Effects of Partial Hepatectomy and Dietary Phenobarbital on Liver and Mammary Tumorigenesis by Two N-Hydroxy-N-acylaminobiphenyls in Female CD Rats

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ABSTRACT

The objective of this study was to investigate the induction of liver tumors by arylhydroxamic acids. The potential involvement of sulfate conjugation was minimized by the administration of a N-hydroxy-4-acylaminobiphenyl to female CD rats. This experimental design provided for the exposure of a target organ that has only a low capacity for activation of hydroxamic acids by sulfate conjugation, with a carcinogen that does not induce tumors in livers that possess a high sulfotransferase activity. A single dose of the N-formyl or N-acetyl derivatives of N-hydroxy-4-aminobiphenyl was given i.p. at 0.4 mmol/kg body weight to 34-day-old animals. In attempts to amplify the hepatocarcinogenic potential of the compounds, partial hepatectomy 24 hr before the chemical injection and subsequent long-term treatment with phenobarbital in the diet were carried out. For comparative purposes, other animals were subjected to three additional partial hepatectomies subsequent to the carcinogen administration instead of the phenobarbital treatment. The experiments were terminated 64 weeks after injection. Both the N-formyl and N-acetyl derivatives of N-hydroxy-4-aminobiphenyl, in conjunction with partial hepatectomy and subsequent treatment of dietary phenobarbital, induced a high incidence of neoplastic nodules and γ-glutamyltranspeptidase-positive foci in the liver. Only one hepato-cellular carcinoma was observed in each treatment group. Repeated partial hepatectomies enhanced the yield of γ-glutamyltranspeptidase-positive foci but were ineffective in producing neoplastic nodules. In addition to the liver lesions, mammary tumors were also induced. Importantly, an inhibitory effect of the subsequent administration of phenobarbital was observed on mammary tumor formation, possibly because of alterations in hormone metabolism resulting from the induction of microsomal enzymes by phenobarbital, which resulted in a decreased promoting effect. There was no difference in the tumorigenicity of the formyl and acetyl derivatives in these experiments.

INTRODUCTION

An initial N-oxidation is believed to be required for tumor induction by aromatic amines (3, 30). Experiments with N-hydroxy-AAF\(^4\) have demonstrated that further activation of this N-hydroxylated metabolite of N-2-acetylaminofluorene is catalyzed by a sulfotransferase to yield a sulfuric acid ester that is associated with both hepatotoxicity and hepatocarcinogenicity in rats (4, 8, 10). The resulting metabolite, the O-sulfate ester of N-hydroxy-AAF, reacts readily with cellular macromolecules (20). The low incidence of liver tumors in female Sprague-Dawley rats treated with N-hydroxy-AAF is believed to be due to low levels of hepatic sulfotransferase (8). This pathway is, however, apparently restricted to rat liver, because other target organs of carcinogenic aromatic amines have little or no capacity to conjugate arylhydroxamic acids with sulfate (4, 12).

An alternative mechanism for the metabolic activation of these compounds is by the formation of reactive N-acetoxyaromatic amines as a consequence of N,O-acetyltransfer (2, 17, 19). Enzymes capable of activating N-hydroxy-AAF by acetyltransfer have been demonstrated in a variety of tissues including the mammary glands of the rat as well as the liver from a number of species that are susceptible to the carcinogenic effects of aromatic amines (17, 23).

The carcinogenic potential of N-4-acylaminobiphenyl and its N-hydroxylated metabolite, N-hydroxy-AAF, has been demonstrated in the mammary gland and Zymbal gland of rats (32) and the liver of newborn male mice (7). These compounds have not been shown to be hepatocarcinogenic in the rat. The covalent binding of N-hydroxy-AAFP to nucleic acid and protein in isolated hepatocytes from rats is independent of sulfate conjugation; the structure of the adducts is compatible with activation via an acetyltransferase mechanism in that the N-acetoxy moiety of the hydroxamic acid has been lost (22). Similarly, the structure of 4-aminobiphenyl derivatives in nucleic acid adducts formed in vivo in rat liver is analogous with those from hepatocytes and is inconsistent with their formation as a consequence of the activation of hydroxamic acids by sulfte conjugation (27). These findings and previous work (1) support the conclusion that the metabolic activation of N-hydroxy-AAFP to electrophilic reactants can be accomplished through N,O-acetyltransfer and that sulfotransferase may not play an important role in the activation of aminobiphenyl derivatives.

