Metabolism of Three Radiolabeled Pancreatic Carcinogenic Nitrosamines in Hamsters and Rats

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ABSTRACT

The in vivo metabolism and disposition of three radiolabeled N-nitrosamines which are carcinogenic for the pancreas of the hamster but not the rat have been examined. N-[1-14C]Nitrosobis(2-oxopropyl)amine (BOP), N-[1-14C]Nitrosobis(2-hydroxypropyl)amine (BHP), and their suggested proximate pancreatic carcinogenic metabolite N-[1-14C]Nitroso(2-hydroxypropyl)(2-oxopropyl)amine (HPOP) were metabolized and exhaled as 14CO2 to various extents somewhat proportional to their carcinogenic potency. More than 50% of the dose of BOP and HPOP was exhaled as 14CO2, whereas 26% of BHP was excreted this way, and 40% of BHP was excreted unchanged in the urine. Administered BOP was excreted to a small extent in the urine of both species as HPOP and BHP. No other nitrosamine metabolites were detected in urine. HPOP and BHP were detected in the pancreatic juice and bile of both species after administration of BOP and BHP. The results suggest that pancreatic ductular carcinogenesis in the hamster as a result of exposure to BOP is not due to secretion of carcinogenic metabolites in the pancreatic juice or reflux of bile containing nitrosamine metabolites into the ducts. Carcinogen metabolic activation appears to be by an oxidative pathway.

INTRODUCTION

When Syrian golden hamsters were given BHP3 by s.c. injection once weekly for life, a high incidence of pancreatic ductular cell tumors was induced, as well as lung, liver, and kidney tumors (16). However, when rats were similarly treated with BHP, tumors occurred in the nasal cavity, lungs, esophagus, liver, and kidneys but not in the pancreas (9, 19). Compared to BOP, BHP was more toxic and induced more pancreatic ductular tumors, fewer liver and kidney tumors, and no lung tumors in hamsters (14). In the rat, the main target organs of BOP were the intestines, liver, and respiratory and urogenital tracts but not the pancreas (13). N-Nitroso-2,6-dimethylmorpholine was also a pancreatic carcinogen in the hamster (6) but not in the rat (8). BOP, BHP, and N-nitroso-2,6-dimethylmorpholine were metabolized in the hamster to HPOP, which can exist in open and cyclic forms, and HPOP has been suggested to be responsible for the pancreatic carcinogenicity of these compounds (3, 4). The metabolic interrelationship of these compounds with the cyclic form of HPOP is illustrated in Chart 1. HPOP itself has a carcinogenic potency similar to that of BOP, further suggesting that it may be the proximate pancreatic carcinogenic metabolite in hamsters (17). The in vivo metabolism and disposition of BOP and BHP in the rat and hamster, especially with respect to HPOP formation, has been examined in an attempt to account for the species differences in organotropism of these compounds and to differentiate between proposed mechanisms of pancreatic carcinogenesis. In preliminary studies, only a relatively small percentage of the administered dose of BOP or BHP could be recovered in hamster urine (4). Consequently, the 14C-labeled compounds were synthesized to perform more detailed studies.

MATERIALS AND METHODS

Chemicals. [1-14C]BHP (0.238 μCi/mg) was synthesized (5) and was >97.5% pure by autoradiography after TLC (Table 1). Most of the radioactive impurity remained at the origin, but 0.5% was present as HPOP. [1-14C]BOP (0.19 μCi/mg) was prepared by chromic acid oxidation of [1-14C]-diisopropylamine followed by nitrosation (10). This was purified by preparative layer chromatography to >96% purity, the major impurity remaining at the origin. [1-14C]HPOP (specific activity, 0.19 μCi/mg) was a by-product in the preparation of [14C]BOP and was purified to >97% as above. These compounds were stored frozen as aqueous solutions at −20° and did not decompose appreciably under these conditions, as determined by TLC followed by autoradiography.

