Biochemical Pharmacology, Vol. 24, pp. 1313–1316. Pergamon Press, 1975. Printed in Great Britain.

EFFECTS OF SHORT-TERM ADMINISTRATION OF NITROSAMINES ON RAT HEPATIC MICROSOMAL ENZYMES

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(*Received* 30 *August* 1974; *accepted* 29 *November* 1974)

Abstract—Pretreatment of male rats with the carcinogenic nitrosamines, dimethylnitrosamine or *N*-nitrosomorpholine, increased pentobarbital sleeping time (PST), and decreased liver weight:body weight ratio, content of hepatic microsomal protein, and level of microsomal cytochrome P-450. Associated with these changes were decreases in metabolism *in vitro* of ethylmorphine, aniline, *p*-nitrobenzoic acid and dimethylnitrosamine. Treatment with non-carcinogenic nitrosamines, diphenylnitrosamine, dicyclohexylnitrosamine or ethyl*tert*-butylnitrosamine decreased PST, and either increased or did not alter each of the other parameters. In addition to the above findings, specificity of the enzyme system that metabolizes dimethylnitrosamine was indicated. Whereas administration of ethyl*tert*-butylnitrosamine induced liver microsomal protein and cytochrome P-450 and increased rates of microsomal metabolism of aniline, ethylmorphine and *p*-nitrobenzoic acid, there was no change in *N*-demethylation of dimethylnitrosamine.

The class of compounds containing the *N*-nitroso group includes some of the most potent carcinogenic and acutely hepatotoxic agents [1, 2]. The presence of the compounds in certain foods [3, 4] and in tobacco smoke [5, 6] and the ability of amines to undergo nitrosation to form the corresponding nitrosamines [7–12] pose a potential environmental hazard.

During attempts in this laboratory to develop a suitable bioassay for these compounds, we noted that administration of any of a series of carcinogenic nitrosamines to mice for 2 or 4 days resulted in an increase in duration of pentobarbital-induced anesthesia; pretreatment with known non-carcinogenic nitrosamines shortened the anesthetic interval [13, 14]. This finding was believed to be the result of inhibition or induction of microsomal enzymes responsible for the metabolic breakdown of pentobarbital.

The above finding and previously reported evidence that some nitrosamines are metabolized by liver microsomal enzymes to active carcinogenic and mutagenic substances [1, 15–19], prompted the investigations described in this report. The effects of short-term administration of two carcinogenic and three non-carcinogenic nitrosamines on hepatic microsomal cytochromes and on the metabolism *in vitro* of various substrates, including dimethylnitrosamine, were studied.

MATERIALS AND METHODS

Male Sprague–Dawley rats (ARS Sprague–Dawley, Madison, Wis.*) weighing 75–100 g were fed Rockland Mouse/Rat Diet (Teklad, Inc., Monmouth, Ill.) and tap water *ad lib.* for 3–4 days after arrival. Nitrosamines, obtained commercially (dimethylnitrosamine and diphenylnitrosamine, Eastman Chemical Co.; dicyclohexylnitrosamine, Aldrich Chemical Co.; *N*-nitrosomorpholine, Schuchardt Gmb. H. & Co.; ethyl*tert*-butylnitrosamine, Parish Chemical Co., Provo, Utah), were dissolved in olive oil and administered per os (p.o.) on 3 consecutive days. Control rats received olive oil (5 ml/kg/day, p.o.). Doses of nitrosamines were based on doses which effectively altered pentobarbital sleeping times in mice [13, 14].

Metabolism *in vivo* of a hypnotic dose of pentobarbital (40 mg/kg, i.p.) was determined 24 hr after the final treatment by measuring the duration of loss of righting reflex. In separate studies *in vitro*, animals were decapitated and livers removed and homogenized in 2 vol. of ice-cold 1·15% KCl. Subsequent centrifugation procedures were carried out at 4°. Homogenates were centrifuged for 20 min at 9000 *g*, and the resulting supernatant was centrifuged for 1 hr at 105,000 *g*. The microsomal pellet was gently resuspended in cold 1·15% KCl and centrifuged again for 1 hr at 105,000 *g*. The washed microsomal pellet was stored overnight at −15° under 2 ml of 1·15% KCl −0·05 M Tris buffer, pH 7·4.

