Synthesis of Hepatotoxic Agents in Germfree and Conventional Mice Which Had Been Fed $NaNO_2$ and Dimethylamine[1] (36614)

MORRIS POLLARD, NEHAMA SHARON, AND CHIENKUO F. CHANG
Lobund Laboratory, University of Notre Dame, Notre Dame, Indiana 46556

Nitrosamine compounds are efficient mutagenic, teratogenic, and oncogenic agents (1–3). The effects in animals of orally administered dialkyl nitrosamines have been described as toxic hepatitis, with subsequent malignant neoplasms in multiple organ systems of the body, depending on the animal and strain used. Carcinogenic nitrosamine compounds have been synthesized in the gastric contents of conventional mice and rats following oral administration of $NaNO_2$ and sources of secondary amines (4). The synthetic reaction has been attributed to the metabolic activity of intestinal microorganisms; however, the implication was not decisive (5, 6). In order to determine the role of microbial flora in the biosynthesis of nitrosamine from $NaNO_2$ and secondary amines, it would be necessary to apply the protocol to germfree animals. This report describes the synthesis of hepatotoxic agent(s), which may be nitrosamine compounds, in germfree (GF) and conventional mice which had been fed, simultaneously, subtoxic doses of $NaNO_2$ and dimethylamine (DMA).

Methods. The initial procedures repeated the protocol described by Asahina *et al.* (7): the toxic dose levels in mice per kilogram body weight were determined by administering increasing quantities of $NaNO_2$ and of DMA to groups of mice through a metal 23 gauge stomach tube. That protocol was modified by depriving the treated mice of commercial diet and drinking water for 6 hr prior to exposure. Drinking water was available 2 hr after feeding, and diet 15 hr later.

Drugs. Sodium nitrite (certified A.C.S. grade) and dimethylamine hydrochloride (Matheson, Cole & Bell) were employed in these experiments. Prior to each experiment, the drug was dissolved in distilled water and passed through a Millipore filter (0.45 μ) into sterile ampoules. The heat-sealed ampoules were sterilized by spraying with 2% peracetic acid in a double-doored entry portal through which they were brought into the sterile plastic isolator system (8). The ampoules were then opened and the drug was fed to the mice. Microbial contaminations have not been detected in GF mice with this test system.

Mice. Male GF, CFW and Swiss-Webster mice, age 30 days, and produced in this Laboratory were used. They were free of bacteria, fungi, and macroparasites (9), but they were infected congenitally with leukemia virus as in all other strains of mice (10). However, only one case of spontaneous leukemia has been observed by us during the past 10 years among these two low leukemia mouse strains. Spontaneous hepatitis has not been observed among the GF and conventional mice in this Laboratory. The mice were observed daily, and monitored for GF status at frequent intervals, and at the termination of each experiment. The control conventional mice were derived from GF CFW and Swiss-Webster stock, after they had acquired a mixed microbial flora by exposure to the "clean" animal house. They were free of pathogenic microorganisms.

The mice were weighed individually and groups of them were fed $NaNO_2$ or DMA. The treated mice were observed for 3 days after treatment; and each mouse that died was examined for gross and microscopic evidence of tissue changes. On the third day the survivors were killed by ether anesthesia and examined for evidence of tissue damage.

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TABLE I. Effects of Oral Administration of Dimethylamine, Sodium Nitrite, or Combinations Thereof to Conventional CFW Mice.[a]

Dose (mg/kg)		Mortality by 3 days		Mice with necrosis	
DMA	$NaNO_2$	No.	(%)	No.	(%)
0	100	0/14[b]	(0)	0/14	(0)
2500	0	0/25	(0)	0/25	(0)
3500	0	3/23	(13)	0/20	(0)
2500	100	3/17	(17)	9/14	(66)
3500	100	17/26	(65)	8/9	(89)

[a] Deprived of food for 6 hr prior to and 15 hr subsequent to the feeding experiment.

[b] Number positive/no. at test.

When toxic levels of each drug were determined, groups of mice were fed maximum subtoxic doses of $NaNO_2$, of DMA, or of combinations thereof. The mice that died as a result of the treatments were examined as soon after death as possible, and all survivors were examined on the third day, as noted above. Tissues were fixed in Bouin's solution and embedded in paraffin, from which hematoxylin and eosin stained sections were processed for microscopic examinations.