Metabolism in Vivo. Male Syrian hamsters, 8 to 10 weeks old, weighing 100 to 120 g, or 9- to 10-week-old male Wistar-derived rats weighing about 200 g, received i.p. injections of [14C]BHP, [14C]BOP, or [14C]HPOP (10 or 100 mg/kg). These doses were similar to those in the carcinogenicity studies and were based on toxicity, as determined by the 50% lethal dose (14, 16). The animals were maintained in sealed glass metabolism cages (Crown Scientific, Riverdale, Ill.) without food, but with water ad libitum. Any volatile metabolites were trapped by first bubbling expired air through 0.1 N HCl or a dry ice/ethanol trap. 14CO2 was trapped by bubbling through a solution of ethanolamine/methanol (1:1), and it was measured at 1, 3, 6, 12 and 24 hr by scintillation counting in Aquasol (New England Nuclear, Boston, Mass.) using a Beckman LS-335 liquid scintillation system (Beckman Instruments, Fullerton, Calif.). Twenty-four-hr urine was collected separately from feces and frozen over dry ice. Aliquots of urine were counted in Aquasol after decolorization with several drops of 30% aqueous hydrogen peroxide.

Modification of Metabolism. The effect of several known modifiers of nitrosamine metabolism on CO2 production from [14C]BOP and [14C]BHP was examined. Hamsters were maintained for 1 week on drinking water containing sodium phe-
nobarbital (1 mg/ml) prior to [¹⁴C]BHP treatment. Pyrazole tautomerization. NDMM, N-nitroso-2,6-dimethylmorpholine. carcinogenic nitrosamines. All steps are metabolic except a, which is a chemical reaction with a heating pad. Aliquots of bile were counted, after decol-olite identification was confirmed by gas-liquid chromatography. Radioactivity was determined by scintillation counting. Metabolites were quantitated by scraping from the TLC plates and adding 1 ml methanol, followed by scintillation counting as before. After filtration, the extracts were concentrated by rotary evaporation at 40°C, and the above process was repeated, if necessary, prior to concentration to a small volume under a N₂ stream for TLC. Silica TLC plates were developed in comparison with authentic standards (Table 1). Autoradiography was performed by exposing TLC plates to Kodak SB54 X-ray film. Metabolites were quantitated by scraping from the TLC plates and adding 1 ml methanol, followed by scintillation counting as before. In other experiments, urine from treated animals was extracted with 3 × 5 volumes methylene chloride and evaporated to a small volume under a stream of N₂, and metabolites were identified by HPLC as detailed in Table 1. Fractions were collected into vials as they eluted from the HPLC detector, and radioactivity was determined by scintillation counting. Metabolite identification was confirmed by gas-liquid chromatography (Table 1).

RESULTS

BHP Metabolism. When hamsters were given i.p. injections of [¹⁴C]BHP (100 mg/kg), approximately 74% of the radioactivity was excreted in the urine and 19% was excreted as CO₂ in 24 hr (Chart 2; Table 2). HPLC of methylene chloride extracts of urine revealed only HPOP and unchanged BHP as urinary metabolites (Chart 3A). TLC of ethanol-precipitated urine on silica gel developed in Solvent 2 and followed by autoradiography revealed that 41% of the administered BHP was excreted unchanged, 1% as HPOP, and an unknown metabolite accounted for 8%. The remainder of the excreted dose (about 20%) stayed at the origin. The urine from hamsters given an equivalent dose of [¹⁴C]bicarbonate also contained this unknown metabolite, suggesting that it was due to 1-carbon-pool intermediary metabolism. Urea has a similar Rf in this solvent, but when the urine was treated with urease (Sigma, St. Louis, Mo.) before preparation for chromatography, the band did not disappear. We were unable to modify TLC profiles by treatment of urine for 1 hr with 1 N HCl, 1 N NaOH, or 5000 units of β-galactosidase at 37°C, indicating that no conjugates were present.

Rats given the same dose of [¹⁴C]BHP excreted 73% in the urine and 4% of the dose as CO₂. The rate of BHP metabolism to CO₂ (Chart 2) was less in the rat than in the hamster. Profiles of rat urine metabolites, however, showed no significant differences from those of hamster urine.

