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SUPPRESSION OF HEPATIC DMN DEMETHYLASE ACTIVITY BY NITROSOSARCOSINE AND OTHER NITROSAMINES*

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Abstract—The effects of three nitroso compounds (namely, nitrososarcosine, dibutylnitrosamine and diethylnitrosamine) on dimethylnitrosamine (DMN) demethylase activity were determined. Nitrososarcosine rapidly inhibited DMN demethylase activity, and induced statistically significant inhibitory effects at doses far below threshold levels for inhibition of aminopyrine demethylase. Nitrososarcosine, administered *in vivo*, induced non-competitive inhibition of DMN demethylase. Dibutylnitrosamine, diethylnitrosamine and even dimethylnitrosamine, itself, inhibited DMN demethylase activity at doses which had no effect on aminopyrine demethylase.

Dimethylnitrosamine (DMN) is a potent carcinogen and mutagen which requires enzymatic activation to exhibit its toxic responses [1]. For example, when bacteria are plated in the presence of DMN, there are no increases in mutations [2]. However, when liver microsomal enzyme preparations are included in these plates, mutation frequencies increase markedly. Similarly, these bacteria can be tested in the host-mediated assay, which also reflects bio-activation of DMN. Mutagenicity of DMN in the host-mediated assay has been reported to be suppressed by concomitant administration of other nitroso compounds [3]. More specifically, pretreatment of mice with nitrososarcosine (NS), diethylnitrosamine (DEN), dibutylnitrosamine (DBN) and even dimethylnitrosamine, itself, suppresses DMN mutagenicity in the host-mediated assay [3].

Dialkylnitrosamines inhibit the oxidative metabolism of DMN in female [4] and male rats [5]. In male rats, DEN and NS also suppress DMN inhibition of liver protein synthesis and liver RNA, DNA and protein alkylation [5]. The potential relationship between metabolism and DMN mutagenicity is further supported by observations that acetoaminonitrile [6, 7] and piperonyl butoxide [8] reduce both DMN metabolism and DMN mutagenicity.

We report here the inhibition of mouse liver DMN demethylase activity by 3 *N*-nitroso compounds, namely, diethylnitrosamine, dibutylnitrosamine and nitrososaccosine. We also show inhibition of DMN metabolism by DMN, itself. In the case of NS, we also report that this is a highly specific phenomenon and we report the Michaelis–Menten kinetics.

MATERIALS AND METHODS

Dimethylnitrosamine and diethylnitrosamine were purchased from the Eastman Chemical Co. Nitrososarcosine and dibutylnitrosamine were synthesized in this laboratory by modifications of the method of Lijinsky *et al.* [9]. Distilled water was employed as solvent for all compounds except dibutylnitrosamine, which was dissolved in corn oil. Solutions of nitrososarcosine were adjusted to pH 7 prior to use.

Male Swiss (ICR) mice weighing between 20 and 30 g were used throughout these studies. Mice were housed six to a cage in plastic shoebox cages and maintained on Purina Laboratory Chow and water, *ad lib.* Mice were obtained from Flow Research Animals, Inc., Dublin, Va.

DMN demethylase activity was assayed in post-mitochondrial supernatant by measuring formaldehyde production from dimethylnitrosamine. Animals were killed by cervical dislocation, livers removed and gall bladders discarded. Livers were homogenized in 3 vol. of 0.25 M sucrose containing 0.1 M phosphate buffer, pH 7.4 [10]. Post-mitochondrial supernatant was prepared from homogenates by centrifugation at 9000 *g* for 15 min at 4°. All DMN demethylase incubation mixtures were buffered at pH 7.4 and contained 72 μmoles nicotinamide (this high level was used because it had no adverse effect on DMN and there is an interaction between DMN and nicotinamide which produces a nicotinamide deficiency [11]), 1.7 μmoles NADP, 30 μmoles $MgCl_2$, 45 μmoles $MnCl_2$, 45 μmoles semicarbazide 18 μmoles D,L-isocitrate and 75 μg isocitrate dehydrogenase in a final volume of 3.0 ml. In assaying for DMN demethylase activity, 0.3 ml of post-mitochondrial supernatant was added to the incubation mixture, and, except in experiments to determine enzyme kinetics, 0.3 m-mole DMN was added as substrate. After incubation at 37° for 60 min in a Dubnoff shaker, the reaction was stopped by the addition of 10% trichloroacetic acid (TCA) and the concentrations of the reaction product, formaldehyde, were determined using the Nash procedure [12]. This reaction is linear with respect to time and post-mitochondrial supernatant protein. Doubling the DMN concentration to 0.2 mM had no effect on enzyme activity. Aminopyr-