For determination of metabolism *in vitro* of various substrates, microsomes were thawed and resuspended in 1·15% KCl − 0·05 M Tris buffer, pH 7·4. Protein content was determined [20] and KCl − Tris buffer added to give a final concentration of 5 mg protein/ml. Incubation mixtures for oxidative reactions contained the following in a final volume of 5 ml: 3·6 ml of 0·1 M phosphate buffer, pH 7·5; $MgSO_4$, 5·0 mM; glucose 6-phosphate, 5·0 mM; NADP, 0·8 mM; glucose 6-phosphate dehydrogenase, 2 units; microsomes. 1·0 ml; and substrate, 0·1 ml. For demethylation of dimethylnitrosamine and ethylmorphine, 0·5 ml of 0·1 M semicarbazide, pH 7·0, was substituted

* Reference to a company or product name does not imply approval or recommendation of the product by the U.S. Department of Agriculture to the exclusion of others that may be suitable.

for an equal volume of phosphate buffer. Substrate concentrations were as follows: aniline, 2·0 mM; dimethylnitrosamine, 10 mM; and ethylmorphine, 10 mM. Incubation flasks containing all reactants except substrate were preincubated for 5 min at 37°, and the reaction started by the addition of substrate. For the reduction of *p*-nitrobenzoic acid, incubation mixtures contained: 3·7 ml of 0·05 M phosphate buffer, pH 7·6, with 1·0 mM EDTA; glucose 6-phosphate, 5·0 mM; NADP, 0·8 mM; glucose 6-phosphate dehydrogenase, 2 units; microsomes, 1·0 ml; and *p*-nitrobenzoic acid, 2·0 mM. Flasks containing all reactants except substrate were preincubated for 5 min at 37°, then transferred to an ice bath. *p*-Nitrobenzoic acid dissolved in 0·1 ml dimethylsulfoxide was added, and the flasks were flushed with deoxygenated nitrogen for 10 min. The flasks were then transferred to the shaking water bath and incubated at 37° under nitrogen.

Incubation times were 20 min for oxidative reactions and 30 min for reduction of *p*-nitrobenzoic acid. All assays were run in duplicate. The reaction rates were linear with time for at least 60 min. Reactions were stopped by addition of 2·0 ml of 20% trichloroacetic acid (TCA) to incubations containing aniline or *p*-nitrobenzoic acid, or by addition of 2·0 ml of 5% zinc sulfate ($ZnSO_4$) followed by 2·0 ml saturated barium hydroxide ($Ba(OH)_2$) for *N*-demethylation studies. Tissue blanks containing complete incubation mixtures, but with either TCA or $ZnSO_4$–$Ba(OH)_2$ added prior to incubating, were included with each experiment. Recovery blanks contained complete incubation mixtures plus either TCA or $ZnSO_4$–$Ba(OH)_2$ except that known quantities of the expected reaction product were substituted for substrate (*p*-aminophenol for aniline, formaldehyde for ethylmorphine and dimethylnitrosamine, and *p*-aminobenzoic acid for *p*-nitrobenzoic acid). Formaldehyde was determined by the method of Nash [21]. Aniline *p*-hydroxylation was assayed by the colorimetric determination of *p*-aminophenol [22], and reduction of *p*-nitrobenzoic acid by azo dye formation using the coupling agent *N*-(1-naphthyl)ethylene diamine HCl [23].

Table 1. Effect of nitrosamines on liver weight:body weight ratio and content of heaptic microsomal protein*

Treatment	Dose (mg/kg)	% Change† Liver wt/ body wt	% Change† Microsomal protein
Dimethylnitrosamine	10	−9·3‡	−12·6‡
N-nitrosomorpholine	50	−23·5‡	−10·4‡
Diphenylnitrosamine	500	+26·5‡	+17·3‡
Dicyclohexylnitrosamine	300	+14·3‡	+4·3
Ethyl*tert*-butylnitrosamine	300	+21·9‡	+23·3‡

* Values obtained from six animals/treatment or control group.

