

# Carcinogenicity of Nitrosation Products of Ephedrine, Sarcosine, Folic Acid, and Creatinine<sup>1</sup>

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## SUMMARY

Carcinogenic activity of several synthetic *N*-nitroso compounds was evaluated in C57BL/6J × C3HeB/FeJ F<sub>1</sub> mice. Test substances, suspended in trioctanoin, were injected i.p. in three equal doses given on Days 1, 4, and 7 after birth and animals were held without further treatment for up to 85 weeks.

Nitrosoephedrine at a total dose of 600 mg/kg induced metastasizing liver cell carcinomas in 28 of 30 animals. Nitrososarcosine (225 mg/kg) induced similar tumors in 8 of 14 animals. Nitrosofolic acid (375 mg/kg) induced lung adenocarcinomas in 4 of 28 mice. Creatinine-5-oxime (600 mg/kg) showed no evidence of carcinogenic activity. Diethylnitrosamine (12 mg/kg given in four doses), included as a positive control, caused metastasizing liver cell tumors in 23 of 25 animals.

## INTRODUCTION

The potency and versatility of *N*-nitroso compounds as carcinogens for experimental animals have been extensively documented (3, 8). In the recent past, increasing attention has been paid to the extent to which such compounds are formed in the human environment through the interaction of nitrite with amines or amides. Reactants for this interaction are widely distributed as food components, preservatives, drugs, and agricultural chemicals (5, 14). It has been established that nitrosation can occur when appropriate conditions exist in nitrite-preserved foods, with the production of compounds with carcinogenic activity (14). It has also been demonstrated that nitrosation reactions can occur *in vivo* under the acidic conditions of the stomach (11), and carcinogenic responses have been produced in animals by simultaneous feeding of nitrite and nitrosatable substrates (10).

In order to contribute to a fuller understanding of the importance of this class of compounds as potential human carcinogens, we have been investigating several aspects of the chemistry and biological effects of nitroso compounds of environmental importance. We report here the results of carcinogenesis evaluations on nitrosation products of ephedrine, folic acid, sarcosine, and creatinine since all are

readily nitrosatable compounds and the probability of human exposure to them is relatively high. Ephedrine is a commonly used drug, and the nitroso derivative is related structurally to methyl(phenylethyl)nitrosamine, a compound shown to be carcinogenic by Druckrey *et al.* (3). The remaining compounds are ubiquitous nitrogen-containing components of foods. Nitrososarcosine and creatinine-5-oxime are major products of reactions between creatine and/or creatinine and nitrite (1) and might therefore be widely distributed in foods. With the exception of nitrososarcosine, which was shown to have carcinogenic activity in 1 previous study (3) none of the other compounds has previously been investigated. The structures of these derivatives are shown in Chart 1.

## MATERIALS AND METHODS

Chemicals tested for carcinogenicity were suspended in trioctanoin (Eastman Organic Chemicals, Rochester, N. Y.). Diethylnitrosamine (Eastman) was included as a positive control. The test compounds were synthesized in our laboratories according to the following methods.

Nitrososarcosine was prepared from sarcosine by the method described by Lijinsky *et al.* (6). The product was purified by elution from a silica gel column with diethyl ether. Nitrosoephedrine was prepared from ephedrine (2-methylamino-1-phenyl-1-propanol) by a method similar to that described by Sander (9). One g of ephedrine-HCl (Merck & Co., Inc., Rahway, N. J.) was dissolved in 25 ml 10% HCl and cooled to 10°. Seven g NaNO<sub>2</sub> in 10 ml H<sub>2</sub>O were added over a period of 5 min with stirring. The

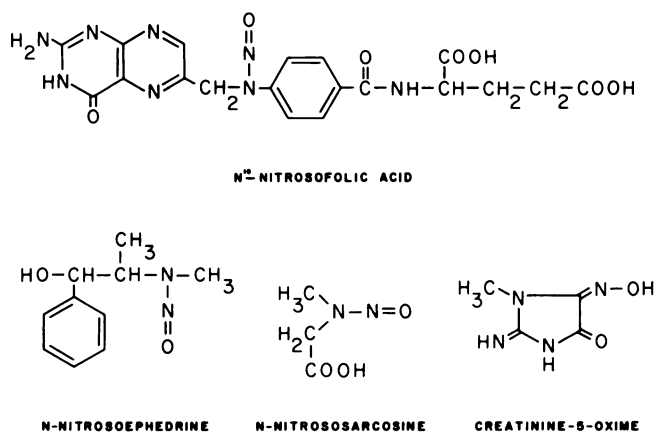


Chart 1. Structures of *N*-nitroso compounds tested for carcinogenicity.