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Table 1. DMN demethylase activity at various times after nitrososarcosine administration*

Treatment	Enzyme activity (mean ± S. E.)
H_2O (5 ml/kg)	0.096 ± 0.008
Nitrososarcosine (1 g/kg)	
0.5 hr prior to sacrifice	0.069 ± 0.25
1 hr prior to sacrifice	0.056 ± 0.009†
1.5 hr prior to sacrifice	0.049 ± 0.013†
2 hr prior to sacrifice	0.052 ± 0.013†
19 hr prior to sacrifice	0.099 ± 0.014

* Nitrososarcosine (1.0 g/kg) was injected, i.p., at times indicated prior to sacrifice. Three mice per group were used in each experiment and livers pooled to prepare post-mitochondrial supernatant for each group. Enzyme activity of each preparation was determined in four replications and is expressed as nmoles H_2CO formed $min^{-1} \cdot mg^{-1}$ liver. The results shown are a summary of four determinations and represent twelve treated animals per group.

† Significantly different from control ($P < 0.01$).

ine demethylase activity was determined on some of these samples as described previously [12]. Treatment of animals prior to preparation of post-mitochondrial supernatant varied, and these alterations in experimental protocol are noted in the Results section.

RESULTS

The effect of time of nitrososarcosine administration on enzyme activity is shown in Table 1. Nitrososarcosine (1.0 g/kg) was administered to mice 19, 2, 1.5, 1 and 0.5 hr before the animals were killed and post-mitochondrial supernatant was prepared. DMN demethylase activity was reduced to 73, 59, 52 and 55 per cent of control when treatment with nitrososarcosine preceded sacrifice by 0.5, 1, 1.5 and 2 hr respectively. Administration of nitrososarcosine 19 hr prior to sacrifice had no effect on enzyme activity.

The dose–response characteristics of DMN demethylase to nitrososarcosine are shown in Table 2. Doses of nitrososarcosine of 100, 250 and 500 mg/kg were administered by intraperitoneal injection to groups of mice 1.5 hr prior to sacrifice. The lowest dose of nitrososarcosine tested, 100 mg/kg, produced inhibition of DMN demethylase activity to 74 per cent of control; 250 and 500 mg/kg nitrososarcosine lowered the enzyme activity to 44.2 and 31.2 per cent of control respectively. Statistically significant inhibition of aminopyrine demethylase was observed at 500 mg/kg of NS but was much less than that observed with DMN demethylase.

Kinetic constants of DMN demethylase were measured 2 hr after treatment with 1000 mg/kg of NS. Figure 1 shows results of one of these determinations. The V_{max} was affected by nitrososarcosine treatment to a greater extent than was the apparent K_m. For example, injection of 1000 mg/kg of NS lowered the V_{max} to 63 per cent of that of untreated animals, while increasing apparent K_m by only 18 per cent.

Effects of nitrososarcosine added *in vitro* on DMN demethylase kinetics are shown in Table 3. These preparations contained 0.00 M, 0.028 M and 0.056 M nitrososarcosine. Various substrate concentrations were employed to permit determination of the kinetics of DMN demethylase inhibition *in vitro*. The inhibition produced appeared largely non-competitive, as the mean V_{max} was decreased to 62 per cent of control by 0.056 M nitrososarcosine, whereas apparent K_m was not significantly altered. Similarly, 0.028 M nitrososarcosine lowered the mean V_{max} obtained to 78 per cent of control without effect on apparent K_m. Nitrososarcosine did appear to undergo demethylation itself under conditions of the assay, as

Table 2. Dose–response of aminopyrine demethylase and DMN demethylase activities to nitrososarcosine*