BOP Metabolism. The quantitative recovery of radioactivity after administration of BOP (10 mg/kg) to hamsters and rats is given in Table 2, and the rates of metabolism to expired CO₂ are given in Chart 2. Hamsters given [¹⁴C]BOP excreted 12% of the dose in the urine and expired 67% as CO₂. TLC of hamster urine on silica gel plates developed in Solvent 3, followed by autoradiography, yielded a chromatographic profile.

Identification of Metabolites. Radioactive metabolites were recovered for chromatography from urine, bile, and pancreatic juice by adding 10 volumes of absolute ethanol and leaving the liquids to stand overnight at −20°C to allow precipitation of debris. We were unable to modify TLC profiles by treatment of urine for 1 hr with 1 N HCl, 1 N NaOH, or 5000 units of β-galactosidase at 37°C, indicating that no conjugates were present.

Chart 1. In vivo metabolic interrelationship of BOP and related pancreatic carcinogenic nitrosamines. All steps are metabolic except a, which is a chemical tautomerization. NDMM, N-nitroso-2,6-dimethylmorpholine.

Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>System 1</th>
<th>System 2</th>
<th>System 3</th>
<th>HPLC</th>
</tr>
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<tbody>
<tr>
<td>BOP</td>
<td>55</td>
<td>45</td>
<td>98</td>
<td>6.6</td>
</tr>
<tr>
<td>HPOP</td>
<td>42, 55</td>
<td>30, 55</td>
<td>94</td>
<td>10.9</td>
</tr>
<tr>
<td>BHP</td>
<td>23</td>
<td>23</td>
<td>83</td>
<td>23.8</td>
</tr>
</tbody>
</table>


5. GC was performed using a Hewlett-Packard 3820A gas chromatograph equipped with a 2-mm x 6-foot glass column packed with 5% SP2100 (Supelco) on 100 to 200 mesh Supelcoport, and flame ionization detector. The column oven was programmed from 80°C to 140°C with temperature raised at 4°C/min, and N₂ carrier gas flow rate was 20 ml/min.

6. These 2 values represent the cyclic (greater) and open forms of HPOP which are separated in these systems.

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with more than 10 distinct radioactive bands. Three metabolites have been identified as BOP, HPOP and BHP. One band matched the Rf of the band derived from bicarbonate metabolism, as for BHP, and is also probably due to 1-carbon-pool intermediates. Less than 1% of the dose was recovered in the feces; this may have been due to contamination with urine. Using the Preussmann spray reagent (18) under conditions which show BHP and HPOP as positive, no other bands gave a positive response, indicating that they are probably not nitrosamines. No other nitrosamine metabolites were detected by HPLC (Chart 3B).

Rats given [14C]BOP i.p. at 10 mg/kg excreted 23% of the dose in urine and expired 55% as CO2 (Table 2; Chart 2). Less than 1% was recovered in the feces. TLC profiles of the rat urine appeared quantitatively similar to those of the hamster; differences were only in the relative amounts of some of the bands.

HPOP Metabolism. The small quantity of [14C]HPOP available permitted only limited metabolic studies. HPOP was rapidly and extensively metabolized to CO2 in the hamster (Chart 2; Table 2) and excreted to a small extent unchanged and as BHP in the urine. Chromatographic profiles of urine extracts were similar to those obtained for BOP, except no BOP was detected.

Inhibition of Metabolism. None of the pretreatments with phenobarbital, pyrazole, disulfiram ethanol, or diethylformamide had any significant effect on rates of CO2 production from [14C]BOP or [14C]BHP.

Biliary and Pancreatic Secretions. The amounts of 14C secreted in the bile and pancreatic juice of hamsters and rats that received 10-mg/kg i.p. injections of [14C]BOP are given in Table 3. Hamsters secreted 0.36% and rats secreted 0.67% of the radioactive dose in the pancreatic juice over a 24-hr period. In the bile, hamsters secreted 1.6% and rats secreted 4.4% in 24 hr. Most of the 14C in bile and pancreatic juice was removed from solution by the ethanol precipitation method or stayed at the origin on TLC, implying that it was mostly present in bile acids and proteins as a result of 1-carbon pool incorporation. Of the administered dose to rats, 0.2% was accounted for as HPOP and 0.05% as BHP in the bile, and 0.1 and 0.02%, respectively, in the pancreatic juice. Similar trace amounts of HPOP and BHP were also detected in hamster pancreatic juice and bile.

