). Nonetheless, a specific isozyme of P-450 may be required which is not present in the S9 mix. However, it should be noted that both hydrolysis and α-hydroxylation of DMENU and TMNU can give rise to methyl or ethyl diazonium ion (or related reactive methylating or ethylating species) and both of these species induce biphasic mutagenic responses in the Salmonella mutation assay [Guttenplan, 1979; Guttenplan and Milstein, 1982; Guttenplan, 1984]. A low sensitivity

TABLE V. Mouse Bone Marrow Micronucleus Assay Testing ENU and DMENU in Male AP Mice

Compound	Dose (mg/kg)	Route	No. of animals	Sample time (hr)	MPE/1000 PE based on 2000 PE assessed per animal		PE/NE ± SD
					Individual animal data	Group mean ± SD	
Corn oil	10 ml	po	2	24	4.5, 6	5.3	0.9
ENU	100	po	2	24	117, 102	109.5	0.95
DMENU	100	po	2	24	43.5, 34.5	39	0.93
Corn oil	10 ml	ip	2	24	6.5, 10.5	8.5	0.85
ENU	100	ip	2	24	72.5, 70	71.3	0.45
DMENU	100	ip	2	24	76, 59.5	68	0.9

The use of the ip route of administration does not ablate the activity of DMENU seen after po administration. Small group sizes prevent the contrary conclusion that activity is increased using the ip route. This strain of mouse has high control MPE levels, but this does not affect the comparisons made.

a)



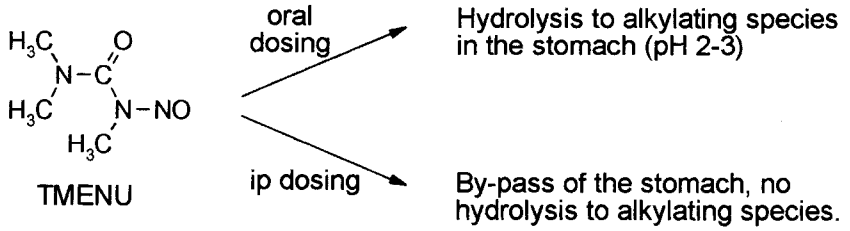
α-Hydroxylation yielding three possible electrophilic species.

b)

	Administered to mouse (mg/kg)	Quantity reaching BM, unhydrolysed (mg/kg)	% decomposition to alkylating species in BM	Quantity of parent compound forming alkylating species in BM (mg/kg)
MNU	100	10	90	9
TMNU	100	90	10	9

Equivalent net alkylation of DNA

c)



Acidic hydrolysis

d)

Analogy between TMNU and DMCC

Fig. 5. Four possible ways to explain the similar activity of MNU, ENU, TMNU and DMENU in the mouse bone marrow (BM) micronucleus assay. **a:** α-Hydroxylation yielding three electrophiles including formaldehyde and methyl isocyanate (MIC). **b:** A chance equivalence between net DNA alkylation due to balanced differences in the rate of delivery to tissue and rate of hydrolysis. **c:** Rapid hydrolysis of DMENU and TMNU in the acidic environment of the stomach. **d:** Loss of methyldiazohydroxide, by analogy with loss of the chlorine atom in dimethylcarbamoyl chloride (DMCC).

region predominates until a threshold exposure is reached. The mutagenic potency increases markedly above this threshold. Such responses are apparent in Table I for MNU and ENU. The reason for the sharp increase in response is the exhaustion of a constitutive O^6-alkyl guanine repair in Salmonella at higher doses [Guttenplan and Milstein, 1982; Guttenplan, 1984]. The high sensitivity threshold for DMENU and TMNU may not have yet been reached because of the relatively slow rate of hydrolysis and/or a slow rate of α-hydroxylation of these compounds. Thus, the differences in mutagenic potencies between MNU/ENU and DMENU/TMNU may be exaggerated with respect to their abilities to alkylate DNA. In order for mispairing to occur, damage to DNA must occur before replication occurs. In the Salmonella assay the time window for DNA damage may only be several hours. In a whole animal system, if DMENU and TMNU are long-lived, their effective time for damaging DNA will mainly be limited by their rate of excretion, which may be considerably longer.