Results. Conventional CFW mice tolerated $NaNO_2$ or DMA up to 150 and 3500 mg, respectively; and mice which had been deprived of food tolerated 100 and 3500 mg, respectively (Table I). All drug weights refer to milligrams per kilogram of body weight. Administration of combined 100 mg $NaNO_2$ and 2500 mg DMA caused deaths in 3 of 17 mice (17%) within 24 hr, and liver necrosis in 9 of 14 (66%) survivors on the third day after treatment. In the mice which died within 24 hr after feeding combined $NaNO_2$ and DMA, the vascular patterns of the livers were markedly dilated; but there was no detectable necrosis in that organ. As indicated in Table I, increased dosages of DMA and $NaNO_2$ resulted in higher acute death rates, and a higher incidence of necrotic lesions in the livers of the survivors. In addition, the peritoneal cavities contained considerable amounts of clear fluid.

GF CFW mice tolerated 75 mg, but not 100 mg of $NaNO_2$, but the toxic effect was enhanced when 75 mg was combined with 2500 mg DMA (Table II). However, clearly defined liver necrosis was not observed in the survivors when they were examined 3 days later.

Conventional and GF Swiss-Webster mice tolerated 100 mg $NaNO_2$ (Table III). When this dose of $NaNO_2$ was administered with 2500 mg and 3500 mg of DMA, many of them died within 24 hr and the 3 day survivors had necrotic lesions in the livers (Fig. 1). The toxicity and extent of liver necrosis were directly related to the dosage of DMA.

The necrotic areas in the livers ranged from small local foci, confined to part of one lobe, to large diffuse areas of amorphous, cell-free degenerated tissue encompassing an entire lobe. Intact liver cells occupied the margins of the necrotic zones, and there was no microscopic evidence of inflammatory reaction surrounding the areas of necrosis. The normal appearing liver cells were in sharp contrast to the necrotic areas (Fig. 1). Mice which survived toxic levels of $NaNO_2$ or of DMA were free of liver lesions, when they were examined 3 days later.

Discussion. The data in this report indicate that a microbiological flora is not required in Swiss-Webster mice for the *in vivo* production of a toxic agent(s) from orally administered combinations of $NaNO_2$ and DMA. Two responses have been observed in the Swiss-Webster and CFW mice following simultaneous feeding of $NaNO_2$ and DMA: (a) an acute toxic response which killed the

TABLE II. Effects of Oral Administration of Dimethylamine, Sodium Nitrite or Combinations Thereof to Germfree CFW Mice.[a]

Dose (mg/kg)		Mortality at 24 hr		Liver necrosis at 3 days	
DMA	$NaNO_2$	No.	(%)	No.	(%)
0	75	0/6[b]	(0)	0/6	(0)
2500	0	1/24	(4)	0/23	(0)
3500	0	8/20	(40)	0/12	(0)
2500	75	2/5	(40)	0/3	(0)
3500	75	5/5	(100)	—	

[a] Deprived of food for 6 hr prior to and 15 hr subsequent to the feeding experiment.

[b] Number positive/no. at test.

TABLE III. Effects of Oral Administrations of Dimethylamine, Sodium Nitrite, or Combinations Thereof to Germfree and Conventional Swiss-Webster Mice.[a]

Dose		Total no. of mice		Mortality at 24 hr		Liver necrosis at 3 days	
DMA	$NaNO_2$	Conv.	GF	Conv.	GF	Conv.	GF
				No. (%)	No. (%)	No. (%)	No. (%)
0	100	11	11	0/11[b] (0)	0/11 (0)	0/11 (0)	0/11 (0)
2500	0	10	10	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)
3500	0	10	15	0/10 (0)	3/15 (27)	0/10 (0)	0/12 (0)
2500	100	17	20	4/17 (24)	6/20 (30)	4/13 (31)	2/14 (14)
3500	100	18	29	7/18 (39)	18/29 (60)	8/11 (73)	9/11 (80)

[a] Deprived of food for 6 hr prior to and 15 hr subsequent to the feeding experiment.
[b] Number positive/no. at test.