† Average control values ± S. D. are as follows: liver wt/body wt × 100 = 4·89 ± 0·20 (four experiments); and microsomal protein = 20·28 ± 2·41 mg/g of liver (four experiments).

‡ Significantly different from controls ($P < 0·05$).

Microsomes, diluted to 2·0 mg protein/ml with 1·15% KCl – 0·05 M Tris buffer, pH 7·4, were assayed for content of cytochrome b_5 and cytochrome P-450 using extinction coefficients of 185 and 91 cm^{-1} mM^{-1}, respectively [24, 25], with an Aminco DW-2 spectrophotometer.

Data were statistically analyzed using the Student's *t*-test, with P values of < 0·05 considered to represent significant differences between control and treatment groups.

RESULTS

Three-day pretreatment with nitrosamines had varying effects on liver weight:body weight ratio and on content of microsomal protein (Table 1). Dimethylnitrosamine and *N*-nitrosomorpholine decreased both parameters. Hepatic hemorrhage and ascites were evident in dimethylnitrosamine-treated rats, whereas livers of *N*-nitrosomorpholine-treated animals were pale in color. Diphenylnitrosamine, dicyclohexylnitrosamine and ethyl*tert*-butylnitrosamine increased liver size. Diphenylnitrosamine and ethyl*tert*-butylnitrosamine, but not dicyclohexylnitrosamine, significantly increased the content of microsomal protein.

Table 2. Effect of nitrosamines on metabolism *in vivo* and hepatic microsomal metabolism *in vitro* of various substrates

Treatment	Dose (mg/kg)	Pentobarbital sleeping time* (min ± S. D.)	% Change† *p*-Hydroxylation of aniline	% Change† Reduction of *p*-nitrobenzoic acid	% Change† *N*-demethylation of ethylmorphine	% Change† *N*-demethylation of dimethylnitrosamine
Control		73 ± 15 (10)				
Dimethylnitrosamine	10	148 ± 31 (10)‡	−33·9‡	−12·9	−40·7‡	−33·1‡
N-nitrosomorpholine	50	292 ± 54 (10)‡	−37·7‡	−34·4‡	−50·5‡	−27·7‡
Diphenylnitrosamine	500	25 ± 6 (9)‡	+3·4	+109·5‡	+21·1	+3·8
Dicyclohexylnitrosamine	300	42 ± 5 (8)‡	+0·5	+15·4	+11·0	+12·9
Ethyl*tert*-butylnitrosamine	300	32 ± 11 (9)‡	+14·1‡	+118·7‡	+67·4‡	+4·7

* Duration of loss of righting reflex after pentobarbital (40 mg/kg, i.p.). Values in parentheses represent number of animals/group.

† Average control values ± S. D. from six animals/treatment or control group are as follows: aniline *p*-hydroxylation = 0·860 ± 0·083 nmole *p*-aminophenol formed/mg of protein/min (seven experiments); *p*-nitrobenzoic acid reduction = 0·151 ± 0·038 nmole *p*-aminobenzoic acid formed/mg of protein/min (seven experiments); ethylmorphine *N*-demethylation = 6·91 ± 1·26 nmoles formaldehyde formed/mg of protein/min (one experiment); and dimethylnitrosamine *N*-demethylation = 2·68 ± 0·56 nmoles formaldehyde formed/mg of protein/min (six experiments).

‡ Significantly different from controls ($P < 0·05$).

Table 3. Effect of nitrosamines on content of hepatic microsomal cytochromes*

Treatment	Dose (mg/kg)	% Change† Cytochrome b_5	% Change† Cytochrome P-450
Dimethylnitrosamine	10	−8·1	−23·0‡
N-nitrosomorpholine	50	−13·3‡	−25·3‡
Diphenylnitrosamine	500	−5·7	+21·1‡
Dicyclohexylnitrosamine	300	+6·1	+10·6
Ethyl*tert*-butylnitrosamine	300	−1·2	+75·8‡

* Values obtained from six animals/treatment or control group.

