On the Sulfate Ester of \(N\)-Hydroxy-\(N\)-2-fluorenylacetamide as a Key Ultimate Hepatocarcinogen in the Rat\(^1\)

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SUMMARY

The problem of the biochemical activation steps required to elicit carcinogenicity to rat liver by \(N\)-2-fluorenylacetamide was investigated by bioassay, competitive inhibition, and biochemical techniques. Acetanilide inhibits not only the hepatocarcinogenicity of \(N\)-2-fluorenylacetamide but also that of the carcinogenic \(N\)-hydroxylated metabolite. With the parent compound, the inhibition was traced chiefly to competition at the \(N\)-hydroxylation step, which should not apply to the \(N\)-hydroxy derivative. With the latter, the addition of sodium sulfate to the diet restores the hepatocarcinogenicity of \(N\)-hydroxy-\(N\)-2-fluorenylacetamide in the presence of inhibitory acetanilide. However, sulfate addition fails to return carcinogenicity in the system \(N\)-2-fluorenylacetamide plus acetanilide, wherein the inhibition lies at the \(N\)-hydroxylation step. The combined results provide evidence that sulfate ester formation constitutes a required second activation step for the expression of carcinogenicity to the liver of rats of \(N\)-hydroxy-\(N\)-2-fluorenylacetamide, obtained by a first activation step. The required sulfotransferase enzyme system is found mainly in liver cytosol, but attempts to locate it in rat liver nuclear fractions resulted in the detection of only questionable amounts.

INTRODUCTION

Carcinogens of the aromatic amine type require biochemical activation for the expression of carcinogenicity. With the agent FAA\(^3\) (or 2-acetylaminofluorene), it was demonstrated unambiguously that the 1st step involves hydroxylation on the nitrogen, yielding N-OH-FAA (or \(N\)-hydroxy-2-acetylamino-fluorene; \(N\)-2-fluorenylacetohydroxamic acid). This material, which is more carcinogenic than the parent compound in sensitive species is also active at the point of injection and in species in which the parent compound is inactive (14, 24, 25, 35).

However, under in vitro conditions, the \(N\)-hydroxy derivative fails to react with cellular macromolecules such as DNA, RNA, or select proteins whereas, in vivo, all of these macromolecules are tagged when labeled carcinogen is injected. Also, the levels of \(N\)-hydroxy compound available in vivo did not always parallel carcinogenic activity, for example as a function of species, sex, or endocrine situation (32). Thus a further activating step appeared essential.

Model studies with a substituted ester, \(N\)-acetoxy-\(N\)-2-fluorenylacetamide, have pointed towards some form of O esterification. The \(O\)-acetyl derivative does indeed react with cellular macromolecules in vitro and is also quite carcinogenic in animal systems (cf. Ref. 25).

A number of proposals have been made regarding the structure of the ester-type metabolite that is active in vivo. Those considered were the \(O\)-acetyl compound itself, the glucosiduronic acid, a phosphate ester, and a sulfate ester (7, 14, 17, 19). We now have secured evidence that the sulfate ester appears to be the “ultimate” carcinogen involved in the chain of reactions leading to liver cancer in rats.

We obtained these results by taking advantage of our discovery that acetanilide inhibited the carcinogenicity of FAA (38, 39). This earlier observation was satisfactorily explained by the demonstration of a competition between acetanilide and the carcinogen for \(N\)-hydroxylation systems and also for cellular or molecular receptors (21). However, we now find that acetanilide also inhibited the carcinogenicity of N-OH-FAA. While competition for receptors is still possible, Miller (24) provided an alternate explanation, namely, that acetanilide could also act, through the metabolite \(p\)-hydroxyacetanilide, by depletion of the sulfate ions required for the activation step (6, 8). This indeed seems likely, since such a depletion was demonstrated and, more importantly, since the carcinogenicity of N-OH-FAA, inhibited by acetanilide, was restored by an increase in the level of dietary sulfate. This paper describes these and corollary findings.

MATERIALS AND METHODS

Chemicals. Acetanilide and \(p\)-hydroxyacetanilide were purchased from Fisher Scientific Co. (Silver Spring, Md.) and FAA was purchased from Aldrich Chemical Co. (Milwaukee, Wis.). N-OH-FAA was synthesized by the method of Miller et al. (cf. Ref. 7) and acquired through the courtesy of Dr. Harry B. Wood of the Cancer Chemotherapy National Service Center, National Cancer Institute.

1Presented in part at the 61st Annual Meeting of the American Association for Cancer Research, Philadelphia, Pa., April 9 to 11, 1970 (36).

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3The abbreviations used are: FAA, \(N\)-2-fluorenylacetamide; N-OH-FAA, \(N\)-hydroxy-\(N\)-2-fluorenylacetamide; TCA, trichloroacetic acid.

