0090-9556/86/1406-0733$00.00/0
DRUG METABOLISM AND DISPOSITION
Vol. 14, No. 6
Printed in U.S.A.

TISSUE LEVELS AND BIOLOGICAL EFFECTS OF *N*-NITROSODIMETHYLAMINE IN MICE DURING CHRONIC LOW OR HIGH DOSE EXPOSURE WITH OR WITHOUT ETHANOL

LUCY M. ANDERSON, GEORGE W. HARRINGTON, HARRY M. PYLYPIW, JR., AKIHIRO HAGIWARA, AND PETER N. MAGEE

Laboratory of Comparative Carcinogenesis, Division of Cancer Etiology, National Cancer Institute, Frederick, Maryland 21701 (L.M.A., A.H.), Department of Chemistry, Temple University, Philadelphia, Pennsylvania 19122 (G.W.H., H.M.P.), and Fels Research Institute, Temple University School of Medicine, Philadelphia, Pennsylvania 19140 (P.N.M.)

(Received April 8, 1986; accepted July 10, 1986)

ABSTRACT:

In a study of the metabolism, disposition, and hepatotoxicity of the environmental carcinogen *N*-nitrosodimethylamine (NDMA), as a function of dose in the drinking water and of concomitant administration of ethanol, outbred Swiss mice were given NDMA for 1–4 weeks at levels of 50–0.5 ppm, with or without 10, 20, or 30% ethanol. NDMA, assayed in blood, liver, kidney, lung, and brain by thermal energy analysis after methylene chloride extraction, was detectable (>0.5 ppb) in tissues of the mice after all doses of NDMA. The 0.5-ppm dose yielded tissue levels of NDMA (1–4 ppb) near the detection limit of 0.5 ppb; this was also found to be the minimal concentration causing significant numbers of lung tumors in strain A mice after treatment for 16–18 weeks. Co-administration of ethanol caused an increase in blood and tissue levels of NDMA at all levels of both chemicals, often by a factor of 10 or more. Ethanol also partially alleviated the morphological hepatotoxic effects of NDMA at 50 ppm (centrilobular hemorrhage and necrosis). These results are consistent with competitive inhibition of metabolic activation of NDMA by ethanol. Ten per cent ethanol did not induce liver NDMA demethylase activity significantly and did not prevent loss of this activity from the livers of mice receiving 5–50 ppm NDMA. Thus, inhibition, rather than induction, of NDMA metabolism was the predominant effect of ethanol, with increased levels of NDMA in blood and other tissues as a consequence.

The potent carcinogen NDMA[1] is present in a variety of human contact sources (1). Furthermore, low levels of NDMA, usually in the 0.1–1-ppb range, have been reported in human blood and urine (2–8). Since the meaning of these human blood NDMA levels for cancer risk is of interest, we have measured amounts of NDMA in blood and other tissues of mice exposed chronically to various concentrations of NDMA in drinking water or liquid diet, and correlated these values with incidence of tumors in the lung.

A second objective of the study was examination of the effect of chronic co-administration of ethanol on tissue levels and hepatotoxicity of chronic NDMA, of concern because of the occurrence of NDMA in some alcoholic beverages (1), frequent concomitant exposure of humans to nitrosamine-containing cigarette smoke along with ethanol, etc. Studies in rodents of the biological interactions of ethanol and NDMA, as related to carcinogenicity and toxicity, have revealed that this and other alcohols have complex effects on the metabolism, pharmacokinetics, and biological effectiveness of NDMA and other nitrosamines. When present simultaneously with NDMA, ethanol inhibits its metabolism by oxidative pathways, *in vivo* (9, 10), in perfused liver (11), in intact liver cells *in vitro* (12), in liver slices (13), and in cell-free preparations of microsomes (14, 15). The inhibition is competitive in nature (14, 15). Conversely, chronic treatment of animals with ethanol results in induction of the low K_m form of NDMA demethylase (14–16). With regard to the biological consequences of these effects of ethanol, liver cells or microsomes from rats pretreated with ethanol were more effective than controls in catalyzing mutagenesis or DNA damage by nitrosamines *in vitro* (17–20), but similar effects were not seen *in vivo* (19, 21). Pretreatment of rats with isopropanol 24 hr before NDMA led to potentiation of hepatotoxicity (22), whereas rats given ethanol in a liquid diet for 3 weeks before NDMA experienced a reduction in hepatotoxicity compared to controls (23). Effects on neoplasia are similarly complex. Ethanol given simultaneously with *N*-nitrosodiethylamine or *N*-nitrosomorpholine caused an increase in number and size of preneoplastic liver foci (24), but ethanol given simultaneously with various nitrosamines reduced the numbers of liver tumors (25–28), sometimes with a concomitant increase in tumors of distal targets such as esophagus (26) or nose (28). Ethanol administered with *N*-nitrosonornicotine to rats resulted in fewer esophageal but more nasal cavity tumors than in rats given the carcinogen alone (29).