† Average control values ± S. D. are as follows: cytochrome b_5 = 0·377 ± 0·031 nmole/mg of protein (seven experiments); and cytochrome P-450 = 0·561 ± 0·057 nmole/mg of protein (eight experiments).

‡ Significantly different from controls ($P < 0·05$).

Dimethylnitrosamine and *N*-nitrosomorpholine administration increased duration of pentobarbital sleeping time (PST) and depressed the activity of the hepatic microsomal enzyme system (Table 2). PST was significantly shorter in rats given non-carcinogenic nitrosamines. Diphenylnitrosamine increased anaerobic reduction of *p*-nitrobenzoic acid, but did not increase the activity of other enzymes studied. Dicyclohexylnitrosamine did not significantly increase any of the pathways studied. Pretreatment with ethyl*tert*-butylnitrosamine resulted in increased metabolism of aniline, *p*-nitrobenzoic acid and ethylmorphine, but had no effect on *N*-demethylation of dimethylnitrosamine.

Content of the primary component of the mixed function oxidase system, cytochrome P-450, was decreased by dimethylnitrosamine and *N*-nitrosomorpholine, and was increased by diphenylnitrosamine and ethyl*tert*-butylnitrosamine administration (Table 3). Dicyclohexylnitrosamine had no significant effect on cytochrome P-450 content. The other major heme component of microsomes, cytochrome b_5, was slightly decreased by treatment with *N*-nitrosomorpholine, but unaffected by other nitrosamines.

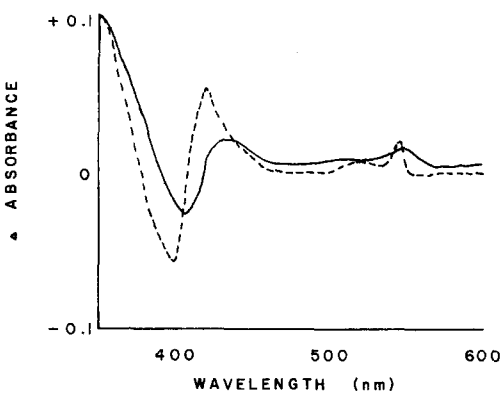

Fig. 1. Difference spectra of dithionite-reduced minus NADH-reduced microsomes. Sample cuvette contained microsomes + 0·1 mM NADH + 2 mg dithionite, and reference cuvette contained microsomes + 0·1 mM NADH. (——) Microsomes from control rats given olive oil (5 ml/kg/day); (– – – – –) microsomes from rats given dimethylnitrosamine (10 mg/kg/day).

Table 4. Effect of nitrosoamines on magnitude of spectral difference between dithionite-reduced microsomes and NADH-reduced microsomes*

Treatment	Dose (mg/kg)	% Change†
Dimethylnitrosamine	10	+94·9‡
N-nitrosomorpholine	50	+40·2‡
Diphenylnitrosamine	500	−13·9
Dicyclohexylnitrosamine	300	−7·5
Ethyl*tert*-butylnitrosamine	300	−75·6‡

* Values obtained from six animals/treatment or control group.

† Average control value ±S.D.: $\Delta E_{426-410}$ = 0·021 ± 0·004 (seven experiments).

‡ Significantly different from controls ($P < 0·05$).

When sodium dithionite was added to NADH-reduced microsomes obtained from dimethylnitrosamine-treated rats, the magnitude of the cytochrome b_5 peak at 426 nm increased. If the sample cuvette contained microsomes + NADH + dithionite and the reference cuvette contained microsomes + NADH, a difference spectrum such as that in Fig. 1 could be recorded. Repeating the experiment with microsomes prepared from rats treated with other nitrosamines (Table 4) demonstrated a similar increase in absorbance at 426 nm for *N*-nitrosomorpholine-treated rats, whereas ethyl*tert*-butylnitrosamine administration decreased the magnitude of the peak.

