[CANCER RESEARCH 42, 54-58, January 1982]

Comparative Metabolism of 2,6-Dimethylnitrosomorpholine in Rats, Hamsters, and Guinea Pigs[1]

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ABSTRACT

The metabolism of *N*-[3,5-^{3}H]nitroso-2,6-dimethylmorpholine (NDMM) was studied in female Sprague-Dawley rats, Syrian golden hamsters, and guinea pigs. NDMM induces tumors in the esophagus in rats, pancreatic cancer in hamsters, and hemangioendothelial tumors of the liver in guinea pigs.

An intragastric dose of NDMM (2 mg, 2 μCi/animal) was rapidly distributed throughout the tissues of both the rat and hamster, with no apparent accumulation of radioactivity in any one tissue. At low dose levels, NDMM was metabolized rapidly by both species. The hamster appeared to metabolize the compound faster than did the rat or guinea pig.

An appreciable amount of radioactivity was excreted in the urine in all three species after 8 hr: approximately 54% in the hamster; 39% in the rat; and 30% in the guinea pig. During the first 24 hr, only a small percentage of the radioactivity excreted by the hamster, rat, and guinea pig was NDMM (0.8, 2, and 0.5%, respectively).

High-pressure liquid chromatography analysis of urine collected 24 hr after administration revealed 12 metabolites. Although the urinary metabolites appeared to be similar in all three species, one large difference was the presence of a major urinary metabolite in hamster urine, which was absent or present in only small quantities in the rat and guinea pig. The guinea pig urine also had relatively more radioactivity present in one major fraction than did the hamster or rat.

INTRODUCTION

The cyclic *N*-nitrosamine NDMM[3] is one of the few carcinogens which induces pancreatic cancer in an experimental animal, namely the Syrian golden hamster (13, 17, 18). When given to Sprague-Dawley (10) and Fischer rats (7, 9) this compound induces mostly esophageal tumors, while in guinea pigs the induction of hemangioendothelial tumors of the liver has been reported (1, 8, 16).

An understanding of the underlying cause of such species specificity may well explain our knowledge of the mechanisms whereby tumors are induced by chemicals. Since NDMM is carcinogenic in all 3 species, it cannot be supposed that there is a failure to metabolize and activate the carcinogen, as occurs with other carcinogens (such as polynuclear hydrocarbons and aromatic amines, which are not carcinogenic in guinea pigs). Instead, it seems that the different responses of the 3 species to the carcinogenic action of NDMM might be due to differences in the way the compound is metabolized in the 3 species or to differences in activation of the carcinogen in the affected organs (esophagus, pancreas, and liver).

We have undertaken a study of the metabolism of NDMM in rats, Syrian hamsters, and guinea pigs, using tritium-labeled NDMM. In view of earlier findings that cyclic nitrosamines are metabolized to a relatively greater extent at low doses (22), all of our experiments were conducted with doses similar to those which gave rise to tumors after repeated administration. Initially, we have examined the urinary metabolites of NDMM in the 3 species, seeking differences that might reflect variations in metabolic pathways related to carcinogenesis.

MATERIALS AND METHODS

Chemicals. NDMM was prepared as previously described (10) and labeled with tritium by exchange with tritiated water in the presence of base. Analogous experiments with deuterium showed that the isotope was incorporated only in positions 3 and 5, *i.e.*, positions α to the nitroso function (3, 12). Labile tritium was removed and the radiochemical purity of the product was determined by HPLC (20, 21). HPOP was a gift from Dr. R. Gingell, Eppley Institute for Research in Cancer, Omaha, Neb. BHP was prepared as described previously (11).

Animals. Female Sprague-Dawley rats, Syrian golden hamsters, and guinea pigs, 8 weeks old and approximately 200, 100, and 470 g, respectively, were maintained in plastic metabolism cages without food but were given water *ad libitum*.

Distribution Studies. After 18 hr of food deprivation, the rats and hamsters were given 2 mg (2 μCi) [^{3}H]NDMM per animal in corn oil by gavage. Urine samples were collected and frozen. At various times, groups of 3 animals were sacrificed by asphyxiation.