Treatment	Enzyme activity (mean ± S. E.) Aminopyrine demethylase	DMN demethylase
H_2O (5 ml/kg)	0.286 ± 0.032	0.077 ± 0.003
Nitrososarcosine		
(100 mg/kg)	0.278 ± 0.010	0.057 ± 0.001†
(250 mg/kg)	0.225 ± 0.020	0.034 ± 0.002†
(500 mg/kg)	0.205 ± 0.013†	0.024 ± 0.002†

* Groups of four mice were treated with H_2O or nitrososarcosine, i.p., 1.5 hr prior to sacrifice. Livers were removed and post-mitochondrial supernatant was prepared. Enzyme activities were determined on individual liver preparations and are expressed as nmoles H_2CO formed $min^{-1} \cdot mg^{-1}$ liver.

† Significantly different from control ($P < 0.01$).

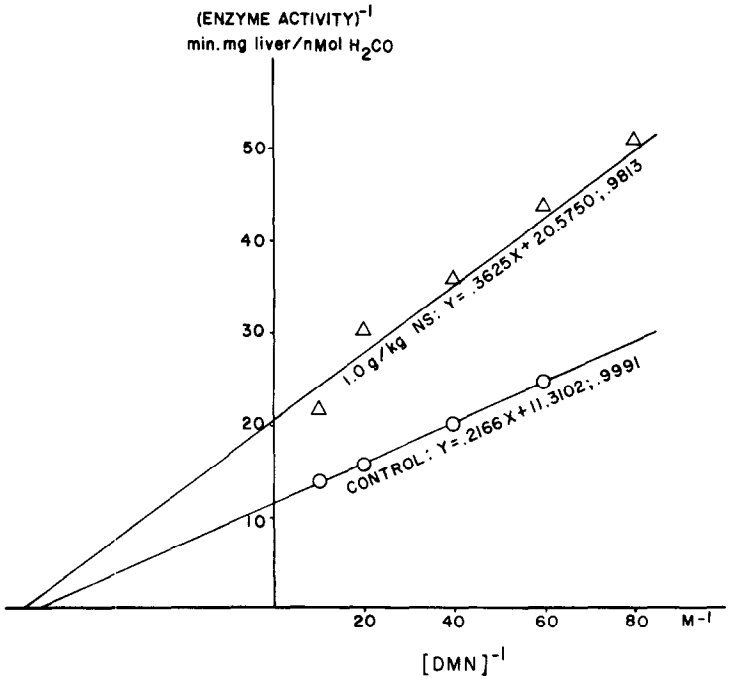

Fig. 1. Lineweaver–Burk plot of DMN demethylase activity as a function of substrate concentration—effect of nitrososarcosine *in vivo*. Groups of six mice were given 5 ml/kg of H_2O (control) or 1.0 g/kg of nitrososarcosine by intraperitoneal injection 2 hr prior to sacrifice. Post-mitochondrial supernatant was prepared for each group and DMN demethylase activity determined at varying substrate concentrations. The lines are labeled with the equation followed by the regression coefficient.

addition of 0.028 M and 0.056 M nitrososarcosine instead of DMN in the incubation mixture resulted in formaldehyde production which was 158 and 196 per cent, respectively, higher than blanks which contained post-mitochondrial supernatant and cofactors but no added substrates.

Preincubation of the reaction mixture with 0.056 M nitrososarcosine for 30 min at 37° prior to the addition of substrate did not markedly increase the extent of inhibition produced. With preincubation, 0.056 M nitrososarcosine lowered DMN demethylase activity to 53 per cent of control; without preincubation, enzyme activity was 61 per cent of control. When NaCl isosmolar with 0.056 M NS was added to the incubation mix, only a 10 per cent decrease in enzyme activity was observed.

Mice were treated with 500 mg/kg of DMN, DEN or dibutylnitrosamine, and DMN demethylase activities were determined on postmitochondrial supernatant prepared from each group 45 min after administration of the respective nitroso compound. These results are summarized in Table 4. Treatment with all three compounds significantly reduced the activity of DMN demethylase. DMN lowered enzyme activity by 35 per cent; DEN and dibutylnitrosamine reduced DMN demethylase activity by 28 and 24 per cent respectively.