**DISCUSSION**

The synthesis of 3 14C-labeled nitrosamines has allowed more detailed metabolic studies of these pancreatic ductular carcinogens. Previously, when only solvent extraction and gas-liquid chromatography were used for detection, less than 25% of the administered 100-mg/kg dose of BHP and HPOP and less than 10% of the same dose of BOP were recovered as BHP and HPOP in the urine (4). Using 14C-labeled compounds, metabolism studies have been possible at the 10-mg/kg carcinogenic dose of BOP and HPOP.

HPOP has been proposed to be the pancreatic carcinogenic metabolite of BOP and BHP in hamsters, since it is a common metabolite of both (4). HPOP has a carcinogenic potency, with respect to the hamster pancreas, similar to that of BOP (17). The rat, which does not develop pancreatic tumors, also is able to metabolize BOP to HPOP. Thus, this resistance of the rat is not due to a species difference in metabolic activity.

The hamster pancreatic ductular cells may be exposed to these carcinogenic nitrosamines, and thus induce tumors, either systemically by secretion in the pancreatic juice or by biliary reflux into the pancreatic duct, a postulated mechanism for human disease (23). Species differences in the nature of metabolites in bile or pancreatic juice might be expected between the rat and hamster if one of the latter 2 mechanisms is important in pancreatic carcinogenesis.

However, trace amounts of HPOP and BHP were detected in bile and pancreatic juice of both species treated with [14C]BOP, and no major differences could be attributed to pancreatic carcinogenesis. Thus, neither reflux of bile containing carcinogenic metabolites into the pancreatic duct nor direct secretion in the pancreatic juice is likely to be a mechanism of pancreatic ductal carcinogenesis in this animal model, which is probably due to systemic exposure to the metabolite HPOP. This result has been previously suggested by carcinogenesis.

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**Table 2** In vivo metabolism of [14C]nitrosamines

<table>
<thead>
<tr>
<th>Species</th>
<th>Compound</th>
<th>% of dose excreted as CO2 in urine</th>
<th>Urinary metabolite (% of dose)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hamster</td>
<td>14C-BOP</td>
<td>67 ± 7</td>
<td>12 ± 2</td>
</tr>
<tr>
<td></td>
<td>14C-HPOP</td>
<td>57 ± 6</td>
<td>24 ± 2</td>
</tr>
<tr>
<td></td>
<td>14C-BHP</td>
<td>26 ± 4</td>
<td>66 ± 6</td>
</tr>
<tr>
<td>Rat</td>
<td>14C-BOP</td>
<td>55 ± 7</td>
<td>23 ± 8</td>
</tr>
</tbody>
</table>

*a* Each compound was administered i.p. at 10 mg/kg, and urine and expired CO2 were collected for 24 hr. No compound was detected in expired air. Results are the mean ± S.D. for at least 3 animals. Amounts of specific metabolites are taken from pooled urines of 3 animals.

*b* Tr, trace.

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**Chart 2.** Metabolism of [14C]nitrosamines to 14CO2. The extent of metabolism of [14C]BOP, [14C]HPOP, and [14C]BHP to 14CO2 was determined at various times after i.p. administration. The 24-hr cumulative dose is the mean ± S.D. (bars) for at least 3 animals. ○ BOP (10 mg/kg) to hamster; □ HPOP (10 mg/kg) to hamster; △ BHP (10 mg/kg) to hamster; ☆ BOP (10 mg/kg) to rat; □ BOP (100 mg/kg) to hamster; △ BHP (10 mg/kg) to hamster; ▲ BHP (100 mg/kg) to hamster; ▽ BHP (100 mg/kg) to rat.

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**Table 3.** Hamsters secreted 0.36% and rats secreted 0.67% of the radioactive dose in the bile and pancreatic juice of hamsters and rats that received 10-mg/kg i.p. injections of [14C]BOP or [14C]BHP.