No studies have been reported on the short-term toxic effects of ethanol given continuously and simultaneously with NDMA, even though this is presumably an exposure mode commonly encountered by the human. Under these conditions, both induction and competitive inhibition of NDMA demethylase would be possible, and either action could influence the activation and/or detoxification of the chemical. The nature of the biological phenomena resolving from this complexity of possibilities required empirical determination. In the experiments reported here, we inquired as to whether co-treatment with ethanol during

This work was supported in part by National Cancer Institute Grant CA-18618, National Cancer Institute Contract NO1-CP-31016 to Litton Bionetics, Inc., and the Japanese Foundation for the Promotion of Cancer Research under the Nakasone Program.

[1] Abbreviation used is: NDMA, *N*-nitrosodimethylamine.

Send reprint requests to: Dr. Lucy M. Anderson, NCI-LCC, Building 538, Room 205-E, Fort Detrick, Frederick, MD 21701-1013.

TABLE 1

Lung tumorigenesis in strain A mice by 500 ppb NDMA

Expt.	Diet	Treatment Time	Treatment	Tumor Bearers/ Total	Average No. of Tumors (±SD)	Forestomach Papillomas: Tumor Bearers/ Total
		weeks				
1: A/J mice	Purina 5002	12	NDMA	8/49	0.16 ± 0.37	7/49
			Control	8/50	0.16 ± 0.37	7/50
		14	NDMA	14/50	0.28 ± 0.45	12/50
			Control	8/49	0.18 ± 0.44	8/49
		16	NDMA	23/50[a]	0.48 ± 0.54[b]	10/50
			Control	10/50[a]	0.22 ± 0.46[b]	9/50
2: A/JCr mice	Purina 5002	18	NDMA	27/99[a]	0.33 ± 0.61	NC[c]
			Control	18/100[a]	0.20 ± 0.45	NC
	Purina 5015	18	NDMA	33/100[a]	0.43 ± 0.70[b]	NC
			Control	17/100[a]	0.18 ± 0.41[b]	NC

[a] Significant differences between NDMA-treated and control mice, $p < 0.01$, χ^2 test.
[b] Significant difference, $p < 0.05$ or better, Student's t test.
[c] NC, not counted.

TABLE 2

Effects of 50 ppm NDMA in the drinking water: water consumption, body weights, NDMA demethylase, and pathological change

Exposure Time	Treatment	Average Water Consumption	Average Body Weight	NDMA Demethylase	Pathological Change (average score)	
					Gross	Histological
weeks		*ml*	*g*	*nmol CH_2O/mg protein/20 min*		
1	None	3.6 ± 0.6 (4)[a]	26.2 ± 1.7 (10)[a]	ND[b]	0	ND
	10% EtOH	4.5 ± 0.4 (4)	26.8 ± 1.9 (10)	ND	0	ND
	50 ppm NDMA	2.4 ± 0.1 (4)	23.4 ± 1.9 (20)	1.0 ± 0.7 (6)[c]	0.8	3.0
	NDMA + EtOH	2.6 ± 0.3 (4)	22.4 ± 2.4 (20)	2.0 ± 1.0 (6)	0	0
2	None	4.0 ± 0.3 (4)	26.8 ± 1.5 (10)	ND	0	0
	10% EtOH	4.0 ± 0.8 (4)	25.9 ± 1.9 (10)	ND	0	0
	50 ppm NDMA	2.3 ± 0.6 (4)	24.6 ± 2.3 (20)[d]	1.8 ± 0.7 (6)	2.2	2.0
	NDMA + EtOH	1.9 ± 0.4 (4)	20.1 ± 2.3 (20)[d]	1.6 ± 0.8 (6)	0.2	1.7
4	None	4.9 ± 1.7 (4)	27.9 ± 1.7 (10)	ND	0	0
	10% EtOH	2.9 ± 0.3 (4)	28.6 ± 1.7 (10)	ND	0	0
	50 ppm NDMA	2.5 ± 0.6 (4)	24.6 ± 2.3 (10)[d]	0.9 ± 0.6 (6)[c]	2.0	2.1
	NDMA + EtOH	1.8 ± 0.3 (4)	22.4 ± 2.0 (10)[d]	0.5 ± 0.4 (6)[c]	0	1.1

[a] All values are ± standard deviation. Numbers in parentheses are numbers of determinations.
[b] ND, not done.
[c] Significantly different from value in untreated mice, 2.4 ± 1.0 ($N = 7$), $p < 0.05$, t test.
[d] Values with and without ethanol significantly different, $p < 0.01$, t test.

exposure of mice to a low but tumorigenic dose of NDMA in the drinking water, or to a high, hepatotoxic dose of this agent, would influence expression of hepatotoxicity or levels of NDMA demethylase in the liver and, concomitantly, circulating levels of NDMA in the blood and in organs distal to the liver.

Materials and Methods

Lung Tumorigenesis Assay. Male strain A/J mice from the Jackson Laboratory, Bar Harbor, ME (experiment 1) or A/JCr from the Animal Production area of the Frederick Cancer Research Facility (experiment 2) were housed in plastic cages with hardwood shavings as bedding at 24 ± 2°C, 40–60% humidity, and with a 12-hr fluorescent light/dark cycle. Solutions of 500 ppb NDMA (Aldrich Chemical Co.) were diluted daily in distilled water from a stock solution of 10 ppm NDMA; the latter was kept at 4°C in the dark and prepared monthly. The drinking water solutions of NDMA were contained in amber bottles. All bottles and sipper tubes were rinsed with distilled water before use. Control animals received distilled water. The concentration of the chemical was confirmed by chemical analysis (extraction with methylene chloride followed by measurement by gas chromatography).