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reaction mixture was maintained at 10° for 2 hr, during which time yellow oil droplets formed. The mixture was then extracted twice with 15-ml portions of CH<sub>2</sub>Cl<sub>2</sub>. The extracts were dried over anhydrous magnesium sulfate and then filtered. Solvent removal yielded a yellow oil that, on standing at room temperature, formed white crystals. The solid was recrystallized from CCl<sub>4</sub>, and dried; the yield was 0.6 g (62%) of white platelets, m.p. 90°. The identity of the compound was established by the following analytical and spectral data.



Calculated: C 61.85, H 7.22, N 14.43, O 16.49

Found: C 61.40, H 7.05, N 14.37, O 6.31

Infrared, (KBr pellet, 1%) 3480 cm<sup>-1</sup> (m, broad); 3370 (m, broad); 3020 (w); 3000 (w); 2990 (w); 2940 (w); 2880 (w); 1490 (w); 1450 (s); 1420 (s); 1375 (m); 1345 (s); 1290 (m); 1270 (m); 1220 (m); 1200 (w); 1110 (m); 1045 (s); 990 (w); 905 (w); 835 (w); 775 (s); 715 (s); 630 (w).

UV (in water), λ<sub>max</sub> 230 nm (ε = 7,230); 205 nm (ε = 11,500). Nuclear magnetic resonance (in CDCl<sub>3</sub>), τ2.7 (s, 5H); τ5.0 (d, 1H, J = 5.0 Hz); τ5.2 (m, 1H); τ7.1 (s, 3H); τ7.3 (s, 1H); τ8.55 (d, 3H, J = 6.0 Hz).

N<sup>10</sup>-Nitrosofolic acid was prepared from folic acid by the method described by Cosulich and Smith (2). The product was purified by recrystallization from water. Creatinine-5-oxime was prepared from creatinine by the method of Archer *et al.* (1) and was purified by recrystallization from water.

Experimental animals were C57BL/6J × C3HeB/FeJ F<sub>1</sub> mice. Both parent strains were purchased from the Jackson Laboratories, Bar Harbor, Maine. The animals were bred in our laboratories and pregnant females were assigned randomly to treatment groups. Mothers were housed together with their offspring in plastic cages with natural bedding (Anderson Cob Mills, Maumee, Ohio). Animals were fed a chow diet (Charles River Breeding Laboratories, Wilmington, Mass.) throughout the experiment.

Initial experiments consisted of determination of the single-dose LD<sub>50</sub><sup>a</sup> value for each test compound. Mice were treated before they were 27 hr old, each receiving a single i.p. injection of compound suspended in trioctanoin in a volume of 0.005 ml/g body weight. A series of 5 to 7 logarithmically spaced doses were used, each being administered to groups of 9 to 17 mice. Mortality was recorded twice daily for 1 week, and LD<sub>50</sub> values were computed by the method of Litchfield and Wilcoxon (7).

The treatment protocol for the carcinogenesis evaluation consisted of 3 i.p. injections/animal, given on Days 1, 4, and 7 after birth, a regimen previously shown to be effective for tumor induction in animals of this strain by other carcinogens (12, 13). Compounds were suspended in trioctanoin as in the previous experiment, and each animal received a cumulative dose of test substance approximately equal to twice the maximum sublethal dose determined in the LD<sub>50</sub> study. Diethylnitrosamine was sufficiently toxic to require a smaller total dose administered at wider intervals (Days 1,

4, 10, and 16) to avoid excessive mortality. Deaths attributed to acute toxicity, *i.e.*, occurring up to 1 week following cessation of dosing, were observed at the following incidence: nitrososarcosine, 14 of 51; nitrosoephedrine, 3 of 49; N<sup>10</sup>-nitrosofolic acid, 3 of 45; creatinine-5-oxime, 5 of 44; diethylnitrosamine, 1 of 47; and trioctanoin, 0 of 33. Mice were left with their mothers for 4 weeks and then were segregated by sex and housed in groups of 5/cage. After 4 months, deaths due to fighting necessitated housing them individually for the remainder of the experiment. Animals surviving after 78 weeks were killed and subjected to complete autopsy, as were those dying in the intervening period. Tissues fixed in buffered formalin and stained with hematoxylin and eosin were subjected to histopathological examination. Lungs were examined for the presence of metastatic lesions by preparing microscopic sections of single blocks removed randomly from each lung after fixation.