After sacrifice, blood was collected from the thoracic vessels and mixed with 1 ml of 1% sodium citrate solution in a weighed vial. The lungs, liver, kidney, pancreas, spleen, esophagus, and squamous portion of the stomach were removed rapidly, washed in 250 ml of cold deionized water, blotted dry, weighed, and frozen. The frozen tissues were homogenized in approximately 10 volumes of 1% sodium carbonate and 0.001 M EDTA in a motor-driven Potter-Elvehjem homogenizer. The homogenates were weighed, and duplicate samples of approximately 150 mg were weighed into scintillation vials. The aliquots were incubated for 1 hr at 50° with 2 ml of Soluene 350 tissue solubilizer (Packard Instrument Co., Inc., Downers Grove, Ill.). The radioactivity of the digested homogenates was determined by adding 10 ml of Dimilume 30 scintillation fluid (Packard) to each vial and counting in a Packard liquid scintillation counter. Appropriate corrections for quenching were obtained by internal standardization with tritiated water.

The remainder of the tissue homogenates, urine, and blood samples were extracted with an equal volume of methylene chloride and centrifuged at 8000 × *g* for 15 min, and an aliquot of the methylene chloride layer was assayed for radioactivity.

Urinary Analysis. After 18 hr of food deprivation, groups of 2 rats, hamsters, and guinea pigs were given by gavage 12 mg (12 μCi) of [^{3}H]NDMM per kg of body weight. At 8 and 24 hr after treatment, the urine samples were collected and analyzed separately. Sodium chloride was added to the urine samples to a final concentration of 10 mM;

[1] This work was supported by Contract NO1-CO-75380, with the National Cancer Institute, NIH, Bethesda, Md. 20205.

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[3] The abbreviations used are: NDMM, *N*-nitroso-2,6-dimethylmorpholine; HPLC, high-pressure liquid chromatography; HPOP, *N*-nitroso-*N*-(2-hydroxypropyl)-*N*-(2-oxopropyl)amine; BHP, *N*-nitrosobis(2-hydroxypropyl)amine; BOP, *N*-nitrosobis(2-oxopropyl)amine.

Received April 21, 1981; accepted September 28, 1981.

the urine was precipitated with 3 volumes of methanol at 0° and centrifuged at 8000 × *g* for 15 min. The pellet was washed in methanol: water (3:1) and centrifuged, and the combined supernatants were concentrated under nitrogen at 0°. Recovery of urinary radioactivity in the methanol supernatants was 90 to 95%. Aliquots of 100 to 150 μl, containing approximately 40,000 cpm, were applied to a Dupont Zorbax-ODS column developed at 1.5 ml/min according to the following program: 0 to 5 min, with 0.01 M sodium acetate buffer (pH 5.6); 5 to 6 min, 0 to 10% methanol in acetate buffer; 6 to 17 min, 10% methanol in acetate buffer; 17 to 20 min, 10 to 60% methanol in acetate buffer; and 20 to 27 min, 60% methanol in acetate buffer. Fractions were collected at 10-sec intervals, and the radioactivity was determined by counting in P.C.S. (Amersham/Searle Corp., Arlington Heights, Ill.) scintillation fluid. Known samples of NDMM, HPOP, and BHP were chromatographed by HPLC and detected at 254 nm, using the same elution program (Chart 1).

Isolation of Metabolites. Urine samples from 10 hamsters receiving 8 mg, (8 μCi) of [^{3}H]NDMM were collected after 24 hr. The hamster urines were precipitated with 3 volumes of methanol at 0° and centrifuged at 10,000 × *g* for 10 min. The methanol supernatant was evaporated and reprecipitated 3 times. Metabolites were isolated as previously described (6). Solutions of Metabolite A at pH 1.0 were extracted with ethyl acetate. The extracts were blown to dryness and then taken up in methanol. The solution was treated with diazomethane to form the methyl ester of the acid. Mass spectral analysis of Metabolite A was obtained on a VG Micromass ZAB-2F mass spectrometer equipped with a VG 2035 data system (VG Micromass, Cheshire, England). Samples were admitted to the mass spectrometer through a Perkin-Elmer Sigma 3 gas chromatograph containing a Tenax G/C (Altech Associates, Deerfield, Ill.) column (6 ft x 2 mm). Similar conditions are being used for the isolation of other metabolites.

