Effect of p-Hydroxyacetanilide on Liver Cancer Induction by N-Hydroxy-N-2-fluorenylacetamide

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SUMMARY

We explored, in rats, the conditions underlying the induction of liver tumors by N-hydroxy-N-2-fluorenylacetamide (N-OH-FAA) in terms of the participation of an ultimate carcinogen in the form of the sulfate ester. Male rats were fed two dose levels, i.e., 0.0213% (0.89 mmole/kg) and 0.032% (1.34 mmole/kg) of N-OH-FAA in the diet, with or without 0.89% (59 mmole/kg) p-hydroxyacetanilide, and 1 or 3 molar equivalents or 0.84% (59 mmole/kg) and 2.52% (178 mmole/kg) dietary sodium sulfate. At the lower dose level of carcinogen, p-hydroxyacetanilide inhibited liver tumor formation, but additional sulfate failed to restore the carcinogenicity. At the higher level of carcinogen, p-hydroxyacetanilide inhibited cancer formation, but to a lesser extent, and additional sulfate had no additional effect. Thus, p-hydroxyacetanilide was a weaker inhibitor than acetanilide, and sulfate had virtually no modifying effect in these two experimental series. We conclude that liver tumor induction with N-OH-FAA is mediated only in part by the sulfate ester of N-OH-FAA and that other activated esters may also be instrumental in the carcinogenic process.

INTRODUCTION

Previously, we reported (15) that acetanilide inhibited the carcinogenicity to the liver of FAA. We accounted for this inhibition, in part, by demonstrating that acetanilide lowered the conversion of FAA to the proximate N-hydroxy derivative (4). However, the fact that acetanilide also depressed the carcinogenicity of the N-hydroxy metabolite certainly could not be explained directly by an effect on the biochemical oxidation of the procarcinogen (12). It was thought that acetanilide counteracted the carcinogenicity of the N-hydroxy derivative by virtue of conversion to its principal metabolite, p-hydroxyacetanilide which, in turn, as shown in several studies (2, 3, 12), decreases the levels of available free sulfate. It was this finding, plus the fact that increasing dietary sulfate levels restored the tumorigenic potential to the liver of these carcinogens, that seemed to resolve the question of the mechanism of action of acetanilide (12).

Nonetheless, it seemed valuable to test directly the ability of the p-hydroxyacetanilide to inhibit the carcinogenicity of N-OH-FAA and to determine whether, as was true with acetanilide, sulfate would restore the carcinogenic potential. This paper deals with the results obtained.

MATERIALS AND METHODS

Chemicals. p-Hydroxyacetanilide of the highest purity grade, m.p. 169–170° (Kofler microstage), was purchased from the Fisher Scientific Co., Silver Spring, Md. Recrystallized N-OH-FAA, m.p. 143–144° (Kofler microstage), was acquired through the courtesy of Dr. Harry B. Wood, Jr., Drug Development Branch, National Cancer Institute.

Treatment of Animals. The experiments were patterned after those already reported (12). Briefly, groups of animals were fed diets containing either 0.0213% (0.89 mmole/kg) or 0.032% (1.34 mmole/kg) N-OH-FAA alone, or 1 of the 2 aforementioned diets together with 0.89% (59 mmole/kg) p-hydroxyacetanilide (a 66 or 44 molar excess, respectively, over carcinogen). Some additional groups received 0.84% (59 mmole/kg, 1 molar equivalent) or 2.52% (178 mmole/kg, 3 molar equivalents) of sodium sulfate. The agents were carefully mixed in a basal diet of pulverized Wayne laboratory meal.

The rats were maintained on the various diets for 16 weeks, then were placed on a control diet of Wayne Lab Blox for another 10 or another 16 weeks, as indicated in Tables 1 and 2. At the end of the total experimental period, the animals were bled by heart puncture under light ether anesthesia and necropsied. The liver, kidney, and spleen were weighed and fixed in a solution of acetic acid:formalin:70% ethanol (5:10:90). Rat livers were routinely processed by accepted histological techniques. The sections, usually stained with hematoxylin and eosin, were studied microscopically. The lesions were classified, as described previously (12), into large or small hepatoma, hyperplastic nodule with malignant atypiality, hyperplastic nodule, hyperplastic area, hyperplastic focus, no hyperplasia, or normal.