In experiment 1, the mice were fed Purina Certified Rodent Chow (#5002). Treatment was started at 4 weeks of age and continued for 12, 14, or 16 weeks. For experiment 2, two diets were formulated by the Ralston Purina Company to be of defined composition with regard to essential nutrients, so that these could be reproducibly manufactured if desired. One of these was similar to Chow 5002, as used in experiment 1. The other was similar to Purina Mouse Chow 5015, a high fat, low fiber diet. Treatment was started at 4 weeks of age and continued for 18 weeks. Lungs and stomachs were fixed in Bouin's solution and were examined under a dissecting microscope for tumors. Lungs were examined grossly and after hand-sectioning into 1-mm slices. Any questionable lesions were subjected to histological verification (7-μm sections, hematoxylin and eosin staining). Statistical analyses included the χ^2 test for differences in percentages of tumor-bearing mice and the two-tailed Student's t test for differences in average number of tumors per mouse.

Subchronic Exposure of Mice. Outbred Swiss Cr:NIH(s) specific pathogen-free mice were obtained from Animal Production, National Cancer Institute-Frederick Cancer Research Facility, Frederick, MD. Female mice were used except where specified in the tables. They were housed in polycarbonate cages with wire inserts and fed NIH autoclavable diet

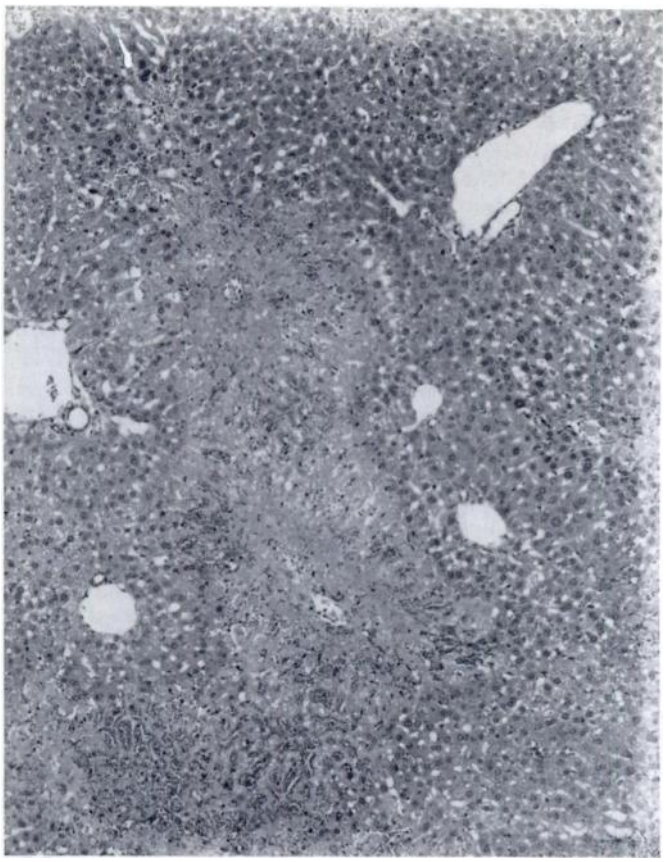

FIG. 1. *Liver from a female mouse that had received 50 ppm NDMA in the drinking water for 1 week, with pronounced centrilobular necrosis.* H & E, × 100.

31. Environmental conditions included a temperature of 25 ± 2°C, humidity of 50 ± 10%, and 12 changes of room air/hr. Fluorescent lights were automatically timed at 12 hr on/12 hr off. Treatment was started when the mice were 5 weeks of age. Drinking water was shielded from room lighting with aluminum foil and was changed and measured twice weekly. NDMA (Sigma Chemical Co.) and ethanol (95%, U. S. Industrial Chemicals) were diluted with deionized water.

At kill, blood was collected after decapitation into cryotubes containing a few crystals of ascorbic acid and 50 units of ammonium heparin. The carcass was placed on ice and the livers, kidneys, lungs, and brains were quickly removed to cryotubes. The tissues were frozen in liquid nitrogen and stored at −20°C until analyzed. In some experiments sections of liver were fixed in 10% formalin for histopathology. Portions of liver to be analyzed for content of NDMA demethylase activity were stored at −80°C prior to analysis.

Gross pathology was estimated semiquantitatively at kill by scoring on the basis of 0–3 [0 = normal; 1 = focal change in liver appearance and/or slight peritoneal fluid; 2 = general liver change (pits, yellowing, or darkening) and/or moderate peritoneal fluid; 3 = pronounced change in liver morphology and extensive peritoneal fluid]. Similarly, histopathological change was estimated semiquantitatively on a basis of 0–4 (0 = normal or minimal focal change; 1 = minimal centrilobular hemorrhage; 2 = mild centrilobular hemorrhage; 3 = moderate centrilobular hemorrhage with focal necrosis; 4 = moderate centrilobular hemorrhage with massive necrosis).