The present experiments were undertaken to explore the possibility that aminobiphenyl derivatives were capable of initiating the hepatocarcinogenic process in rats but were unable to induce tumors, because they did not elicit a hepatotoxic response, possibly as a result of the relatively low reactivity of the sulfate conjugate of N-hydroxy-AAFP (4, 22). Thus, this compound, or its formyl analog, was administered to female CD rats, which have low sulfotransferase activities (10) and

\(\text{PB, phenobarbital; N-hydroxy-FABP, N-hydroxy-4-formylaminobiphenyl; GGT, }\gamma\text{-glutamyltranspeptidase.}\)
formyl derivative was included in this study to provide comparable acyltransferase levels that are comparable to males (18). The formyl derivative was included in this study to provide comparable data on the tumor induction ability of a structurally related arylhydroxamic acid that differs in its potential for metabolic activation (42). In an effort to amplify the possible initiating activity of the 4-aminobiphenyl derivatives, the compounds were administered 24 hr after PH and subsequently given PB. This protocol is basically the same as that used by Pitot et al. (35) by combining the procedure of Scherer and Emmelot (39) with that of Peraino et al. (34). The effectiveness of this combination procedure on the enhancement of hepatocarcinogenicity was also confirmed in a recent study by Miller et al. (31). In some animals, as an alternative to using PB as a tumor promoter, multiple PH were performed as described by Pound and McGuire (36).

MATERIALS AND METHODS

Chemicals. N-Hydroxy-AABP and N-hydroxy-FABP were synthesized, and their purity was established as described.6

Animals. Weanling female CD rats were purchased from the Charles River Breeding Laboratories, Inc. (Wilmington, Mass.), housed 3 or 4 per plastic cage on hardwood chips, and maintained at 24° on a 12-hr light-dark cycle. Animals were fed a commercial diet (Wayne Lab Blox; Allied Mills, Inc., Chicago, Ill.) prior to the experiment.

Determination of the Effect of PH on the Arylhydroxamic Acid A cyltransferase Activity of Rat Liver. Approximately 70% of the livers of 4 animals (average weight, 155 g) was removed by the procedure described by Higgins and Anderson (9). The acyltransferase activities of the resected liver at PH and the regenerating liver 24 hr after PH were determined by measuring the ability of 105,000 x g supernatants of tissue homogenates to introduce the N-2-fluorenylamine moiety of N-hydroxy-[9-14C]AAF into tRNA by N,O-acyltransfer as described previously (16). Duplicate assays were carried out.

Tumor Induction Experiments. The fundamental treatment of the animals in this study consisted of PH prior to administration of the carcinogen and subsequent treatment with PB or repeated PH. Appropriate control animals, that are described in Table 1, were treated so that the effects of these 4 procedures could be evaluated. Initially, there were 26 animals in Group 1, 24 in Group 13, and 20 in each of the other 13 groups.

The rats were 34 days old and weighed approximately 128 g at the start of the experiment. All animals were fed a pelleted antioxidant-free semisynthetic diet from which ethoxyquin had been omitted (AIN 76 purified diet; Teklad Test Diets, Madison, Wis.) and water ad libitum. PB (Sigma Chemical Co., St. Louis, Mo.) was incorporated into this diet at a concentration of 0.05% where indicated. The diet was stored at –20° to retard autoxidation. The animals were weighed weekly for the first 5 weeks and at monthly intervals thereafter.

PH was carried out under light anesthesia with ethyl ether. A single injection of N-hydroxy-AABP or N-hydroxy-FABP was given i.p. 23 to 24 hr post operation at a dose of 0.4 mmol per ml dimethyl sulfoxide per kg of body weight. Animals in the control groups were given injections of dimethyl sulfoxide alone. Two days after the injection, PB diet was started as indicated. All other animals received the PB-free diet.

Three groups of animals (Groups 13, 14, and 15) underwent 3 additional PH after the injection instead of treatment with PB. The PH were performed at 1-week intervals. The first PH consisted of the removal of the median and left lateral lobe. The right anterior lateral, caudal, and right posterior lateral lobes were removed at the second, third, and fourth PH, respectively. Silk sutures were used to ligate the liver. Stainless steel clamps were used to close the incisions. All rats were sacrificed 64 weeks after the beginning of the experiment and were subjected to routine autopsies which included inspection of mammary glands and Zymbal glands. Animals that died earlier or were moribund were also autopsied. The mammary tumors, mammary glands, and liver were fixed in 10% buffered formalin and then sectioned. The sections (4 to 5 μm) were stained with hematoxylin and eosin. Thick pieces (2 mm) of the livers were fixed in cold acetone (4°) at the time of sacrifice for the histochemical demonstration of GGT according to the methods described by Ogawa et al. (33). The number of GGT-positive foci was counted microscopically, and the area of the liver sections (approximately 1.5 to 3.0 sq cm per liver) was measured by an Omnicam Image Analyser 500 (Bausch and Lomb, Rochester, N.Y.) so that the number of GGT-positive foci could be expressed as the number of foci per sq cm. Statistical analysis of differences in the incidences of neoplastic nodules and the numbers of GGT-positive foci between groups were done by Fisher’s exact test and t test, respectively. Differences in mammary tumor incidence were compared by χ2 analysis.