RESULTS

The distribution of radioactivity in tissue, blood, and urine from rats and hamsters at various times after administration of a single dose of [^{3}H]NDMM is shown in Table 1. After intragastric administration, radioactivity was detected in all tissues examined. The variation between tissues within a species was quite small, suggesting that there was no large concentration of the compound or its metabolites in any one site which could explain its organotropy. However, there could be specific reactions within the target organs which might be responsible for the induction of neoplasia. The blood and the urine generally contained the highest proportion of the administered radioactivity. The liver consistently had the highest proportion of total radioactivity among the tissue homogenates, although the liver is not a target organ in the rat.

[^{3}H]NDMM is fully extractable from aqueous media with methylene chloride. The amount of remaining unmetabolized starting material and nonpolar metabolites in the tissues, blood, and urine extractable with methylene chloride is shown in Table 2. At all times, the rat tissues had a significantly greater proportion of radioactivity which was methylene chloride extractable in the tissue homogenates, blood, and urine than did hamster tissues.

The nitrosamine was metabolized rapidly by both rats and hamsters, since only a small amount of radioactivity remained after 24 hr. Previous studies have indicated that the proportion of a cyclic nitrosamine converted to water-extractable compounds can be used as a measure of its metabolism (6). Therefore, our results indicate that the hamster metabolizes [^{3}H]NDMM at a faster rate than does the rat, especially since the hamster received a larger dose per unit body weight than did the rat in these experiments (2 mg/animal).

An examination of the pattern of urinary metabolites excreted in the urine in the first 8 hr showed many similarities, but there were some differences among the species (Chart 1). In the urine of each, a small amount of unchanged NDMM remained after 8 hr, with the largest proportion found in rat urine. Metabolites with similar retention times were excreted in all 3 species, but the proportions varied. For example (Chart 1), Fraction B represented a much larger proportion of the urinary metabolites of the guinea pig than of the rat or hamster, whereas Fraction C was prominent in the hamster urine but almost absent from urine of the other 2 species.

The pattern of excretion of NDMM metabolites by the 3 species between 8 and 24 hr did not show any very large differences from the pattern during the first 8 hr.

Gingell *et al.* (4) identified HPOP and BHP as minor urinary metabolites in Syrian golden hamsters after administration of NDMM and BOP. Metabolites I, J, and L cochromatographed with BHP, HPOP, and NDMM, respectively. None of the urinary metabolites of NDMM in rats, hamsters, or guinea pigs coincided in retention time with BOP, which does not seem to be a metabolite excreted in the urine; it is not likely that a tritium label α to the nitroso function could be lost from this compound by exchange at neutral pH. Known samples of BHP and HPOP occur as 2 isomers which are separated by HPLC. The combined isomers of BHP and HPOP did not exceed, respectively,

Table 1

Percentage of radioactivity in rat and hamster tissues after [^{3}H]NDMM administration in tissues, blood, urine, and feces

Each animal received, by gavage, 2 mg (2 μCi) [3,5-$^3H_2$]NDMM in corn oil. Values usually represent the mean of 3 animals.

Time (hr)	Blood	Liver	Kidney	Lung	Spleen	Esophagus	Pancreas	Urine	Feces
Hamster									
1	7.4	6.6	1.0	0.5	0.1	1.6	0.5	3.6	
2	19	11	2.0	1.0	0.2	2.0	0.8	34	
5	17	11	2.1	1.0	0.2	3.2	0.7	65	
24	8.0	5.2	1.2	0.5	0.1	0.3	0.3	70	
48	6.7	4.4	0.8	0.5	0.1	0.2	0.2	66	0.4
Rat									
1	14	11	2.5	1.2	0.8	2.0	1.0	1.5	
2	16	12	2.7	2.1	0.8	3.0	1.0	2.8	
5	14	12	2.5	2.2	0.8	0.8	0.9	16	
24	7.7	9.0	1.5	1.4	0.4	0.4	0.5	47	
48	6.3	5.5	1.4	0.9	0.4	0.3	0.4	62	

Table 2

Proportion of methylene chloride-extractable radioactivity in tissue homogenates, urine, and blood

Methylene chloride extracts contain unmetabolized [^{3}H]NDMM and nonpolar metabolites. Values represent the mean of 3 animals.