DISCUSSION

Nitrososarcosine markedly inhibited DMN demethylase activity both *in vivo* and *in vitro*. The doses used here are considerably below the LD_{50} (3500 mg/kg) and are accompanied by no gross histological damage. DMN demethylase activity was inhibited within 1 hr and the kinetics of this inhibition were non-competitive. These inhibitory effects are also shared by other nitrosamines. Diethylnitrosamine, dibutylnitrosamine and DMN itself also inhibited the activity of this enzyme.

Several nitrosamines inhibit the conversion of DMN-[^{14}C] to $^{14}CO_2$ *in vivo* in female rats [4].

Table 3. Effect of nitrososarcosine *in vitro* on kinetic parameters of DMN demethylase*

Nitrososarcosine concn (M)	No. of determinations	K_m (M)	V_{max} (nmoles H_2CO $min^{-1} \cdot mg^{-1}$ liver)
0.000	4	0.038 ± 0.003	0.210 ± 0.036
0.028	4	0.028 ± 0.004	0.163 ± 0.007†
0.056	4	0.032 ± 0.007	0.129 ± 0.011†

* Six mice/determination were sacrificed and livers were used to prepare post-mitochondrial supernatant. Enzyme activity of the preparation was determined at varying substrate concentrations in the absence and the presence of indicated concentration of nitrososarcosine. Nitrososarcosine was added to incubation mixtures in aqueous solutions adjusted to neutrality.

† Significantly different from control ($P < 0.025$).

Table 4. Effect of dialkylnitrosamine on DMN demethylase activity *in vivo**

Group	No. of replications	Enzyme activity (mean ± S. E.)
Control (5 ml/kg H_2O)	6	0.072 ± 0.003
DMN (500 mg/kg)	6	0.047 ± 0.002†
DEN (500 mg/kg)	6	0.052 ± 0.002†
DBN (500 mg/kg)	6	0.055 ± 0.002†

* Groups of three mice were injected intraperitoneally with the compounds indicated 45 min prior to sacrifice. Hepatic post-mitochondrial supernatant was assayed for DMN demethylase activity. DMN and diethylnitrosamine (DEN) were administered in aqueous solutions; dibutylnitrosamine (DBN) was dissolved in corn oil. Enzyme activity is expressed as nmoles H_2CO $min^{-1} \cdot mg^{-1}$ liver.

† Significantly different from control ($P < 0.001$).

These nitrosamines in order of potency include DEN, butylmethylnitrosamine, *t*-butylmethylnitrosamine, bis-2-hydroxyethylnitrosamine and ethyl-2-hydroxyethylnitrosamine. NS but not DMN, DBN or DEN inhibited mouse liver aminopyrine demethylase under the conditions used here [12]. However, DMN demethylase is more sensitive to NS than is either aminopyrine demethylase or aniline hydroxylase. Several nitroso compounds, including nitrososarcosine methylester, inhibit pentobarbital sleeping time in mice [13].

DMN analogues of the dimethylacylamide series are potent competitive inhibitors *in vitro* of DMN demethylase activity [14]. This inhibition decreases with increasing chain length of the acyl constituents with the butyl derivative inactive. Similarly in the present studies, the 2-carbon nitrosamine is a more potent inhibitor than the 8-carbon compounds, but the 8-carbon derivative still has considerable activity.

The inhibitory effects observed on DMN demethylase are both qualitatively and quantitatively consistent with inhibition of DMN mutagenicity in the host-mediated assay [3]. Maximal inhibition of DMN mutant frequency was observed 30 min after nitrososarcine treatment and persisted for 2 hr; it was back to control levels at 19 hr after treatment. As was the case in these studies, the inhibitory effects on mutagenicity were approximately 55 per cent. Similarly, the effects of diethylnitrosamine and dibutylnitrosamine are quantitatively and qualitatively similar on DMN demethylase activity and mutant frequency.

It is not clear at this point as to why nitrososarcosine is a non-competition inhibitor of DMN demethylase. Slight formaldehyde production *in vitro* was observed from nitrososarcosine incubations with microsomal enzymes, indicating the possibility of enzymatic activation. However, these mechanisms are still not clear.

There seems to be no question about the intrinsic oncogenic activity of *N*-nitroso compounds. However, human environmental exposure appears to be to several types of nitrosamines in contrast to single purified compounds. It appears from these data that there is a significant interaction between nitroso compounds which merits further consideration.

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