RESULTS

Effects of PH on the Metabolic Activation of Arylhydroxamic Acids by A cyltransfer

The use of prior PH to enhance the biological effects of aromatic amine derivatives may not succeed if the procedure decreases the metabolic capacity of the liver to activate the carcinogen. While the choice of animal and compound circumvented both the requirement for N-oxidation and the involvement of the sulfate conjugation system, the experimental design did not preclude the possibility that PH could alter the level of the hepatic arylhydroxamic acid acyltransferase. This possibility was ruled out by experiments that showed cytosols prepared from liver at or 24 hr following PH had essentially equivalent acyltransferase activities, i.e., 3.68 ± 0.49 and 3.83 ± 0.43 nmol fluoropyridamino bound to tRNA/min/cytosol equivalent to 1 g liver, respectively.

Regeneration of the Liver following Repeated PH

In a preliminary experiment to establish the feasibility of subjecting rats to repeated PH, 22 female CD rats weighing approximately 145 g were subjected to this procedure 4 times at weekly intervals. The rate of regeneration of the liver 1 week after each PH was examined by comparison of the liver weights of 3 animals, represented as percentage of body weight to those of nonhepatectomized age-matched control animals. It was shown that the liver could be restored to approximately 100, 98, 96, and 99% of that of the control animals within 1
week after removal of approximately 70% at the first PH, 32% at the second, 22% at the third, and 21% at the fourth.

Tumor Induction Experiment

Toxicity and Mortality. Animals given injections of either of the hydroxamic acids showed transitory signs of methemoglobinemia from which they usually recovered overnight. During the first month of the experiment, the body weights of the animals that had been given injections of N-hydroxy-AABP were no more than 5% lower than were those from comparable treatment groups that received only the solvent.

The rats that were treated with N-hydroxy-FABP exhibited a slower growth than did solvent-treated controls. Those animals that had been subjected to PH (Table 1, Groups 5, 6, and 14) were the only ones to lose weight during the first week following the injections. While those treated with N-hydroxy-FABP without prior PH gained weight during this period, they weighed only as much as 89% of the comparably treated controls after 1 week. The average weight of all animals given the aminobiphenyl derivatives in Groups 1 through 9 essentially reached that of their controls in the first 3 weeks of the experiment.

Mortality in the rats that had been subjected to multiple PH was relatively high because of the combined effects of the repeated invasive procedures and the toxicity of the chemicals. One week after the fourth PH, the cumulative postoperative deaths were 25% in Groups 13 and 14 and 5% in Group 15. With the exception of an 18% weight loss 1 week after the injection of N-hydroxy-FABP in Group 14, the body weights of those subjected to repeated PH steadily increased during the operative period and reached those of the controls within the first 6 weeks of the experiment. The average weights of the liver removed at the second, third, and fourth PH were estimated to be 46, 35, and 43% of the regenerated livers, respectively. These figures were calculated from the amount of liver removed as compared with the expected normal liver weight, which was assumed to be 4.3% of the body weight at this age as judged from the preliminary experiment described above.

Liver Lesions. The effective number of rats, average final body weights, liver lesions including the incidences of cellular-altered foci, neoplastic nodules, and hepatocellular carcinoma, and the number of GGT-positive foci are shown in Table 1. Fewer rats were available for the evaluation of liver lesions than for mammary lesions (Table 3) because of autolytic changes in some animals which died with mammary tumors prior to sacrifice.

