

## SHORT PAPERS

## EFFECT ON GUINEA-PIGS OF FEEDING NITROSOMORPHOLINE AND ITS PRECURSORS IN COMBINATION WITH ASCORBIC ACID

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**Summary**—Nitrosomorpholine (70 mg/litre drinking-water) or morpholine (6.33 g/kg food) plus sodium nitrite (1.0 g/litre drinking-water) was administered to guinea-pigs, which were fed a diet containing either a high or a low level of ascorbic acid or no ascorbic acid at all. Animals on the ascorbic acid-deficient diet developed scurvy before toxic effects of either the nitrosamine or the nitrosamine precursors were evident. Precancerous liver changes occurred in guinea-pigs receiving nitrosomorpholine together with the high or low levels of ascorbic acid. Animals receiving the nitrosamine precursors together with ascorbic acid were not affected. Guinea-pigs and other species that require exogenous sources of ascorbic acid may be protected from the hazard of *in vivo* nitrosation by the presence of ascorbic acid in the stomach.

**Introduction**

Nitrosamines have been detected in a wide variety of consumer products, including grains, meats, alcohol and tobacco (Lijinsky & Epstein, 1970; Magee & Barnes, 1967; Rhoades & Johnson, 1972). Several recent studies indicate that, under suitable conditions, secondary amines can react with nitrites to form nitrosamines *in vivo* (Greenblatt, Mirvish & Sc, 1971; Lijinsky & Greenblatt, 1972; Wolff, 1972), suggesting that the potential carcinogenic hazard of nitrosamines may be even greater than was originally suspected. Experiments with mice (Greenblatt, 1973) and rats (Kamm, Dashman, Conney & Burns, 1973) have shown that the presence of ascorbic acid or ascorbate in the stomach prevents the hepatotoxicity resulting from the ingestion of secondary amines plus nitrite, apparently by blocking the nitrosation reaction. These animals, however, normally synthesize ascorbic acid rather than depend on a dietary source. The risk of nitrosamine formation from environmental precursors in species, such as man, that require a daily intake of ascorbic acid has been difficult to evaluate.

The present report describes an attempt to study nitrosamine toxicity and the nitrosation process in guinea-pigs, which resemble man in requiring an exogenous source of ascorbic acid (Chatterjee, 1973). Nitrosomorpholine was selected as the test nitrosamine in these preliminary studies because of its demonstrated capacity to induce hepatotoxicity and liver tumours in rodents within a short period of time (Druckrey, Preussmann, Ivanovic & Schmähl, 1967).

## Experimental

**Animals and materials.** Male and female guinea-pigs of the Duncan Hartly strain, weighing 200–250 g, were obtained from Carworth Laboratories, New York\*. The basic diet (ground oats, 40.0%; ground bran, 15.0%; alfalfa, 8.0%; sodium chloride, 0.5%; calcium carbonate, 1.0%; magnesium sulphate, 0.5%) was purchased from Nutritional Biochemicals, Cleveland, Ohio. L-Ascorbic acid, certified morpholine and sodium nitrite (crystalline) were obtained from Fisher Scientific Co., Atlanta, Ga. Nitrosomorpholine was prepared from morpholine and sodium nitrite in an acid solution (Pensabene, Fiddler, Dooley, Doerr & Wasserman, 1972). Ascorbic acid (at a high level of 6.7 g/kg food or a low level of 150 mg/kg) and/or morpholine (6.33 g/kg) were blended into the basic diet. Nitrosomorpholine (70 mg/litre) or NaNO<sub>2</sub> (1.0 g/litre) was added to the drinking-water, which was supplied to each cage in amber bottles. The guinea-pigs were allowed free access to the food and water supply.

**Treatment.** Guinea-pigs were randomly divided into nine groups, each consisting of three males and three females. Each group was fed one of the levels of ascorbic acid or an ascorbic acid-free diet and one of the three treatment regimens, as shown in Table 1. All possible combinations of ascorbic acid and nitrosamine or precursors were administered. Animals were killed after the feeding periods listed. Sections of liver and lung and occasional samples from other organs were fixed in buffered formalin. Histopathological examination was performed by Dr. T. E. Murchison, Dawson Research Corp., Orlando, Fla.