In order to assess the effects of ethanol on tissue levels of NDMA under conditions of controlled nutrition, Lieber-DeCarli Liquidiets were employed (BioServ, Inc., Frenchtown, NJ). Weanling female mice were allowed to become accustomed to the diet for 5 weeks, until their weights stabilized. The mice were then divided into two groups and those of one group were offered diet with gradually increasing amounts of ethanol, according to the instructions of the supplier. The mice accepted the ethanol-containing diet only poorly. After an additional 6 weeks of attempted accommodation to the diet, it was decided to carry out the experiment with two-thirds of the recommended ethanol level (4.3% of the diet, 24% of total calories). NDMA was included in both ethanol and control diets at 10 ppm, and the mice were pair-fed for 2 weeks so that mice were given NDMA-control diet in amounts equal to that consumed by the mice given NDMA-ethanol diet.

Estimation of Tissue Content of NDMA. The amount of NDMA in the samples of blood and other organs (pooled from 5–10 mice) was estimated by methylene chloride extraction-thermal energy analysis as described previously (30). The limit of detection of NDMA by this procedure was 0.5–1 ppb, depending on the amount of starting material. Water blanks and tissues from control mice were repeatedly assayed and did not contain measurable levels of NDMA (<0.5 ppb).

Assay of NDMA *N*-Demethylase. *N*-Demethylase activity toward NDMA was measured using 9000*g* supernatants prepared from homogenates of samples of frozen liver as described previously (31). The incubation mixture contained 0.5 μmol of NADP, 10 μmol of glucose 6-phosphate, 10 μmol of $MgCl_2$, 8 μmol of nicotinic acid, 20 μmol of semicarbazide hydrochloride, 0.2 mM NDMA, and supernatant from 50 mg of liver in a total of 2 ml of 0.1 M potassium phosphate buffer, pH 7.3. Blanks contained all reagents except the NADPH-generating system (NADP, glucose 6-phosphate, and nicotinic acid). After incubation for 20 min, the reaction was stopped by addition of 1.5 ml of cold 2.5% $ZnCl_2$ and 0.5 ml of 0.5 N NaOH. After centrifugation, formaldehyde was determined by the Nash reaction (32). The amount of protein was estimated by the method of Lowry *et al.* (33). Under these conditions, rate of formation of formaldehyde was linear with time and amount of supernatant protein.

Results

Tumorigenesis by 500 ppb NDMA. These experiments were designed to test whether a significant increase in lung tumors could be reproducibly obtained after exposure of male strain A mice to 500 ppb NDMA in the drinking water for 16–18 weeks as previously reported (34). The results presented in table 1 answer this question affirmatively: in three separate trials involving strain A mice from different sources and fed widely differing

TABLE 3

Content of NDMA in tissues of mice given 50 ppm NDMA with or without ethanol

Exposure Time	Treatment	NDMA Content				
		Blood	Liver	Kidney	Lung	Brain
weeks				*ppb*		
1	NDMA	30	4	21	27	27
	NDMA + EtOH	64	7	54	51	66
2	NDMA	12	4	7	4	6
	NDMA + EtOH	175	45	203	171	204
4	NDMA	65	6	51	38	32
	NDMA + EtOH	218	72	64	444	182

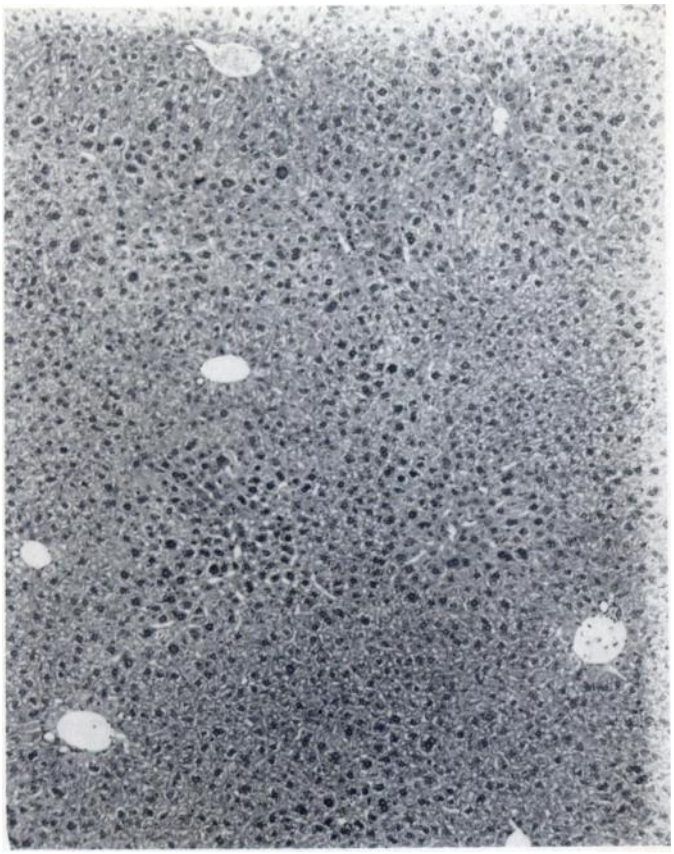

FIG. 2. *Liver from a mouse that had received 50 ppm and 10% ethanol in the drinking water for 1 week, with normal appearance.*

H & E, × 100.

diets, exposure to 500 ppb NDMA resulted in significant increases in incidences of lung tumors compared with controls. In experiment 1 it was found that 16 weeks of exposure was the minimum time required for a significant difference to be manifested. The strain A mice obtained from the Frederick Cancer Research Facility Animal Production Area, experiment 2, exhibited somewhat fewer tumors after NDMA treatment than did those in experiment 1. In spite of the large differences in diet composition for the two groups in experiment 2, resulting in those given the 5002 diet weighing on average 19% less and drinking 34% more than those given the 5015 diet (both differences significant; data not shown), the diet composition did not significantly affect the incidences of lung tumors in the control or NDMA-treated mice.