Continuous feeding of PB induced a transitory increase in body weight in the early experimental period that was not evident by the time of sacrifice (Table 1). However, PB distinctly increased the liver weight, which was about 3.3% of the body weight in the groups fed PB and about 2.7% in those given the basal diet. The liver of rats placed on PB diet was histologically characterized by hypertrophy of hepatocytes at Zone 3. In the multiple PH groups, the body and liver weights were not different from those of rats in other groups, with the exception of a slightly lighter liver weight in the animals of Group 15. The 3 liver lesions, cellular-altered foci, neoplastic nodules, and hepatocellular carcinoma were diagnosed according to the criteria described by Squire and Levitt (Ref. 41; Table 1). One hepatocellular carcinoma developed in both Groups 1 and 5 in which rats were given N-hydroxy-AABP or N-hydroxy-FABP coupled with PH and placed on the PB diet. Both were histologically well differentiated. In contrast to the low incidence of hepatocellular carcinoma, cellular-altered foci and neoplastic nodules, which are the putative precursors of hepatocellular carcinomas (5, 41), were frequently observed.

The highest incidence of neoplastic nodules was in Groups 1 and 5 in which rats were treated with the carcinogen after PH and subsequently treated with PB. Hepatocellular carcinoma also developed in these groups. Moreover, the frequency of GGT-positive foci per unit area of the liver section generally paralleled the incidence of neoplastic nodules. Although the present experiment yielded only a few hepatocellular carcinomas, the high incidence of precancerous lesions indicates that a single i.p. dose of 0.4 mmol of N-hydroxy-AABP or N-hydroxy-FABP per kg body weight to hepatectomized rats appeared to be sufficient to initiate liver carcinogenesis. In agreement with previous reports (31, 35), statistical analyses in this

Table 1
Body and liver weight and incidences of tumors and number of GGT-positive foci per sq cm in the livers of rats induced by a single i.p. injection of N-hydroxy-AABP or N-hydroxy-FABP

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Effective no. of rats</th>
<th>Av. final body wt (g)</th>
<th>Av. liver wt (% of body wt)</th>
<th>Cellular altered foci</th>
<th>Neoplastic nodules</th>
<th>Hepatocellular carcinoma</th>
<th>No. of GGT-positive foci/sq cm liver section</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PH, C, PB</td>
<td>19</td>
<td>419 ± 98*</td>
<td>3.2 ± 0.6</td>
<td>21 (91)*</td>
<td>17 (74)*</td>
<td>1</td>
<td>1.97 ± 1.49 (20)*</td>
</tr>
<tr>
<td>2</td>
<td>PH, C</td>
<td>19</td>
<td>444 ± 95</td>
<td>2.8 ± 0.6</td>
<td>12 (63)</td>
<td>2 (11)</td>
<td>0</td>
<td>0.13 ± 0.33 (11)*</td>
</tr>
<tr>
<td>3</td>
<td>C, PB</td>
<td>19</td>
<td>415 ± 74</td>
<td>3.2 ± 0.5</td>
<td>15 (79)</td>
<td>4 (21)</td>
<td>0</td>
<td>0.74 ± 0.72 (11)*</td>
</tr>
<tr>
<td>4</td>
<td>C</td>
<td>20</td>
<td>417 ± 69</td>
<td>2.8 ± 0.4</td>
<td>10 (50)</td>
<td>1 (5)</td>
<td>0</td>
<td>0.06 ± 0.16 (15)</td>
</tr>
<tr>
<td>5</td>
<td>PH, C, PB</td>
<td>16</td>
<td>413 ± 58</td>
<td>3.4 ± 1.3</td>
<td>15 (94)</td>
<td>11 (69)</td>
<td>1</td>
<td>2.30 ± 1.31 (13)</td>
</tr>
<tr>
<td>6</td>
<td>PH, C</td>
<td>15</td>
<td>444 ± 97</td>
<td>3.0 ± 0.3</td>
<td>8 (53)</td>
<td>4 (25)</td>
<td>0</td>
<td>0.86 ± 0.12 (11)</td>
</tr>
<tr>
<td>7</td>
<td>C, PB</td>
<td>17</td>
<td>402 ± 59</td>
<td>3.4 ± 0.5</td>
<td>10 (59)</td>
<td>4 (24)</td>
<td>0</td>
<td>0.98 ± 1.82 (11)</td>
</tr>
<tr>
<td>8</td>
<td>C</td>
<td>19</td>
<td>447 ± 93</td>
<td>2.9 ± 0.4</td>
<td>3 (16)</td>
<td>0</td>
<td>0</td>
<td>0 ± 0 (13)</td>
</tr>
<tr>
<td>9</td>
<td>PH, S, PB</td>
<td>18</td>
<td>470 ± 97</td>
<td>3.3 ± 0.3</td>
<td>8 (44)</td>
<td>3 (17)</td>
<td>0</td>
<td>0.60 ± 0.74 (13)</td>
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<tr>
<td>10</td>
<td>PH, S</td>
<td>18</td>
<td>460 ± 84</td>
<td>2.7 ± 0.3</td>
<td>1 (6)</td>
<td>0</td>
<td>0</td>
<td>0.10 ± 0.22 (11)</td>
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<tr>
<td>11</td>
<td>S, PB</td>
<td>19</td>
<td>431 ± 67</td>
<td>3.2 ± 0.3</td>
<td>5 (26)</td>
<td>1 (5)</td>
<td>0</td>
<td>0.43 ± 0.58 (15)</td>
</tr>
<tr>
<td>12</td>
<td>S</td>
<td>19</td>
<td>454 ± 78</td>
<td>3.0 ± 0.3</td>
<td>2 (10)</td>
<td>0</td>
<td>0</td>
<td>0.21 ± 0.36 (14)</td>
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<tr>
<td>13</td>
<td>PH, C, (PH)</td>
<td>15</td>
<td>472 ± 100</td>
<td>2.8 ± 0.4</td>
<td>13 (67)</td>
<td>2 (13)</td>
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<td>0.67 ± 0.71 (13)</td>
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<tr>
<td>14</td>
<td>PH, C, (PH)</td>
<td>14</td>
<td>460 ± 105</td>
<td>3.0 ± 0.5</td>
<td>12 (68)</td>
<td>3 (21)</td>
<td>0</td>
<td>1.70 ± 1.39 (13)</td>
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<tr>
<td>15</td>
<td>PH, S, (PH)</td>
<td>16</td>
<td>423 ± 97</td>
<td>2.4 ± 0.3</td>
<td>2 (11)</td>
<td>0</td>
<td>0</td>
<td>0.08 ± 0.19 (13)</td>
</tr>
</tbody>
</table>