Table 1. Liver and lung alterations in guinea-pigs fed nitrosomorpholine or precursors in combination with ascorbic acid

Dietary level of ascorbic acid*	Treatment†	Duration of feeding (wk)	Type of tissue alteration‡	
			Liver	Lung
High	None (control)	25		
High	Nitrosomorpholine	25	Focal giant liver cells (3)	
High	Morpholine + NaNO <sub>2</sub>	25		
Low	None (control)	9		
Low	Nitrosomorpholine	9	Occasional giant cells (3) and liver-cell hyperplasia (2)	Lymphoid hyperplasia (1)
Low	Morpholine + NaNO <sub>2</sub>	9		
None	None (control)	3		
None	Nitrosomorpholine	3	Liver-cell hyperplasia (1)	
None	Morpholine + NaNO <sub>2</sub>	3		

\*High, 6.7 g/kg; low, 150 mg/kg.

†Nitrosomorpholine, 70 mg/litre drinking-water; NaNO<sub>2</sub>, 1.0 g/litre drinking-water; morpholine, 6.33 g/kg diet.

‡Numbers in parentheses indicate the numbers of animals affected/group of six.

## Results and Discussion

Guinea-pigs in the three groups fed the high level of ascorbic acid remained healthy throughout the 25 wk of trials. A routine analysis (Schaffert & Kingsley, 1954) during wk

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

4 confirmed the presence of ascorbic acid in the urine of these animals, indicating tissue saturation with ascorbic acid. All the guinea-pigs fed diets devoid of ascorbic acid exhibited classical symptoms of vitamin C deficiency (including weight loss, diarrhoea and loss of leg mobility) after 2 wk. Survivors in these groups were killed during wk 3. Several guinea-pigs on the low-level ascorbic acid diet developed deficiency symptoms after 6-8 wk and the survivors were killed at 9 wk.

Pathological findings in the liver and lung tissue from guinea-pigs receiving the various treatment and diet combinations are summarized in Table 1. Lesions indicative of a precancerous condition were observed in livers from about half of the animals receiving nitrosomorpholine. Concomitant feeding of ascorbic acid did not prevent the hepatotoxicity, which occurred in both sexes. The most pronounced hepatotoxicity developed in guinea-pigs that ingested the nitrosamine for the full 25 wk. However, those receiving nitrosomorpholine for only 9 wk showed moderate liver alterations. Thus, in guinea-pigs, nitrosomorpholine, like diethylnitrosamine (Argus & Hoch-Ligeti, 1963), appears to have an effect primarily on the liver. The occurrence of lung hyperplasia in a single animal receiving treatment for 9 wk and the liver-cell hyperplasia seen in one animal that survived for only 3 wk cannot definitely be attributed to the nitrosamine treatments since the incidence was too small.

Carcinogenic nitrosamines and nitrosoureas have been recovered from the stomach contents of rats that received, by intubation, the precursors of these *N*-nitroso compounds (Lijinsky & Greenblatt, 1972; Mirvish & Chu, 1973). Mice that ingested morpholine together with  $\text{NaNO}_2$  have been shown to develop a high incidence of lung tumours, presumably as a result of nitrosamine formation (Greenblatt *et al.* 1971). Morpholine has been shown to react with nitrite *in vitro* to form nitrosomorpholine when pH and other conditions simulated those of mammalian stomach contents; nitrosation did not occur in the presence of ascorbic acid (Mirvish, Wallcave, Eagen & Shubik, 1972).

In the present study, the feeding of morpholine and  $\text{NaNO}_2$  together with ascorbic acid, in concentrations that caused tumours in mice, did not cause any of the toxic responses seen with nitrosomorpholine. Although we speculate that the presence of ingested ascorbic acid prevented nitrosamine formation, this fact could not be proved unequivocally, since removal of ascorbic acid from the diet of guinea-pigs resulted in their early death. Nevertheless, man and other species requiring dietary sources of ascorbic acid may have a built-in protection against *in vivo* nitrosation. We are currently investigating other methods of measuring nitrosamine formation in guinea-pigs.

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