Free and Conjugated Urinary Sulfate. Three rats from each test diet series were housed in stainless steel metabolism cages (Acme Metal Company, Cincinnati, Ohio) in which urine and feces could be collected separately over a 24-hr period. Urine...
Ultimate Carcinogen from N-OH-FAA

Table 1

Effect of p-hydroxyacetanilide on N-OH-FAA hepatocarcinogenesis and restoration by dietary sulfate

Groups of 6-week-old male Fischer F344 rats were fed the experimental diets containing carcinogens with and without p-hydroxyacetanilide and additional sodium sulfate, then were continued on the control regimen of Wayne Lab Blox meal for 10 weeks longer. After necropsy, the liver lesions were evaluated by the criteria described in the text.

<table>
<thead>
<tr>
<th>Experimental diets#</th>
<th>Final no. of rats</th>
<th>Final body wt. (g)</th>
<th>Liver wt.</th>
<th>Hyperplasia</th>
<th>Hepatoma</th>
<th>Rats with liver neoplasms (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>None</td>
<td>Area</td>
<td>Nodule</td>
</tr>
<tr>
<td>N-OH-FAA</td>
<td>10</td>
<td>281 ± 5b</td>
<td>19.9 ± 7.1</td>
<td>7.1</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>N-OH-FAA + p-hydroxyacetanilide</td>
<td>20</td>
<td>307 ± 4</td>
<td>12.3 ± 0.3</td>
<td>4.0</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>N-OH-FAA + p-hydroxyacetanilide + SO₄²⁻</td>
<td>20</td>
<td>311 ± 6</td>
<td>11.4 ± 0.3</td>
<td>3.7</td>
<td>11</td>
<td>4</td>
</tr>
<tr>
<td>p-Hydroxyacetanilide</td>
<td>5</td>
<td>336 ± 8</td>
<td>10.3 ± 0.1</td>
<td>3.1</td>
<td>1c</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>5</td>
<td>321 ± 9</td>
<td>9.9 ± 0.4</td>
<td>3.1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

# Ingredients were added in the following concentrations: N-OH-FAA, 0.0213% (0.89 mmole/kg); p-hydroxyacetanilide, 0.89% (59 mmoles/kg); sodium sulfate (SO₄²⁻), 2.52% (178 mmoles/kg).

b Mean ± S.E.

c One rat had abnormal bile duct proliferation; the remainder of the control or p-hydroxyacetanilide-fed rats had normal livers.

Table 2

Effect of p-hydroxyacetanilide on hepatocarcinogenesis of high concentration of N-OH-FAA and its restoration by dietary sulfates

The protocols used were the same as those described in Table 1, except for higher levels of carcinogen, 2 levels of sodium sulfate, and a lengthened holding period of 16 weeks, instead of 10 weeks, on the control diet.

<table>
<thead>
<tr>
<th>Experimental diets#</th>
<th>Final no. of rats</th>
<th>Final body wt. (g)</th>
<th>Liver wt.</th>
<th>Hyperplasia</th>
<th>Hepatoma</th>
<th>Rats with liver neoplasms (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>None</td>
<td>Area</td>
<td>Nodule</td>
</tr>
<tr>
<td>N-OH-FAA</td>
<td>5</td>
<td>274 ± 16b</td>
<td>21.8 ± 1.3</td>
<td>8.1</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>N-OH-FAA + p-hydroxyacetanilide</td>
<td>12</td>
<td>333 ± 5</td>
<td>11.5 ± 0.4</td>
<td>3.4</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>N-OH-FAA + p-hydroxyacetanilide + SO₄²⁻</td>
<td>6</td>
<td>338 ± 8</td>
<td>12.3 ± 0.6</td>
<td>3.6</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>N-OH-FAA + p-hydroxyacetanilide + SO₄²⁻ (3 times)</td>
<td>12</td>
<td>328 ± 7</td>
<td>12.4 ± 0.4</td>
<td>3.8</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>p-Hydroxyacetanilide</td>
<td>6</td>
<td>374 ± 9</td>
<td>10.7 ± 0.4</td>
<td>2.8</td>
<td>6c</td>
<td></td>
</tr>
</tbody>
</table>

# Ingredients were added in the following concentrations: N-OH-FAA, 0.032% (1.34 mmoles/kg); p-hydroxyacetanilide, 0.89% (59 mmoles/kg); sodium sulfate (SO₄²⁻), 0.84% (59 mmoles/kg); and [SO₄²⁻ (3 times)], 2.52% (178 mmoles/kg).

b Mean ± S.E.

c All 6 animals had normal livers.