* C, N-hydroxy-AABP; C, N-hydroxy-FABP; S, solvent; (PH), multiple PH.

* Mean ± S.D.

* Numbers in parentheses, percentage.

* Numbers in parentheses, number of rats examined.
Liver, Mammary Tumorigenesis by N-Hydroxy-N-acylamminobiphenyls

Table 2

<table>
<thead>
<tr>
<th>Group</th>
<th>Incidences of neoplastic nodules</th>
<th>No. of GGT-positive foci</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 (PH, C1, PB)</td>
<td>+ + + + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>Group 2 (PH, C1)</td>
<td>+ + + + + + +</td>
<td>+</td>
</tr>
<tr>
<td>Group 3 (C1, PB)</td>
<td>+ + + + +</td>
<td></td>
</tr>
<tr>
<td>Group 4 (C1)</td>
<td>+ + + + +</td>
<td></td>
</tr>
<tr>
<td>Group 5 (PH, C1, PB)</td>
<td>+ + + + + + +</td>
<td></td>
</tr>
<tr>
<td>Group 6 (PH, C1)</td>
<td>+ +</td>
<td></td>
</tr>
<tr>
<td>Group 7 (C1, PB)</td>
<td>+ + + + +</td>
<td></td>
</tr>
</tbody>
</table>

Table 3

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. of rats</th>
<th>Effective no. of rats</th>
<th>Total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PH, C1, PB</td>
<td>24</td>
<td>7 (0)</td>
<td>8 (33)</td>
</tr>
<tr>
<td>2</td>
<td>PH, C1</td>
<td>20</td>
<td>12 (19)</td>
<td>14 (70)</td>
</tr>
<tr>
<td>3</td>
<td>C1, PB</td>
<td>20</td>
<td>4 (4)</td>
<td>5 (25)</td>
</tr>
<tr>
<td>4</td>
<td>C1</td>
<td>20</td>
<td>6 (12)</td>
<td>7 (35)</td>
</tr>
<tr>
<td>5</td>
<td>PH, C1, PB</td>
<td>17</td>
<td>9 (9)</td>
<td>8 (47)</td>
</tr>
<tr>
<td>6</td>
<td>PH, C1</td>
<td>16</td>
<td>12 (15)</td>
<td>10 (63)</td>
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<td>8</td>
<td>C1</td>
<td>19</td>
<td>10 (14)</td>
<td>10 (53)</td>
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<tr>
<td>9</td>
<td>PH, S, PB</td>
<td>18</td>
<td>1 (1)</td>
<td>1 (6)</td>
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<tr>
<td>10</td>
<td>PH, S</td>
<td>19</td>
<td>5 (5)</td>
<td>5 (26)</td>
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<td>S, PB</td>
<td>19</td>
<td>1 (1)</td>
<td>1 (5)</td>
</tr>
<tr>
<td>12</td>
<td>S</td>
<td>19</td>
<td>4 (4)</td>
<td>4 (21)</td>
</tr>
<tr>
<td>13</td>
<td>PH, C1, (PH)3</td>
<td>16</td>
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<td>9 (56)</td>
</tr>
<tr>
<td>14</td>
<td>PH, C1, (PH)3</td>
<td>14</td>
<td>7 (8)</td>
<td>7 (50)</td>
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<tr>
<td>15</td>
<td>PH, S, (PH)3</td>
<td>18</td>
<td>1 (1)</td>
<td>1 (6)</td>
</tr>
</tbody>
</table>