Time (hr)	Blood	Liver	Lungs	Pancreas	Kidney	Spleen	Esophagus	Urine
Hamster								
1	13	5.2	9.0	21	14	21	40	4.0
2	3.9	2.6	3.7	13	3.5	8.7	35	2.6
5	3.3	0.8	0.6	1.6	0.9	5.0	27	2.2
24	0	0	0	0	0	0	0	1.7
Rat								
1	42	16	29	36	24	48	59	19
2	32	23	37	30	37	49	30	8.0
5	29	8.0	22	23	22	24	22	9.0
24	0.12	0.03	0.08	0.3	0.12	0.34	0.21	6.6

10 and 6% of the urinary metabolites excreted by any of the 3 species. The proportions did vary, however, from one species to another (Table 3). The hamster excreted more BHP (approximately 10%) after 24 hr than did either the rat or guinea pig (approximately 4%). The amounts of BHP we found in the hamster urine 24 hr after treatment were more than those reported by Gingell *et al.* (4).

We have not yet identified completely any of the urinary metabolites of NDMM, other than BHP and HPOP.

Metabolite A was extractable with ethyl acetate at pH 1.0. This compound was not volatile but could be converted to a volatile product by methylation with diazomethane, suggesting that it is an acid. The mass spectrum of this methylated product is shown in Chart 2.

DISCUSSION

There were obvious differences in the pharmacokinetics of NDMM among the 3 species studied. The doses given provided information about carcinogenesis by NDMM, since they were similar to the levels which give rise to tumors after repeated administration (1, 10, 18).

There did not seem to be any particular concentration of NDMM or its metabolites in any one organ which could explain the specificity of the compound for the esophagus in the rat and the liver and pancreas in the hamster. However, further studies using electron microscopic autoradiography have shown that the radioactivity from NDMM is localized in the acinar and ductal cells of the hamster pancreas, while other cells of the organ are largely unlabeled (19).

In all 3 species, a relatively large proportion of the radioactivity appeared in nonvolatile products in the urine. Virtually none of the radioactivity in any of the fractions was lost on evaporation to dryness, showing that the radioactivity was not tritiated water or other volatile compounds. The high recovery of tritium in clearly separated and reproducible products also precluded the possibility that extensive exchange of tritium occurs in the course of the experiments, either in the animals or during isolation of the urinary products.

A comparison of the proportion of administered radioactivity recovered in the urine showed that metabolism in the hamster is much more rapid than in the rat or guinea pig. Almost 50% of the dose appeared in the urine of the hamster during the first 8 hr, compared with approximately 30% in the rat or guinea pig.

Hamster liver S9 fractions and intact hepatocytes are much more effective than rat liver preparations in activating many nitrosamines to mutagenic forms (15). Although the rate of metabolism may be an important factor in both carcinogenesis and mutagenesis, this does not explain the organotropy of NDMM.

In all 3 species, the metabolism of NDMM appears to be almost complete, since very little of the parent compound was found in the urine. Gingell *et al.* (4) failed to detect NDMM in the 24-hr urine of hamsters treated with this compound.

An examination of the metabolites in the urine showed that all 3 species convert NDMM to some extent into BHP and HPOP. With the exception of the relatively large proportion of BHP in the hamster urine, the amounts of these 2 metabolites formed were similar. Presumably, as Gingell *et al.* (4) proposed, NDMM is oxidized at the β position, giving rise to the cyclic form of HPOP, which is then reduced to BHP. Perhaps β oxidation is a relevant metabolic pathway for the hamster in relation to carcinogenesis, since the hamster did convert a larger amount of NDMM to BHP than did either the rat or the guinea pig. However, the minor differences in the excretion of HPOP in the urine do not explain the difference in tumor response among the rat, hamster, and guinea pig or support the concept that HPOP is the "proximate" pancreatic carcinogenic metabolite of NDMM in the hamster (14).