A more plausible explanation (Fig. 5b) is that the similar clastogenic activity in vivo of the methylated compounds and their parent compounds is due to equivalent net alkylation of DNA. The hydrolytic instability of ENU and MNU implies that the majority of the administered dose would be hydrolysed to the active species *before* reaching the bone marrow. Thus, rapid hydrolysis in the bone marrow of only a small proportion of the administered dose would lead to the observed clastogenic response. In contrast, DMENU and TMNU are more stable, thus a greater proportion of the administered dose would be available to induce genotoxicity in the bone marrow. However, as the rate of hydrolysis of the analogues is greatly reduced compared to the parent compounds, it is possible that during the course of the assay only a small proportion of the analogues would decompose to yield alkylating species in the bone marrow. The net effect, by chance, could therefore be that equivalent administered doses of parent compound and analogue result in similar levels of DNA damage and clastogenesis. There is some evidence that, by analogy, supports this explanation. It has previously been shown that weakly acidic conditions stabilize the short-lived nitrosamine metabolites (α-hydroxy-N-nitrosamines) leading to enhanced mutagenesis in Salmonella [Guttenplan, 1980; Negishi and Hayatsu, 1980]. This enhancement is probably caused by a greater fraction of the active metabolite being able to permeate the bacteria before decomposition compared to at neutral pH. It has also been shown that, if a sufficiently long preincubation is carried out, MNU is more mutagenic under weakly acidic conditions than at pH 7.4 [Guttenplan, 1980]. Thus, conditions that increase the ratio of intracellular to extracellular decomposition of an ultimate mutagen result in increased mutagenesis. The hypothesis advanced for the MN assay results fits these previous results, because the stability of the methylated derivatives may allow more intracellular decomposition. The relatively low carcinogenic potency of MNU in the rodent liver compared to dimethylnitrosamine [Preussman and Stewart, 1984] is probably the result of similar effects. Thus, much of the MNU decomposes extrahepatically, in contrast to the nitrosamine which is preferentially activated to methyldiazonium ions intrahepatically. Also, as expected from the extrahepatic decomposition of MNU, it is a more potent carcinogen than dimethylnitrosamine in most other organs.

A third possibility is that DMENU and TMNU are rapidly hydrolysed in the acidic environment of the stomach following their oral administration to rodents (Fig. 5c). This is supported, in part, by the hydrolysis data of Lijinsky and Taylor [1975] and by the fact that DMENU and TMNU were shown to be carcinogenic following their administration to rats in drinking water [Andrews et al., 1978]. However, administration of DMENU and ENU by intraperitoneal injection failed to modify their relative clastogenic activity in the mouse bone marrow MN assay (Table V). This explanation therefore seems to be unlikely.

The final explanation has already been considered by Lijinsky, namely, that *some reaction, at present unknown, is the important one, and that the extent of this reaction varies with the chemical structure of the nitrosamides* [Lijinsky and Taylor, 1975]. One possible "unexpected" reaction is shown in Figure 5d. That involves the $CH_3N—NO—$ group of DMENU and TMNU acting as a simple leaving group, by analogy with the chlorine atom in the mutagenic carcinogen dimethylcarbamoyl chloride (DMCC). If that were to be established, these nitrosoureas would be acting as carbamoylating agents, as opposed to alkylating agents [c.f. Serebryani et al., 1990; Guttenplan, 1993; Elespuru et al., 1993].

The present data confirm that extrapolation of effects observed in vitro (even when chemically rationalized) to the situation likely to prevail in mammals, should proceed with caution. Such advice is not, however, novel [see, inter alia, Lumley, 1990; Ashby et al., 1994b].

ACKNOWLEDGMENTS

We are grateful to R. D. Callander for the provision of the Salmonella data, and to the European Environmental Program for financial support.

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Accepted by—
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