Forestomach papillomas were counted in experiment 1 as a possibly useful indicator of tumorigenesis. The incidences of these tumors were not treatment related (table 1).

Effects of Subchronic Administration of 50 ppm NDMA with and without Ethanol. In order to test whether exposure to a high, hepatotoxic dose of NDMA would result in measurable tissue levels of NDMA, and whether this level might be influenced by the simultaneous presence of ethanol, mice were given 50 ppm NDMA with or without 10% ethanol for 1, 2, or 4 weeks. The NDMA was clearly hepatotoxic, as expected, giving clear gross and histopathological signs of centrilobular liver damage and leading to a decline in liver NDMA *N*-demethylase activity between 1 and 4 weeks of exposure (table 2, fig. 1). NDMA was measurable in blood and all organ samples (table 3). Inclusion of 10% ethanol alone in the drinking water had no effect on water consumption, body weight, or liver histopathology (table 2). However, the ethanol significantly affected the interactions of NDMA with liver. The mice who received ethanol along with the NDMA experienced noticeably less liver damage, as indicated by both gross appearance and histopathology, especially after 1 or 4 weeks (figs. 1 and 2). Furthermore, the amounts of NDMA recovered from blood and organs were consistently greater, by factors of 2–40, in the mice given ethanol as well, at all three time points (table 3).

Subchronic Administration of Low Doses of NDMA with and without Ethanol. In male and female mice given 5 ppm NDMA in the drinking water for 2 weeks, low levels (1–4 ppb) of NDMA were detected in the blood samples and in one sample each of liver and brain (table 4). Co-treatment with 30% ethanol resulted in a dramatic increase in amounts of the NDMA in blood and organs, to about 20 ppb in most cases; livers, especially of the treated males, were the only exception to this pattern. Similar results were obtained in experiment 2 (table 4) when 10% ethanol was used. In this case the amounts of NDMA appearing in the blood and tissues of the mice co-treated with ethanol were less than after 30% ethanol, indicating dose dependency of the effect of ethanol. In experiment 3 of this series, a dose of 0.5 ppm NDMA was given with or without 20% ethanol. In the absence of ethanol, NDMA was detected at low levels in half of the samples analyzed. When ethanol was present, all samples contained NDMA at a rather uniform concentration of about 2–4 ppb.

TABLE 4

Tissue levels of NDMA in mice given low doses of NDMA in drinking water with or without ethanol

Expt.	NDMA Treatment	Ethanol	Average Water Consumption[a]	Average Body Weight[a]	NDMA Content				
					Blood	Liver	Kidney	Lung	Brain
	ppm		*ml/mouse/day*	*g*			*ppb*		
1a (males)	5		3.8 ± 0.7	25.4 ± 1.9	1	0.6	<0.5	<0.5	<0.5
	5	30%	4.7 ± 1.1	20.1 ± 2.7	22	<0.5	25	20	18
1b (females)	5		4.2 ± 0.7	19.2 ± 1.5	4	<0.5	<0.5	<0.5	0.7
	5	30%	4.7 ± 0.4	17.6 ± 1.7	26	2	25	29	19
2 (females)	5		3.9 ± 0.7	20.5 ± 2.1	0.6,2[b]	<0.5	2	<0.5	<0.5
	5	10%	4.5 ± 1.1	20.5 ± 1.9	4,12[b]	3	13	5	11
3 (females)	0.5		4.2 ± 1.5	20.7 ± 4.3	<0.5,2[b]	<0.5	<0.5	4	1
	0.5	20%	5.4 ± 1.2	22.5 ± 2.0	3,2[b]	4	2	4	2

[a] Values are ± standard deviation.

[b] Duplicate samples were analyzed.

TABLE 5

Tissue levels of NDMA in mice pair-fed liquid diet containing NDMA with or without ethanol

Treatment	No. of Mice	Average Body Weight[a]	Average Amount Consumed[a]	NDMA Content				
				Blood	Liver	Kidney	Lung	Brain
		g	*ml/mouse/day*			*ppb*		
10 ppm NDMA	20	19.4 ± 2.2	5.4 ± 1.2	<1	<1	2	<1	<1
10 ppm NDMA + 4% ethanol	14	19.8 ± 1.5	5.4 ± 1.2	5	16	12	7	10

[a] Average at end of 2-week exposure period. Values are ± standard deviation.