It was recently shown (14) that HPOP is carcinogenic in hamsters, giving rise to pancreatic tumors. It is possible that HPOP is a more potent carcinogen in the hamster than are BHP, BOP, and NDMM, but work of ours[4] indicates that, in rats, HPOP is a more potent carcinogen than is BHP but is not more potent than NDMM. It is unlikely that HPOP is the proximate carcinogenic form of NDMM in the rat, since the former gives rise to both esophageal and liver tumors. Furthermore, differences in the activation of NDMM between rats and hamsters were indicated, since substitution of deuterium for hydrogen in the positions (positions 3 and 5) α to the nitroso function decreases carcinogenic activity in the rat (9) but increases carcinogenic activity in the hamster (17). Similarly, in the rat, the *cis* isomer of NDMM is a considerably weaker carcinogen than is the *trans* isomer (7), while the *trans* isomer appears to be less carcinogenic than is the *cis* isomer in the hamster (17) and guinea pig (8). The urinary metabolites from animals treated with the 2 separate isomers differ quantitatively in all 3 species.[5]

Fraction C, a major metabolite appearing in hamster urine, was virtually absent from the urine of the rat and guinea pig,

[4] B. Underwood and W. Lijinsky, unpublished data.

[5] B. Underwood, unpublished data.

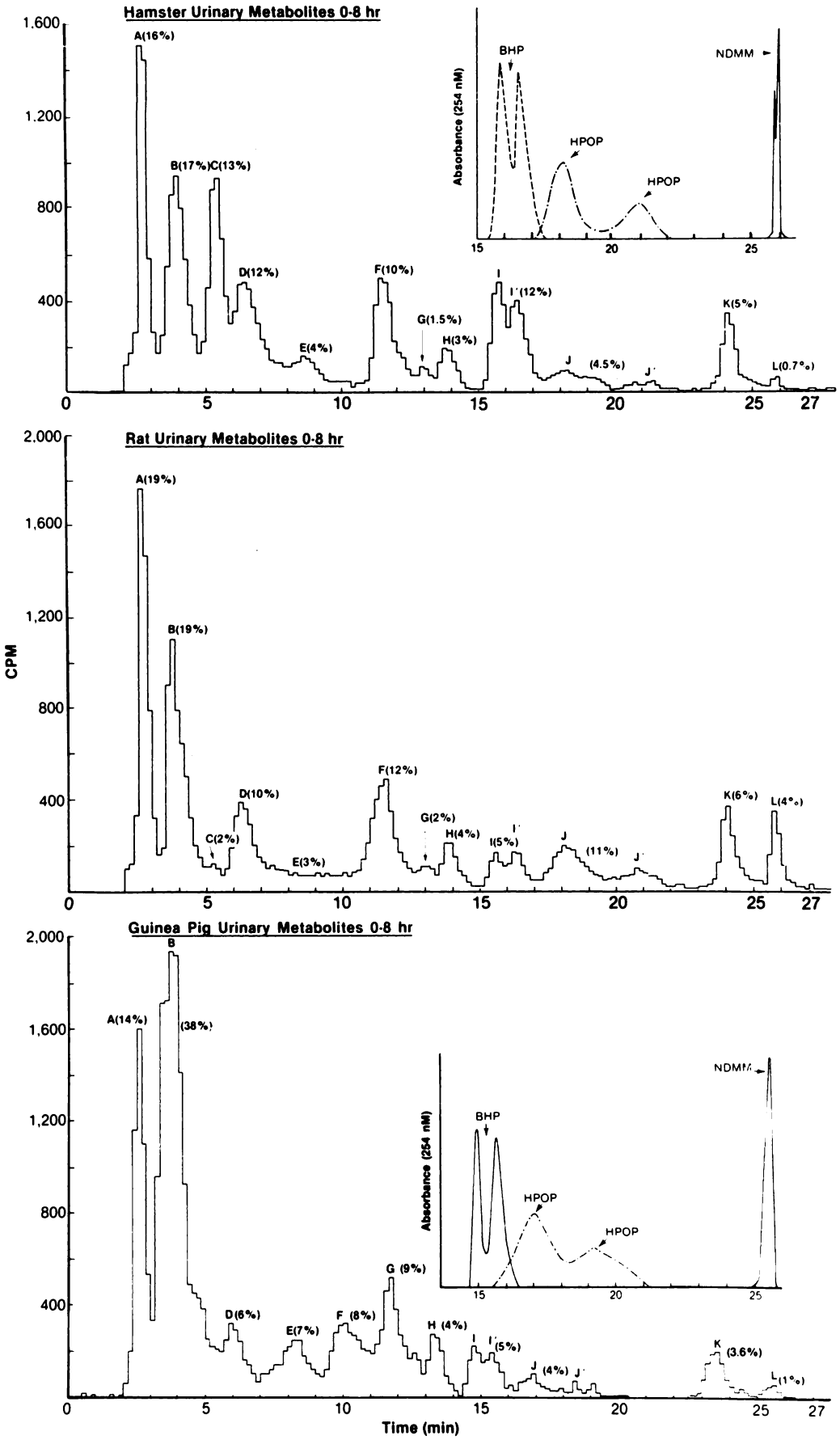

Chart 1. HPLC chromatograms of hamster, rat, and guinea pig 0- to 8-hr urinary metabolites. Aliquots of approximately 40,000 cpm were applied to a Dupont Zorbax-ODS column and eluted for 0 to 5 min with 0.01 M sodium acetate buffer (pH 5.6); 5 to 6 min with 0 to 10% methanol in acetate buffer; 6 to 17 min with 10% methanol in acetate buffer; 17 to 20 min with 10 to 60% methanol in acetate buffer; and 20 to 27 min with 60% methanol in acetate buffer at 1.5 ml/min. The percentage of each metabolite represents the percentage of the total radioactivity eluted from HPCL. The hamster 0- to 8-hr urine was 54% of the total dose given; the rat, 39%; and the guinea pig, 32% of the dose.