DISCUSSION

Previously observed effects of 23 different nitrosamines administered to mice for 2 or 4 days [13, 14] have indicated the ability of carcinogenic nitrosamines to increase pentobarbital-induced sleeping time (PST), whereas pretreatment with non-carcinogenic nitrosamines shortens PST. The present study with rats may explain effects on PST for four of the five compounds tested on the basis of changes in liver weight:body weight ratio, microsomal protein content, or cytochrome P-450 content, and by associated increases or decreases in the activity of liver enzyme systems. The carcinogenic compounds, dimethylnitrosamine and *N*-nitrosomorpholine, effectively decrease activity of the liver enzyme system as evidenced by depression of each of the above parameters. This depression may be related to general hepatotoxic effects of the compounds, with associated hepatic lesions and depression of protein synthesis [2, 26–28].

The non-carcinogens, ethyl*tert*-butylnitrosamine and diphenylnitrosamine, induced formation of microsomal protein and cytochrome P-450. Specific enzyme pathways (Table 2) were, however, affected differently by the agents, and suggest specificity in inducing ability. The other non-carcinogen, dicyclohexylnitrosamine, does not appear to be a liver enzyme inducer in rats at the doses used. The decrease in PST observed in our laboratory in mice and rats treated with dicyclohexylnitrosamine may be due to enhanced extra-hepatic metabolism of the barbiturate, decreased absorption across the blood–brain barrier, or to an indirect action of the compound on sensitivity of laboratory animals to pento-

barbital. Further studies with higher doses of dicyclohexylnitrosamine, measurement of brain levels of pentobarbital upon regaining righting reflex, or testing for possible CNS stimulant properties of dicyclohexylnitrosamine may indicate a mechanism of shortening PST for this compound.

Addition of NADH to microsomes results in the selective reduction of cytochrome b_5, whereas addition of dithionite serves to non-selectively reduce other microsomal components [24]. The increase in absorbance in the 420–430 nm range noted upon addition of dithionite to NADH-reduced microsomes prepared from dimethylnitrosamine-treated rats (Fig. 1) indicates the presence of a component(s), other than cytochrome b_5, which is present in only limited quantities in microsomes from control animals. This component is probably the soluble, inactive form of cytochrome P-450, cytochrome P-420 [24] which is released during thawing and resuspending operations from the protective environment of the microsomal membrane. If the component is cytochrome P-420, then it appears that administration of dimethylnitrosamine and *N*-nitrosomorpholine weakens the microsomal membrane, whereas ethyl*tert*-butylnitrosamine pretreatment strengthens the membrane and retards loss of cytochrome P-450 (Table 4). An alternative explanation is that the component is residual hemoglobin, although this is unlikely in view of the careful washing of the microsomes prior to final centrifugation.

Czygan *et al.* [29] have demonstrated the dependency of *N*-demethylation of dimethylnitrosamine by mouse liver microsomes on the cytochrome P-450-dependent mixed function oxidase system. However, studies of the metabolism of dimethylnitrosamine as well as of other nitrosamines have led Druckrey [15] to conclude that non-specific demethylase is not involved in nitrosamine metabolism, but that highly specific enzymes are responsible for α-carbon hydroxylation of the compounds, ultimately leading to formation of the proximal carcinogen. The present study indicates that *N*-demethylation proceeds by a different pathway with dimethylnitrosamine than with ethylmorphine. Whereas administration of ethyl*tert*-butylnitrosamine induced formation of microsomal protein and cytochrome P-450 and enhanced metabolism of aniline, *p*-nitrobenzoic acid and ethylmorphine, it had no effect on the *N*-demethylation of dimethylnitrosamine. Venkatesan *et al.* [30, 31] found that pretreatment of rats with polycyclic hydrocarbons, which induce synthesis of a number of hepatic mixed function oxidases, actually decreases dimethylnitrosamine demethylation activity, thus providing further evidence that different enzyme systems are involved.

Ability of a compound to alter biotransformation mechanisms could enhance or decrease potential carcinogenicity of the compound by numerous interrelated mechanisms involving proximal carcinogen formation, detoxification and distribution and excretion of the compound [32]. The above observations demonstrate the ability of individual compounds having the nitrosamine group to affect microsomal enzymes differently, but the role of these changes to potential tumorigenicity of the compounds is unknown.

Acknowledgement—The excellent technical assistance of Mary B. Luce is gratefully acknowledged.

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