## RESULTS

The LD<sub>50</sub> values for each compound and the statistical characteristics of the dose-response curves are summarized in Table 1. All of the compounds can be classified as highly to moderately toxic by conventional toxicological criteria. On the basis of these data, it was possible to estimate with reasonable accuracy a total dose level for each compound that would be tolerated without excessive mortality in the multiple-dose carcinogenesis evaluations.

The treatment regimen and results of the carcinogenesis experiments are shown in Table 2. Data presented summarize pathological lesions found in animals surviving for 50 weeks or longer. The negative control (trioctanoin) group showed a low incidence of hepatocellular carcinoma and hyperplasia characteristic of this strain. No other tumors or pathological changes were found. The positive control (diethylnitrosamine) groups also responded as expected from previous reports, showing a final incidence of nearly 100% of liver cell carcinomas in both sexes by 78 weeks, with the 1st tumors appearing within 1 year after treatment.

Nitrosoephedrine, nitrososarcosine, and nitrosofolic acid all elicited carcinogenic responses in this assay. The highest tumor incidence (nearly 100%) was induced by nitrosoephedrine administered at a total dose of 600 mg/kg body weight. In this case, hepatocellular carcinomas were the only tumors observed, and a significant proportion of them had metastasized to the lungs. Nitrososarcosine also in-

Table 1  
Lethal potency of test compounds following a single i.p. injection 24 hr after birth

| Compound           | LD <sub>50</sub><br>(mg/kg) | 95% confidence<br>limit | Slope<br>function |
|--------------------|-----------------------------|-------------------------|-------------------|
| Diethylnitrosamine | 42                          | 36-49                   | 1.35              |
| Nitrososarcosine   | 184                         | 166-204                 | 1.30              |
| Nitrosoephedrine   | 392                         | 347-443                 | 1.43              |
| Nitrosofolic acid  | 327                         | 255-419                 | 1.50              |
| Creatinine-5-oxime | 529                         | 487-575                 | 1.21              |

<sup>a</sup> The abbreviation used is: LD<sub>50</sub>, dose lethal to 50% of the animals.

Table 2  
*Tumors and other pathological lesions in mice surviving from 50 to 85 weeks after dosing*

| Treatment  | Survivors |     |       | Termination (wk) | Pathological Lesions   | Incidence <sup>a</sup> | Earliest tumor (wk) |
|--|-----------|-----|-------|------------------|------------------------|------------------------|---------------------|
|  | Sex       | No. | At Wk |                  |                        |                        |                     |
| Trioctanoin (5 ml/kg) given 3 times                                    | M         | 14  | 78    | 84               | Liver cell hyperplasia | 2/14                   | 85                  |
|  | F         | 9   | 79    | 85               | Liver cell carcinoma   | 1/12                   |                     |
| Diethylnitrosamine (3 mg/kg) given 4 times                             | M         | 12  | 50    | 64               | Liver cell carcinoma   | 11/12                  | 50                  |
|  | F         | 9   | 70    | 79               | Liver cell carcinoma   | 12/13 (4) <sup>b</sup> | 52                  |
| Nitrososarcosine (75 mg/kg) given 3 times                              | M         | 12  | 78    | 78               | Cholangio carcinoma    | 1/13                   | 74                  |
|  |           |     |       |                  | Lung adenocarcinoma    | 1/13                   | 78                  |
|  |           |     |       |                  | Liver cell carcinoma   | 8/12                   | 73                  |
|  |           |     |       |                  | Liver cell hyperplasia | 3/12                   |                     |
| Nitrosoephedrine (200 mg/kg) given 3 times                             | M         | 11  | 64    | 72               | Liver cell carcinoma   | 15/15 (1)              | 50                  |
|  | F         | 13  | 78    | 78               | Liver cell carcinoma   | 13/15 (8)              | 78                  |
| <i>N</i> <sup>10</sup> -Nitrososulfonic acid (125 mg/kg) given 3 times | M         | 12  | 79    | 85               | Lung adenocarcinoma    | 3/14                   | 79                  |
|  |           |     |       |                  | Liver cell carcinoma   | 1/14                   | 85                  |
|  |           |     |       |                  | Neurofibrosarcoma      | 1/14                   | 79                  |
|  | F         | 13  | 79    | 85               | Lung adenocarcinoma    | 1/15                   | 84                  |
|  |           |     |       |                  | Liver cell hyperplasia | 1/15                   |                     |
|  |           |     |       |                  | Liver cell carcinoma   | 1/10                   | 85                  |
| Creatinine-5-oxime (300 mg/kg) given 3 times                           | M         | 8   | 79    | 85               | Liver cell hyperplasia | 3/10                   |                     |
|  | F         | 13  | 78    | 83               | No lesions             | 0/15                   |                     |