mice within 24 hr and resembled the toxic death following a large dose of nitrite, and (b) liver necrosis which was observed 3 days later. It should be determined if these two manifestations are related and sequential, or unique. We assume that animals which consume both $NaNO_2$ and DMA synthesize nitrosamine compounds and develop hepatic lesions, and that eventually they will develop tumors. To prove this assumption we are now observing treated mice for longer periods of time. It has been demonstrated in conventional animals that tumors developed after administration of $NaNO_2$ and dimethylurea (3, 11); but a similar pathogenic pattern has not yet been reported in germfree mice. The biological process described here with $NaNO_2$ and DMA differs from that reported by Laqueur, McDaniel and Matsumoto (12) in rats with cycasin. With cycasin it was found that an enzyme of bacterial origin (β-glucosidase) was required for its conversion to the carcinogenic aglycone, and the carcinogenic effect was manifested only in conventional rats, but not in GF. In our experiments the effects were manifested in conventional and GF mice. Mouse strains can differ in susceptibility to the toxic effects of

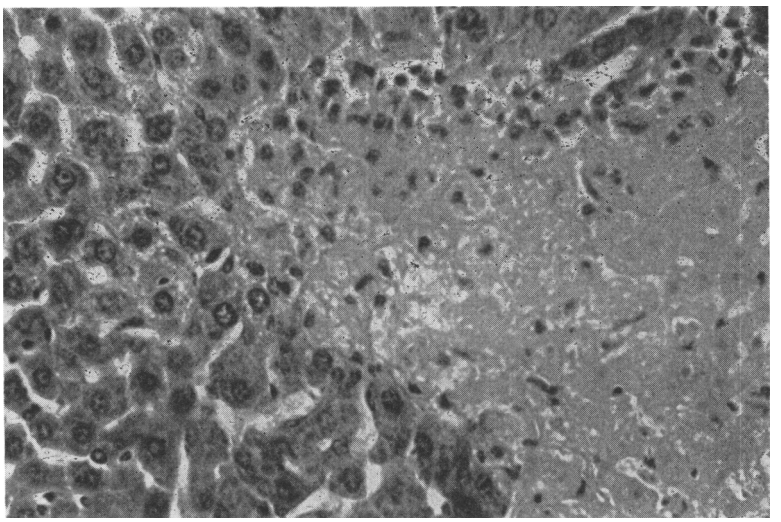

FIG. 1. Liver section from germfree Swiss-Webster mouse at 3 days following oral administration of $NaNO_2$ and dimethylamine. Note the sharply delineated zones of necrosis and intact parenchyma. Hemotoxylin and eosin stain. ×200.

$NaNO_2$, as was demonstrated in GF CFW mice which died acutely prior to the production of necrotic lesions in the livers. However, some of the mice at test survived the toxic stage, but did not manifest evidence of disease, for which we cannot offer an explanation. The GF Swiss-Webster mice survived the acute toxic effect of the combination and subsequently developed the liver lesions. This may be governed by the amount of $NaNO_2$ in the gastrointestinal tract.

Since $NaNO_3$ is a more common constituent of the diet than $NaNO_2$ it would be of importance to determine if specific bacterial species in the gastrointestinal tract can reduce the nitrate to nitrite. In view of the high susceptibility of GF CFW mice to nitrite, they can be used as test animals for detecting conversion of NO_3 to NO_2. Since it is known that specific microorganisms can reduce nitrate to nitrite *in vitro,* it would be of interest to determine if they could do it *in vivo* in gnotobiotic (monocontaminated) mice. In such animals, the newly formed nitrite could induce an acute toxic effect, or with DMA, produce a nitrosamine compound as was described above. The only foreseeable limitation to such a scheme may be the low pH of the stomach which would support the synthesis of nitrosamines, but in which few microorganisms could live and function.

The nitrosamines demonstrate a great disease potential in experimental animals (1–3). Although the full extent of their responsibility for cancer in man is not known, they should be detected and excluded from contact with man. GF animals provide a controlled medium in the search for specific microorganisms which might immobilize nitrosamines in the intestine and thus provide a protective barrier against the absorption of such biological hazards.

Summary. In vivo production of toxic agent(s) was demonstrated in GF and conventional mice by feeding them subtoxic doses of $NaNO_2$ and dimethylamine. Two effects were demonstrated in the mice: acute deaths in some of them within 24 hr after administration of the two drugs; and extensive necrosis in the livers of survivors which were examined 3 days later. GF CFW mice were more sensitive to the toxic effects of $NaNO_2$ than conventional CFW mice, and conventional and GF Swiss-Webster mice. The microbial flora has been excluded as having a role in the *in vivo* synthesis of this toxic agent(s), which we assume to be nitrosoamine.

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