TABLE 6

NDMA demethylase activity in livers of mice given various concentrations of NDMA in the drinking water with or without 10% ethanol

NDMA Concentration	Ethanol (10%)	Water Consumption[a]	Average Body Weight	nmol of Formaldehyde/ mg of Protein/ 20 min
ppm		*ml/mouse/day*	*g*	
0	−	4.3	20.0 ± 1.8[b]	3.1 ± 1.6[b] (*N* = 5)
	+	6.3	18.5 ± 1.8	3.9 ± 1.5 (*N* = 5)
1	−	6.0	18.6 ± 2.2	4.1 ± 0.8 (*N* = 4)
	+	5.5	20.1 ± 1.1	4.4 ± 1.8 (*N* = 5)
5	−	5.7	20.5 ± 1.4	1.3 ± 1.1 (*N* = 4)
	+	4.3	18.3 ± 1.6	1.1 ± 0.5[c] (*N* = 4)
20	−	4.3	20.9 ± 1.4	3.8 ± 0.7 (*N* = 4)
	+	4.0	20.8 ± 1.5	3.3 ± 0.7 (*N* = 5)
50	−	3.0	22.0 ± 2.2	1.7 ± 1.0 (*N* = 4)
	+	2.7	19.9 ± 0.8	0.8 ± 0.7[c] (*N* = 4)

[a] Average amount consumed per mouse for the 4 days prior to kill.

[b] Values are ± standard deviation.

[c] Significantly less than the value for mice given ethanol only, $p < 0.01$, Student's *t* test.

Effects of Ethanol on NDMA Tissue Levels in Mice Pair-Fed a Liquid Diet. In order to ascertain whether the effects of ethanol in the drinking water might be due to nutritional differences between these mice and those not receiving ethanol, two groups of mice were pair-fed a liquid diet containing 10 ppm NDMA with or without 4% ethanol. Inclusion of ethanol resulted in the appearance of significant levels of NDMA in all tissues sampled, whereas, in the absence of ethanol, NDMA was detected at a low level only in kidney (table 5).

NDMA Demethylase Activity in Livers of Mice Given Various Doses of NDMA with or without Ethanol. The results presented in table 2 suggested that 10% ethanol in the drinking water had little inductive effect on NDMA demethylase activity in the presence of 50 ppm NDMA, and that chronic treatment with this high dose of NDMA resulted in a loss of enzyme activity. These findings were confirmed in the more detailed experiment presented in table 6. Treatment with 10% ethanol alone for 2 weeks resulted in only a small, insignificant increase in NDMA demethylase activity, compared with controls. Levels of enzyme activity decreased in the mice given 5 or 50 ppm NDMA, significantly so in those receiving ethanol also. Lack of a similar decrease in those given 20 ppm NDMA may have been related in part to reduction in water consumption at the higher doses of chemical (an average of 4.3 ml/day compared with 5.7 ml/day for the mice given 5 ppm NDMA).

Discussion

In a previous investigation it was found that exposure of male strain A mice from the Jackson Laboratories to 500 ppb NDMA for 16 weeks resulted in lung tumors in 44%, compared with 8% of controls; increases in lung tumor incidence with lower doses of NDMA were not of statistical significance (34). The findings of the present study are in good agreement: 500 ppb NDMA for 16 weeks yielded lung tumors in 48% of the mice from Jackson Laboratories. The results further showed that a significant effect of 500 ppb NDMA can be demonstrated with a different substrain of A mice and with different diets. Thus, 500 ppb NDMA may be the lowest level that gives an easily measurable tumorigenic effect in adult strain A mouse lung, but it does so reproducibly. It is of considerable interest that this was also a minimally effective concentration, in our subchronic trials, in giving rise to detectable levels of NDMA in mouse blood and other organs. In mice given 500 ppb NDMA for 2 weeks, about half of the tissue sample assayed had measurable NDMA, in the low ppb range. Although great caution must be employed in extrapolating from mice to man, these results suggest consideration of the possibility that humans with blood levels of 0.1–1 ppb NDMA may be at risk of tumor initiation in genetically sensitive peripheral target organs.

At 500 ppb and at all higher doses, inclusion of ethanol in the drinking water led to consistent increases in the amounts of NDMA in the blood and other organs. Although there was quantitative variation, as might be expected in an experiment where the chemical dose was received in the drinking water, the differences in NDMA levels with and without ethanol were in many cases of considerable magnitude. The obvious explanation for this effect of ethanol is competitive inhibition of the metabolism of NDMA, as reported by other workers (9–15). This inhibition, causing a reduction in the rate of formation of cytotoxic metabolites, probably also accounts for the alleviation of morphologically apparent hepatotoxic effects of NDMA as well. The ethanol did not, however, prevent the loss in NDMA demethylase activity caused by the higher doses of NDMA but, in fact, seemed to contribute to this loss. Although ethanol is an inducer of NDMA demethylase activity in rats after administration in drinking water (14, 18), at most, only a small inductive effect was seen in our Swiss mice. Inhibition of NDMA metabolism was clearly the dominant action.

In studies employing ethanol in the drinking water, the argument may be raised that the observed effects are due to nutritional imbalance secondary to ethanol consumption, rather than the chemical itself; pair-feeding of liquid diets is thus recommended (35). In our experiments the Lieber-DeCarli diet, formulated for rats, was not very successful. Consumption by the mice was poor, especially after addition of ethanol. Nevertheless, the experiment confirmed that the observed effects of ethanol on blood and organ content of NDMA were due directly to the ethanol and not to nutritional differences, since both groups consumed the same amount of nutritionally equivalent diet. Other workers have similarly found that administration of

ethanol in the drinking water is a sound experimental approach (36).