RESULTS

Experiment 1. The administration of 0.0213% N-OH-FAA to male Fischer rats for 16 weeks, followed by control conditions for another 10 weeks, led to appreciable liver enlargement, and all 10 animals in this group had cancer of the liver (Table 1). When p-hydroxyacetanilide was administered simultaneously, the liver weight was increased nominally over that of untreated controls. Only 20% of a group of 20 rats had cancer, and 40% had hyperplastic nodules. The administration of 3 molar equivalents of dietary sulfate, in addition to carcinogen and p-hydroxyacetanilide, appeared to inhibit the carcinogenicity even more, for no cancers were seen in this group, and fewer animals had hyperplastic nodules. Control animals on Wayne laboratory meal supplemented by p-hydroxyacetanilide had normal livers.

Experiment 2. In this series, the male rats were fed a higher level of 0.032% N-OH-FAA as the basal carcinogenic regimen, and the total test was run for 32 weeks. All animals had greatly enlarged livers and hepatomas. The simultaneous

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administration of p-hydroxyacetanilide led to slightly larger livers in these rats than in animals that received the control diet. Furthermore, the group that received N-OH-FAA, p-hydroxyacetanilide, and 1 or 3 molar equivalents of dietary sulfate also exhibited a very slight liver enlargement. Whereas the hematocrit of animals on carcinogen alone was appreciably depressed (to 36.0 ± 3.3), the groups of animals that received N-OH-FAA and p-hydroxyacetanilide had a hematocrit value in the normal range (about 45).

When the higher amount of carcinogen was used in this series, the addition of p-hydroxyacetanilide led to a decrease in the carcinogenic effect, but the supplement of sulfate had no real additional effect. This is in contrast to the results of the previous experiment, in which, with a lower dose of carcinogen, p-hydroxyacetanilide somewhat more effectively inhibited the development of liver tumors.

Rats on a diet containing only N-OH-FAA excreted about 17 mg of sulfate per day, and virtually all of this was as free, inorganic sulfate (Table 3). Rats that received a supplement of p-hydroxyacetanilide eliminated about the same total amount of sulfate but, in contrast, most of this was conjugated, and very little was in the free form. Animals that received diets supplemented with 1 or 3 equivalents of dietary sulfate had higher levels of total and free urinary sulfate. The difference between the 2 values (after the amount conjugated with p-hydroxyacetanilide was measured) was of the same order as that in the groups with diets not supplemented with sulfate, which is as might be expected.

**DISCUSSION**

Unexpectedly, this study showed that p-hydroxyacetanilide, the main metabolite of acetanilide in rats, is a weaker inhibitor of the carcinogenicity of N-OH-FAA. Indeed, p-hydroxyacetanilide efficiently depletes the animal of free sulfate ion, as was reported by Büch et al. (2) and as has been fully confirmed under our own conditions (12). Also, p-hydroxyacetanilide, as well as acetanilide and the isomeric acetotoluidides, protected rats against the toxicity of FAA or N-OH-FAA (3, 14, 15). If the mechanism of liver tumor formation by N-OH-FAA rested solely on the endogenous formation in the liver cell of the sulfate ester of N-OH-FAA, p-hydroxyacetanilide should have been a more powerful inhibitor than acetanilide. It was not. Also, the restoration of sulfate to the diet should compensate for that withdrawn by the inhibitor and might be expected to be an efficient means of restoring the carcinogenic process. Again, it was not.

Thus, one must postulate alternative ways to define the specific molecular conditions by which N-OH-FAA induces liver cancer.