Table 3

Percentage of total dose excreted as urinary metabolites 24 hr after administration

Summary of administered dose excreted as separate metabolites as established from HPLC. Values represent the mean of 2 animals.

Metabolite	Hamster	Rat	Guinea pig
A′	0.5	0.5	0
A	10	10	6.3
B	13	11	19
C	8.6	1.2	0
D	8.7	7.0	5.3
E	3.0	2.5	2.4
F	8.4	10	6.8
G	1.2	1.6	5.2
H	3.5	3.8	3.8
I	9.5	4.4	3.6
J	2.8	5.5	2.4
K	3.3	3.9	1.5
L	0.8	1.7	0.5

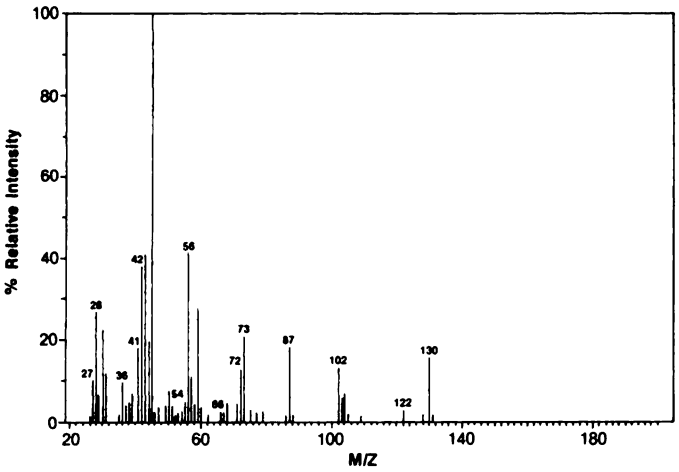

Chart 2. Mass spectrum of methyl ester of Metabolite A.

suggesting that it is produced by a metabolic pathway prominent in the hamster. The high-resolution mass spectrum of Metabolite A following treatment with diazomethane and gas chromatography at a fairly high temperature indicated a compound with a molecular formula of $C_6H_{10}O_3$. This would be the formula of 2,6-dimethyl-3-oxo-1,4-dioxane, the lactone of 2-hydroxy-1-methyl-2-ethoxypropionic acid (which would be derived from the acid or its ester), and is analogous to hydroxy acids found as metabolites of other cyclic nitrosamines. These include γ-hydroxybutyric acid from nitrosopyrrolidine (2, 6) and 2-hydroxyethoxyacetic acid from nitrosomorpholine (5). As in those cases, we believe that Metabolite A arises from α oxidation of NDMM, followed by ring opening and elimination of nitrogen.

ACKNOWLEDGMENTS

The authors thank Dr. J. E. Tomaszewski for the preparation of [^{3}H]NDMM and Dr. G. A. McClusky for the mass spectral information.

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