In sum, chronic co-treatment of mice with ethanol, along with NDMA, resulted in a lowering of one hepatotoxic effect of a high dose of NDMA, centrilobular necrosis, but enhancement of another effect, destruction of NDMA demethylase activity. At all doses the ethanol resulted in marked increases in circulating levels of NDMA. The contribution of this effect to tumorigenesis by low doses of NDMA in peripheral target organs will be an interesting subject for future experiments.

Acknowledgments. We acknowledge with appreciation the important contributions of Dan Logsdon, Rosemary Riggs, Areitha Smith, Ann Jones, Pat Vanatta, Larry Salm, Larry Claggett, Lee Dove, and Joyce Vincent.

References

1. D. H. Fine: Nitrosamines in the general environment and food. In "Nitrosamines and Human Cancer" (Banbury Report 12) (P. N. Magee, ed.), pp. 199–210. Cold Spring Harbor Laboratory, New York, 1982.
2. D. H. Fine, R. Ross, D. P. Rounbehler, A. Silvergleid, and L. Song: Formation *in vivo* of volatile *N*-nitrosamines in man after ingestion of cooked bacon and spinach. *Nature (Lond.)* **265**, 753–755 (1977).
3. M. Yamamoto, T. Yamada, and A. Tanimura: Volatile nitrosamines in human blood before and after ingestion of a meal containing high concentrations of nitrate and secondary amines. *Food Cosmet. Toxicol.* **18**, 297–299 (1980).
4. L. Lakritz, M. L. Simenhoff, S. R. Dunn, and W. Fiddler: *N*-Nitrosodimethylamine in human blood. *Food Cosmet. Toxicol.* **18**, 77–79 (1980).
5. A. A. Melikian, E. J. LaVoie, D. Hoffmann, and E. E. Wynder: Volatile nitrosamines: analysis in breast fluid and blood of nonlactating women. *Food Cosmet. Toxicol.* **19**, 757–759 (1981).
6. W. A. Garland, H. Holowaschenko, W. Kuenzig, E. P. Norkus, and A. H. Conney: A high resolution mass spectrometry assay for *N*-nitrosodimethylamine in human plasma. In "Nitrosamines and Human Cancer" (Banbury Report 12) (P N. Magee, ed.), pp. 183–198. Cold Spring Harbor Laboratory, New York, 1982.
7. L. Lakritz, R. A. Gates, A. M. Gugger, and A. E. Wasserman: Nitrosamine levels in human blood, urine and gastric aspirate following ingestion of foods containing potential nitrosamine precursors or preformed nitrosamines. *Food Chem. Toxicol.* **20**, 455–459 (1982).
8. T. A. Gough, K. S. Webb, and P. F. Swann: An examination of human blood for the presence of volatile nitrosamines. *Food Chem. Toxicol.* **21**, 151–156 (1983).
9. J. C. Phillips, B. G. Lake, S. D. Gangolli, P. Grasso, and A. G. Lloyd: Effects of pyrazole and 3-amino-1,2,4,-triazole on the metabolism and toxicity of dimethylnitrosamine in the rat. *J. Natl. Cancer Inst.* **58**, 629–633 (1977).
10. E. B. Johansson and H. Tjalve: The distribution of ^{14}C-dimethylnitrosamine in mice. Autoradiographic studies in mice with inhibited and noninhibited dimethylnitrosamine metabolism and a comparison with the distribution of ^{14}C-formaldehyde. *Toxicol. Appl. Pharmacol.* **45**, 565–575 (1978).
11. J. F. Tomera, P. L. Skipper, J. S. Wishnok, S. R. Tannenbaum, and H. Brunengraber: Inhibition of *N*-nitrosodimethylamine metabolism by ethanol and other inhibitors in the isolated perfused rat liver. *Carcinogenesis* **5**, 113–116 (1984).
12. G. Hauber, R. Frommberger, H. Remmer, and M. Schwenk: Metabolism of low concentrations of *N*-nitrosodimethylamine in isolated liver cells of the guinea pig. *Cancer Res.* **44**, 1343–1346 (1984).
13. P. F. Swann, A. M. Coe, and R. Mace: Ethanol and dimethylnitrosamine and diethylnitrosamine metabolism and disposition in the rat. Possible relevance to the influence of ethanol on human cancer incidence. *Carcinogenesis* **5**, 1337–1343 (1984).
14. R. Peng, Y. Y. Tu, and C. S. Yang: The induction and competitive inhibition of a high affinity microsomal nitrosodimethylamine demethylase by ethanol. *Carcinogenesis* **4**, 1457–1461 (1984).
15. N. A. Lorr, Y. Y. Tu, and C. S. Yang: The nature of nitrosamine denitrosation by rat liver microsomes. *Carcinogenesis* **3**, 1039–1043 (1982).
16. Y. Y. Tu, R. Peng, Z. Chang, and C. S. Yang: Induction of a high affinity nitrosamine demethylase in rat liver microsomes by acetone and isopropanol. *Chem. Biol. Interact.* **44**, 247–260 (1983).