One such concept, which would account in part for the current results, is based on the known overall metabolic pathway of FAA and N-OH-FAA. In rats, a large portion of a given dose, metabolized in the liver, is secreted as metabolites into the bile (6, 7, 9). In the lower portion of the intestinal tract, bacterial enzymes hydrolyze one of the main metabolites (the glucosiduronic acid of N-OH-FAA) and convert it to FAA, which is reabsorbed (13). Hence, the organism (and, specifically, the liver) is exposed to FAA, which is metabolized effectively, especially under chronic conditions, to the active N-OH-FAA (13). This reaction probably is not inhibited by p-hydroxyacetanilide, but it may be inhibited by acetanilide (4). This scheme would account for the fact that p-hydroxyacetanilide (compared with acetanilide) less effectively inhibits the carcinogenic effect on the liver of N-OH-FAA. In part, this concept is supported by data that demonstrate that additional sulfate failed to restore carcinogenicity but that it had a more neutral effect with the higher level of carcinogen. It is improbable that either acetanilide or p-hydroxyacetanilide would affect the level of sulfotransferase or the formation of the intermediate phosphoadenosine phosphosulfate, since both drugs failed to affect the formation of the sulfate ester of N-(7-hydroxy-2-fluorenly)acetamide (Ref. 4; unpublished observations). Also, Büch et al. (2) and Grantham et al. (4) noted that the urinary excretion of conjugated sulfate esters after the chronic intake of acetanilide was relatively constant, which also supports the view that acetanilide or its main hydroxylated metabolite, p-hydroxyacetanilide, do not interfere with the formation of sulfate esters. On the other hand, glucuronic acid conjugates may be increased under some conditions; this suggests that

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**Table 3**

Intake and excretion of sulfate in urine of rats on various dietary regimens in a 24-hr period

<table>
<thead>
<tr>
<th>Experimental diets</th>
<th>Intake (μmoles) of the following ingredients</th>
<th>24-hr inorganic sulfate excretion (μmoles)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Food (g) N-OH-FAA p-Hydroxyacetanilide SO₄⁻</td>
<td>Total Free Conjugated</td>
</tr>
<tr>
<td>N-OH-FAA</td>
<td>10.0 ± 0.5a 8.9 778</td>
<td>118 ± 14 114 ± 18 4</td>
</tr>
<tr>
<td>N-OH-FAA + p-hydroxyacetanilide</td>
<td>13.2 ± 0.2 11.8 852</td>
<td>165 ± 8 30 ± 6 135</td>
</tr>
<tr>
<td>N-OH-FAA + p-hydroxyacetanilide + SO₄⁻</td>
<td>14.1 ± 0.7 12.6 832</td>
<td>508 ± 8 412 ± 16 95</td>
</tr>
<tr>
<td>N-OH-FAA + p-hydroxyacetanilide + SO₄⁻ (3 times)</td>
<td>12.3 ± 0.9 11.0 726</td>
<td>1068 ± 78 967 ± 77 101</td>
</tr>
</tbody>
</table>

a Mean ± S.E.
p-hydroxyacetanilide may modify the response to FAA or N-OH-FAA by increasing the excretion of glucosiduronic acid [although certainly less effectively than does phenobarbital (9, 10) or butylated hydroxytoluene (5), for example].

Another possibility is that liver tumor formation by N-OH-FAA is mediated not only by the sulfate ester but also through the newly discovered N/O-acetyltransferase which led to an O-acetylated hydroxyamine derivative as a possible ultimate carcinogen (1, 8). This enzyme appears to be powerfully inhibited by aniline (8). Aniline would be available when exogenous acetanilide is supplied, but it obviously is not available when p-hydroxyacetanilide is fed. That this mode may be relevant stems from our earlier demonstration that exogenous sulfate only partially restored carcinogenicity when acetanilide was used as an inhibitor.

Thus the combined data of these experiments can be interpreted to imply that liver tumor formation by N-OH-FAA and related agents is mediated not only by the previously described sulfate ester but also and simultaneously by other activated forms of this carcinogen, such as an O-acetyl-N-2-fluorenylhydroxyamine derivative.

ACKNOWLEDGMENTS

We are grateful to Mr. P. H. Grantham and Miss L. Mohan for performing the measurements and analyses of sulfate intake and excretion for Table 3.

REFERENCES

Effect of \( p \)-Hydroxyacetanilide on Liver Cancer Induction by \( N \)-Hydroxy-\( N \)-2-fluorenylacetamide

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