a C1, N-hydroxy-AABP; C2, N-hydroxy-FABP; S, solvent; (PH)3, multiple PH.
b See Table 1.
c +++, p < 0.005; +, 0.005 < p < 0.05.

The experiment also revealed that the combination of prior PH and subsequent long-term administration of PB effectively amplified the hepatocarcinogenic potency of both chemicals used in this study (Table 2). It was also shown that continuous administration of PB increased the frequency of precancorous liver lesions even in control rats given injections of solvent alone.

Multiple PH were not as effective a promoting stimulus as PB. Multiple PH significantly increase the number of GGT-positive foci as compared to Groups 2 and 6, which had not received multiple PH, whereas the incidence of neoplastic nodules was not increased. Histologically, the liver architecture of rats subjected to multiple PH did not differ much from that of normal control rats, except for slightly increased fibrous tissue and oval cells at the periportal area in a few cases.

Mammary Tumors. The mammary tumors induced in this study were classified as either fibroadenoma or adenocarcinoma (Table 3). Except for a case of adenocarcinoma in Group 1 in which the interstitial tissue of the tumor had a sarcomatous appearance, neither adenoma nor sarcoma was found. Single injections of either N-hydroxy-AABP or N-hydroxy-FABP were clearly demonstrated to induce mammary tumors. There was, however, no difference in the tumorigenicity of these compounds for this organ in this experiment.

Interestingly, PH prior to the carcinogen treatment or PB feeding after treatment clearly affected the induction of mammary tumors. The incidences of mammary tumors are presented in Table 4 according to the presence or absence of PH or PB treatment. Since PH reduces the liver volume to approximately one-third normal, it is reasonable to speculate that the total hepatic capacity for the metabolism of the carcinogen would be reduced in the hepatectomized rat. Thus, it is anticipated that the amount of carcinogen which reached the mammary gland through the liver when given i.p. might depend on whether the animal had undergone a PH.

Partially hepatectomized rats given N-hydroxy-AABP (Group 2) had a significantly higher incidence of mammary tumors (p < 0.05) than did nonhepatectomized rats in Group 4, and Table 4 shows that there was a marginally significant increase in mammary tumor induction by PH in groups given N-hydroxy-AABP (χ2 = 3.48, p < 0.1) but not in groups given N-hydroxy-FABP. This marginally significant difference was also observed when incidences of mammary tumors by N-hydroxy-AABP and by N-hydroxy-FABP were summed.

Continuous administration of PB after the carcinogen treatment distinctly suppressed the induction of mammary tumors, despite its enhancing effect on hepatocarcinogenesis. This inhibitory effect was observed in groups treated with N-hydroxy-AABP (p < 0.05) and even in groups given solvent alone (p < 0.05) but not in the groups given N-hydroxy-FABP (p < 0.2). The inhibitory effect of PB on mammary tumor induction was more clearly notable in the incidences of fibroadenoma than in those of adenocarcinoma. Furthermore, the total number of fibroadenomas in the rats fed PB was about one-half of those in rats maintained with basal diet. This 2-fold difference...
was also seen in groups given N-hydroxy-FABP in which there was no significant difference in the incidence of mammary tumors between the animals fed PB and basal diets. The mammary tumors tended to occur more often in the lower half (i.e., abdominal) than in the upper half (i.e., thoracic) of the rat. The actual number of mammary tumors was: N-hydroxy-AABP (upper, 29; lower, 32); N-hydroxy-FABP (upper, 21; lower, 39); control (upper, 3; lower, 10). This finding is not in agreement with data of Shellabarger et al. (40) which showed that the gastric installation of 7,12-dimethylbenz[a]-anthracene or procarbazine to Sprague-Dawley rats tended to induce more mammary tumors in the thoracic than in the abdominal region, whereas total-body X-irradiation induced tumors in a random fashion.