17. G. D. McCoy, C. B. Chen, S. S. Hecht, and E. C. McCoy: Enhanced metabolism and mutagenesis of nitrosopyrrolidine in liver fractions isolated from chronic ethanol-consuming hamsters. *Cancer Res.* **39**, 793–796 (1979).
18. A. J. Garro, H. K. Seitz, and C. S. Lieber: Enhancement of dimethylnitrosamine metabolism and activation to a mutagen following chronic ethanol consumption. *Cancer Res.* **41**, 120–124 (1981).
19. H. Glatt, L. DeBalle, and F. Oesch: Ethanol- or acetone-pretreatment of mice strongly enhances the bacterial mutagenicity of dimethylnitrosamine in assays mediated by liver subcellular fraction, but not in host-mediated assays. *Carcinogenesis* **2**, 1057–1061 (1981).
20. Olson, M. J., J. G. Pounds, and D. A. Casciano: Potentiation of dimethylnitrosamine genotoxicity in rat hepatocytes isolated following ethanol treatment *in vivo*. *Chem. Biol. Interact.* **50**, 313–326 (1984).
21. M. Schwartz, G. Wiesback, H. Hummel, and W. Kunz: Effect of ethanol on dimethylnitrosamine activation and DNA synthesis in rat liver. *Carcinogenesis* **3**, 1071–1075 (1982).
22. N. A. Lorr, K. W. Miller, H. R. Chung, and C. S. Yang: Potentiation of the hepatotoxicity of *N*-nitrosodimethylamine by fasting, diabetes, acetone, and isopropanol. *Toxicol. Appl. Pharmacol.* **73**, 423–431 (1984).
23. J. Gellert, F. Moreno, M. Haydn, H. Oldiges, H. Frenzel, R. Teschke, and G. Strohmeyer: Decreased hepatotoxicity of dimethylnitrosamine (DMN) following chronic alcohol consumption. In "Alcohol and Aldehyde Metabolizing Systems, IV" (R. G. Thurman, ed.), pp. 237–243. Plenum Press, New York, 1979.
24. M. Schwartz, A. Buchmann, G. Wiesbeck, and W. Kunz: Effect of ethanol on early stages in nitrosamine carcinogenesis in rat liver. *Cancer Lett.* **20**, 305–312 (1983).
25. D. Schmahl, C. Thomas, W. Sattler, and G. F. Scheld: Experimentelle Untersuchungen zur Syncarcinogenesis. 3. Mitteilung Versuch zur Krebserzuegung bei Ratten bei gleichzeitiger Gabe vun Diäethylnitrosamin und Tetrachlorkohlenstoff bzw. Athylalkohol: zugleich ein experimenteller Beitrag zur Frage der Alkoholzirrhose. *Z. Krebsforsch.* **66**, 526–532 (1965).
26. W. Gibel: Experimentelle Untersuchungen zur Synkarzinogenese beim Ösophaguskarzinom. *Arch. Geschwulstforsch.* **30**, 181–189 (1967).
27. M. Habs and D. Schmähl: Inhibition of the hepatocarcinogenic activity of diethylnitrosamine by ethanol in rats. *Hepatogasteroenterology* **28**, 242–244 (1981).
28. L. Griciute, N. Castegnaro, and J. C. Bereziat: Influence of ethyl alcohol on carcinogenesis with *N*-nitrosodimethylamine. *Cancer Lett.* **13**, 345–352 (1981).
29. A. Castonguay, A. Rivenson, N. Trushin, J. Reinhardt, S. Spathopoulos, C. J. Weiss, B. Reiss, and S. S. Hecht: Effects of chronic ethanol consumption on the metabolism and carcinogenicity of *N*-nitrosonornicotine in F344 rats. *Cancer Res.* **44**, 2285–2290 (1984).
30. H. M. Pylypiw, F. Zimmerman, G. W. Harrington, and L. M. Anderson: Apparatus for trace analysis of volatile *N*-nitrosamines in small samples. *Anal. Chem.* **52**, 2996–2997 (1985).
31. L. M. Anderson and M. Angel: Induction of dimethylnitrosamine demethylase activity in mouse liver by polychlorinated biphenyls and methylcholanthrene. *Biochem. Pharmacol.* **29**, 1375–1381 (1980).
32. T. Nash: The colorimetric estimation of formaldehyde by means of the Hantsch reaction. *Biochem. J.* **55**, 416–420 (1953).

33. O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall: Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265–275 (1951).
34. L. M. Anderson, K. Van Havere, and J. M. Budinger: Lung tumorigenesis in strain A mice by low doses of dimethylnitrosamine. In "Nitrosamines and Human Cancer" (Banbury Report 12) (P. N. Magee, ed.), pp. 531–542. Cold Spring Harbor Laboratory, New York, 1982.
35. C. S. Lieber and L. M. DeCarli: The feeding of alcohol in liquid diets: two decades of applications and 1982 update. *Alcohol. Clin. Exp. Res.* **6**, 523–531 (1982).
36. J. S. Prasad, D. L. Crankshaw, R. R. Erickson, C. E. Elliott, A. D. Husby, and J. L. Holtzman: Studies on the effects of chronic consumption of moderate amounts of ethanol on male rat hepatic microsomal drug-metabolizing activity. *Biochem. Pharmacol.* **34**, 3427–3431 (1985).