<sup>a</sup> In animals surviving 50 or more weeks.

<sup>b</sup> Numbers in parentheses, animals with pulmonary metastases.

duced only liver cell carcinomas (and hyperplasia as well). Tumors were found only in males and occurred at smaller incidence than the response to nitrosoephedrine. However, this apparent difference in potency may be in large part attributable to the higher toxicity of nitrososarcosine and the consequently lower dose used in the carcinogenesis study.

Responses to nitrososulfonic acid were qualitatively and quantitatively different from those induced by the other 2 compounds. Whereas liver carcinoma was induced in only 1 animal, lung adenocarcinomas were produced in 4 mice and a neurofibrosarcoma in 1 additional animal. The latter tumor types were not observed in any other treatment groups, nor have they been reported to occur spontaneously in this mouse strain.

Creatinine-5-oxime showed no evidence of carcinogenicity in this assay. The only abnormalities appearing in animals treated with it were liver lesions comparable to those found in the negative control group.

## DISCUSSION

Information previously available on the biological properties of the compounds studied in this investigation is very limited. With respect to toxicity, the LD<sub>50</sub> values reported here fall in the lower portion of the wide range of comparable values previously determined for the *N*-nitroso compounds as a class in various animal systems. This range extends from 18 mg/kg for benzylmethyl nitrosamine to more than 7500 mg/kg for ethyl-2-hydroxyethyl nitrosamine (8).

Nitrosoephedrine at a dose of 600 mg/kg induced the highest incidence of tumors among the test substances; liver cell carcinomas appeared in animals treated with it at an

incidence approximately the same as that of the positive controls treated with diethylnitrosamine at a dose of 12 mg/kg. A high proportion of these tumors had metastasized to the lungs. Although nitrosoephedrine has not been studied previously in carcinogenesis bioassays, methyl-(phenylethyl)nitrosamine, a structurally related compound, had earlier been shown to be an esophageal carcinogen for rats (3). Carcinomas or papillomas were induced by daily intakes of 0.5 to 2.0 g/kg body weight.

In this study, nitrososarcosine also produced an elevated incidence of liver cell carcinomas. Its carcinogenicity had already been indicated by 1 earlier investigation in which it was shown to induce esophageal carcinomas in rats (3). The compound has a very low toxicity in rats (LD<sub>50</sub> > 5000 mg/kg), and the animals tolerated a daily intake of 200 mg/kg to a total dose of 57 g/kg. A small incidence of esophageal carcinomas was induced by that level. Comparable results were also produced by the ethyl ester of nitrososarcosine, a finding that led to the conclusion (3) that sarcosine derivatives are organ-specific carcinogens for rat esophagus. No evidence of esophageal lesions was seen in the mouse bioassay used in these experiments.

Results obtained with nitrososulfonic acid are less decisive. Whereas the compound produced no elevation of liver cell carcinoma incidence, lung adenocarcinomas were found in 4 of 28 animals. These tumors were not observed in other treatment or control groups. The results are therefore suggestive of a carcinogenic effect, but they will require verification and extension before a firm conclusion can be drawn. The compound has not been investigated in any other assay system.

The mouse bioassay model used in these experiments was selected because of its established sensitivity to several carcinogens and the requirement for only small amounts of

test compounds, a factor of great importance for some synthetic materials. The animal model is particularly sensitive to liver carcinogenesis. Hepatocellular carcinomas have been induced by benzidine, by diethylnitrosamine and benzo(a)pyrene (4), and by aflatoxin B<sub>1</sub> (12). A broad spectrum of tumor types is induced by ethylnitrosourea (13). The assay system is therefore particularly useful for initial screening studies; but due to its limited previous application to a wider variety of carcinogens, certain results of this study will require confirmation in other bioassay systems. Appropriate experiments are in progress.

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