<table>
<thead>
<tr>
<th>Species</th>
<th>Compound</th>
<th>% excreted in bile</th>
<th>% excreted in pancreatic juice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hamster</td>
<td>BOP</td>
<td>0.02%</td>
<td>0.05%</td>
</tr>
<tr>
<td></td>
<td>HPOP</td>
<td>0.1%</td>
<td>0.02%</td>
</tr>
<tr>
<td></td>
<td>BHP</td>
<td>0.1%</td>
<td>0.02%</td>
</tr>
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</table>
bioassays of BOP in surgically pretreated hamsters (15). No other nitrosamine metabolites that could be related to pancreatic carcinogenesis were detected in urine, pancreatic juice, or bile.

BOP induced colorectal tumors in rats (13), but secretion of BOP metabolites in bile does not seem to be the mechanism of their induction, since the rate and nature of biliary metabolite secretion was similar in rats and hamsters. Only trace amounts of $^{14}$C were detected in the feces of rats given BOP, indicating that colorectal tumors are also probably due to systemic nitrosamine exposure.

The major finding of these studies using $^{14}$C-labeled nitrosamines was that much of the administered dose was metabolized to exhaled CO$_2$. BOP and HPOP, the more toxic and potent carcinogens, were extensively metabolized to CO$_2$, and the less toxic and weaker carcinogen BHP was less readily metabolized to CO$_2$. This was true at both the 10 mg/kg and 100 mg/kg doses although, as noted by Snyder et al. (21), the extent of metabolism to CO$_2$ for each compound was dose dependent. The effect of various reported modifiers of nitrosamine metabolism on BHP and BOP disposition was investigated in an attempt to identify potential modifiers of carcinogenesis. Pyrazole had been reported to decrease the metabolism of DMN to CO$_2$ in rats and also protect the liver from the
toxic effects of DMN administration, due to a direct effect on DMN demethylase (11, 12). Diethylformamide was also reported to inhibit protein methylation and DMN metabolism to CO₂ in vivo (7). Disulfiram treatment markedly decreased the hepatocarcinogenicity of DMN in rats (20), presumably by inhibiting hepatic metabolism (22). However, in our studies, no significant decrease in the rate of metabolism of BOP or BHP was detected with these inhibitors. Possibly, these reported inhibitors of oxidative metabolism and toxicity of simple dialkyl nitrosamines have no effect on BOP and BHP metabolism because these are already partially oxidized.

N-Nitrosamines are currently thought to be metabolically activated to carcinogens by α-oxidation ultimately to give carbonium ions which react with DNA to initiate tumors (1). Other decomposition products of α-oxidized nitrosamines are aldehydes and alcohols, which may be metabolized in vivo via 1-carbon pool intermediates to CO₂. All 3 nitrosamines studied are labeled in the carbon atom α to the nitroso group. The fact that the rate of metabolism of these compounds to CO₂ correlated somewhat with their relative toxicity and carcinogenic potency suggests that α-oxidation is also a mechanism of their activation. This possibility is being examined in various target and nontarget organs using in vitro techniques.

ACKNOWLEDGMENTS

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REFERENCES


Table 3

<table>
<thead>
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<th>Compound</th>
<th>Species</th>
<th>Time (hr)</th>
<th>% of dose</th>
</tr>
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<tbody>
<tr>
<td>Pancreatic juice</td>
<td>Hamster</td>
<td>0–6</td>
<td>0.32 ± 0.06*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6–24</td>
<td>0.13 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>0–6</td>
<td>0.48 ± 0.33</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6–24</td>
<td>0.19 ± 0.02</td>
</tr>
<tr>
<td>Bile</td>
<td>Hamster</td>
<td>0–6</td>
<td>1.29 ± 0.20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6–24</td>
<td>0.31 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>0–6</td>
<td>3.68 ± 2.10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6–24</td>
<td>0.72 ± 0.12</td>
</tr>
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</table>

* Mean ± S.D. for 3 animals.

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