DISCUSSION

The induction of liver tumors in rats by N-hydroxy-AAF has been correlated with the ability of the organ to activate this compound by conjugation with sulfate to yield a metabolite that is capable of reaction with nucleic acids and protein (4, 8). The formation of N-2-acetylaminofluorene-substituted adducts with macromolecules of rat hepatocytes has been shown to be dependent on the concentration of sulfate in the media (22). Furthermore, a close relationship between liver cell necrosis and sulfate-dependent activation has been shown in a recent study which disclosed that the periportal necrosis observed following the i.p. injection of N-hydroxy-AAF occurred in the same region as the greatest accumulation of the tritiated acetyl group of N-hydroxy-AAF as evidenced by autoradiography.6

Other arylhydroxamic acids, while carcinogenic in extrahepatic organs of the rat that are not capable of conjugating the carcinogens with sulfate, yield relatively more stable sulfate conjugates and do not induce tumors in rat liver (3, 11). Paradoxically, the major structures bound to DNA by both hepatocarcinogenic and nonhepatocarcinogenic hydroxamic acids are not compatible with their formation as a consequence of activation by sulfate conjugation (13, 14, 21, 25, 26).

One characteristic common to the carcinogenic arylhydroxamic acids thus far studied is that they are substrates for N,O-acyltransferases of their target tissues in the rat (17). Moreover, nucleic acid adducts formed in these tissues are consistent with an acyltransferase-dependent activation mechanism (13, 14, 21, 25, 26).

A possible exploration for these apparent anomalies is that arylhydroxamic acids may induce liver tumors only when they produce a hepatotoxic response as a consequence of the production of a reactive sulfate conjugate in conjunction with an independent gene-altering event. This hypothesis is supported by the failure to demonstrate, directly, that the O-sulfate ester of N-hydroxy-AAF is carcinogenic.

The present study was undertaken in an attempt to determine whether the combined effects of prior PH and subsequent PB administration could circumvent this requirement for the enhancing effect of the sulfate-induced hepatotoxicity. Viewed alternatively, the demonstration of hepatic tumor initiation by a sulfate-independent pathway would further implicate, indirectly, the role of acyltransferase in this process.

The experimental design of this study incorporated both animals and compounds that decreased the possibility for the involvement of sulfate conjugation. In a positive vein, N-hydroxy-AABP can be activated by acyltransferase of both target tissues, and the nucleic acid adducts detected in rat liver are consistent with their formation by an acyltransferase mechanism.

The data presented in this report support the idea that arylhydroxamic acids may be metabolized to initiating agents by hepatic acyltransferases. Although there were few hepatocellular carcinomas induced in the present experiment, the formation of neoplastic nodules and GGT-positive foci indicates that both N-hydroxy-AABP and N-hydroxy-FABP are capable of initiating the hepatocarcinogenic process. It is likely that the incidence of liver tumors would have eventually been greater had it not been necessary to terminate the study because of the increasing appearance of life-threatening mammary tumors. This situation was analogous to that previously reported in that female Sprague-Dawley rats subjected to PH during the oral administration of N-2-acetylaminofluorene yielded hyperplastic nodules and a high incidence of mammary and Zymbal gland tumors, but not liver cancer, after 31 weeks (15). In future experiments, the use of older animals that are less susceptible to mammary tumor formation may permit longer observation periods.

The enhancing effect of prior PH on the production of liver.

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**Table 4**

| Effects of prior PH and PB on the incidences of mammary tumor induced by a single i.p. injection of N-hydroxy-AABP or N-hydroxy-FABP in female CD rats |
|---|---|---|---|---|---|---|---|
| | N-Hydroxy-AABP | N-Hydroxy-FABP | Solvent | Total |
| | No. of rats with tumors | No. of rats with tumors | No. of rats with tumors | No. of rats with tumors | No. of rats with tumors | No. of rats with tumors |
| PH | | | | | | |
| + | 44 | 22a | 33 | 18b | 77 | 40c |
| - | 40 | 12 | 36 | 16 | 76 | 28 |
| PB | | | | | | |
| + | 44 | 13c | 34 | 14d | 37 | 2c |
| - | 40 | 21 | 35 | 20 | 38 | 9 |

* a Marginally significantly different from the PH (−) at P < 0.1.
* b Not significantly different from the PH (−) at P < 0.5.
* c Significantly different from the PB (−) at P < 0.05.
* d Marginally significantly different from the PB (−) at P < 0.2.
* e Significantly different from the PB (−) at P < 0.01.

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lesions could have resulted from exposure of the residual liver to a proportionately greater quantity of carcinogen than in the unoperated animals. Alternatively, prior PH may result in the transcription of altered DNA prior to its repair. Resolution of this question may be approached in subsequent dose-response and temporal studies in attempts to identify and clarify the importance of repairable and nonrepairable lesions in initiation that have been reported for aromatic amines (28, 43).

The PH performed after carcinogen treatment was less effective than PB in producing GGT-positive foci and was ineffective in inducing neoplastic nodules. Pound and McGuire (36) have reported a slight promoting activity of repeated PH on hepatocarcinogenesis in rats which were initiated by a single dose of diethylnitrosamine. They performed the repeated PH on Weeks 5, 10, and 15 in the 52-week experiment. Since the responsiveness of neoplastic nodules to PH decreases gradually as their size increases (24), multiple PH were carried out in the early stages of the present experiment, i.e., within the first 4 weeks, in order to better stimulate cell proliferation. The relative lack of effect of the multiple PH may be due to the loss of large numbers of initiated cells, which were eventually reduced to about 22% by the 3 additional PH. The morphological sequence of liver carcinogenesis in the rat has been shown to be cellular-altered foci, neoplastic nodules, and then hepatocellular carcinomas (6, 37, 41). Only a few cancers arise from $10^2$ to $10^3$ focal proliferative lesions (6). Therefore, the potential usefulness of the promoting action of multiple PH may be attenuated by the loss of initiated cells in this procedure.

Studies have now demonstrated that cytosols of rat liver and mammary gland possess relatively acyl-specific enzymes that can metabolize arylhydroxamic acids to derivatives that are capable of reacting with nucleic acids. One enzyme produces adducts with N-formyl substrates; the other is most effective with N-acetyl or N-propionyl derivatives. Liver cytosols are most active with acetylated substrates while cytosols from mammary gland utilize formyl derivatives more efficiently. In contrast to rat liver cytosol, microsomes can convert N-formyl containing arylhydroxamic acids to nucleic acid-bound derivatives 6 to 8 times more readily than acetylated compounds. Unlike the relatively acetyl-specific cytosolic enzymes, the activation of arylhydroxamic acids by rat liver microsomes is inhabitable by the esterase inhibitor, paraxoxin.

In a recent study, the N-acetyl and N-formyl derivatives of N-hydroxy-4-aminobiphenyl were given i.p. at 0.1 mmol per kg of body weight to female CD rats twice weekly for 4 weeks resulting in a higher incidence of cellular-altered foci in the liver and 4-fold higher incidence of mammary tumors with the acetyl derivative. Unexpectedly, in the present study there were no differences in the tumorigenic activities of these two compounds for either liver or mammary gland. The reason for this discrepancy is not clear. The lower dose used in the previous study (i.e., 25% that of the present experiment) may have been subject to a different biochemical disposition. Alternatively, the prolonged time period over which the carcinogen was administered may have provided the opportunity for the expression of a tumor-promoting activity that is unique to the acetylated compound.

Of particular interest is the enhancing effect of PH prior to chemical administration and the suppressive effect of subsequent long-term PB feeding on mammary tumor induction by the 2 hydroxamic acids. Even though the balance between activation and detoxification may be altered by PH, it is likely that the net effect is for a greater quantity of compound to be distributed systemically and, consequently, to affect extrahepatic tissues to a greater degree.

The reason(s) for the suppressive effect of PB is probably less direct. Since PB administration was not started until 2 days after carcinogen treatment, it is reasonable to assume that PB did not alter the initiation events by modifying the activation of carcinogen in either the liver or the mammary gland. It is more probable that PB affected the promotion stage of mammary carcinogenesis; the observation that PB also inhibited the development of spontaneous mammary tumors supports this idea. It should be noted, however, that, in contrast to our data, a life span experiment of PB in female Wistar rats by Rossi et al. (38) did not show an inhibitory effect of PB on mammary tumor incidence as compared to untreated animals. While a direct action of PB on the growth of normal and neoplastic mammary tissue could be envisioned, a more probable explanation is that PB alters steroid metabolism so as to produce a hormonal balance that is a less effective endogenous tumor-promoting system. For example, the chronic treatment of female rats with PB stimulated the metabolism of $17\beta$-estradiol by liver microsomes and this effect was paralleled by a decreased action of the estrogen on the uterus (29).

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