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Treatment of Animals. Weanling male Fischer F344 rats were procured from Charles River Breeding Laboratories (Wilmington, Mass.). They were housed in groups of 6 in stainless steel hanging cages, with free access to Wayne Lab Blox (Allied Mills, Inc., Chicago, Ill.) and drinking water containing veterinary grade Terramycin powder, 2.64 gliter, or with a calculated Terramycin content of 0.146 gliter (Chas. Pfizer & Co., Inc., New York, N. Y.) for 1 week. Subsequently, the animals were maintained under the same conditions, except that tap water was provided for drinking.

At 6 weeks of age, the rats were weighed and housed in groups of 6, with an equal average weight per cage.

The experiments were conducted in 3 series of tests as detailed in Tables 1 to 3. In the 1st series, rats were fed diets containing 0.02% FAA, or the molar equivalent of 0.0213% N-OH-FAA, or these levels of carcinogens together with 0.8% acetaldehyde. In the 2nd and 3rd series, the diets had 0.03% FAA, 0.032% N-OH-FAA, or these higher carcinogen levels with 0.8% acetaldehyde (38, 39). Also, in some groups, the diets with carcinogens and acetaldehyde were supplemented with the further addition of anhydrous sodium sulfate at 2 dosages, equimolar or 3 times equimolar to the level of acetaldehyde (0.84 or 2.52%, respectively). Other groups received additions of 2.25% disodium hydrogen phosphate which, together with the phosphate titr in the diet, yielded a phosphate level approximately equal to 3 moles of acetaldehyde.

We incorporated the carcinogens in the diets by first preparing a concentrate in pulverized Wayne laboratory meal by thorough grinding in a mortar. Designated amounts of these concentrates were then mixed with the remainder of the diets and also, as indicated, with powdered acetaldehyde or with sulfate or phosphate salts in a V-blender (The Patterson-Kelly Co., Inc., East Stroudsburg, Pa.).

The rats were maintained on their respective diets for a total of 16 weeks. At that time they were placed on a control diet of Wayne Lab Blox for another 10 weeks or, in the experimental series recorded in Table 3, for a further 16 weeks.

At the end of 26 or 32 weeks, the animals were lightly etherized and bled completely by heart puncture, and a careful necropsy was conducted.

Liver, kidney, and spleen were weighed and fixed in acetic acid: formalin: 70% ethanol (5:10:90). The livers of all rats were sectioned at 2- to 4-mm intervals after fixation. All visible lesions were sampled, and a section from each lobe was taken to include representatives of smaller lesions on a random basis. In addition, sections were routinely taken from spleen, kidney, pancreas, and intestine. All sections were processed in a conventional manner and stained with hematoxylin and eosin. The classification of lesions follows that of Firminger and Reuber (11) and Reuber (26), with the additional category of focal hepatoma in a preexisting hyperplastic nodule (Figs. 1 and 2). This diagnosis includes the hyperplastic nodule with malignant atypicality (9, 10), as well as the frank hepatocellular carcinoma occupying a portion of a nodule. In previous reports from this laboratory, such lesions were recorded as hepatocellular carcinomas (40), and this practice is followed by others (4). However, because of the accumulating evidence that the hyperplastic nodule is a precursor lesion to liver cancer (4, 5, 9–11, 22, 26, 30), it seems desirable to identify these possible transitional lesions. Small hepatomas (Figs. 3 and 4) are still identified, because their homogeneous histology makes it possible that these small lesions are developing de novo cancers. The separation of morphological types of cancers is made to further histogenetic understanding and does not influence the assessment of tumorigenicity, because the 3 diagnoses are considered together in the analysis.

The data on the incidence of various types of liver lesions and tumors were analyzed by appropriate statistical methods (29) through the courtesy of Dr. J. Gart of the National Cancer Institute.

Free and Conjugated Urinary Sulfate. Rats (3 from each test series) on the various experimental regimens were housed in stainless steel metabolism cages (Acme Metal Co., Cincinnati, Ohio), permitting the separate collection of urine and feces. Urines were collected over 24-hr periods at weekly intervals in ice-cold receivers, filtered, and diluted to a volume of 20 ml. Duplicate aliquots of the diluted urines were assayed for inorganic sulfate, before and after acid hydrolysis, by the method of Wainer and Koch (31), with the use of barium chloranilate (Fisher Scientific Co.). Briefly, to a column of Dowex 50W, with a bed volume of 1 ml, was added 1 ml of diluted urine. The urine sample was allowed to pass through the column into a tube containing 1 ml of 0.1 M acetate buffer, pH 4.6. The column was washed with 3 ml of water. To the tube containing the eluates (5 ml) were added 5 ml of ethanol plus 30 mg of barium chloranilate. The tube was shaken once a min for 10 min and centrifuged at 2000 rpm for 10 min. Five ml of the supernatant was added to a tube containing 2 ml of 8.5 M acetic acid. The absorbance was read at 530 nm on a Beckman DB spectrophotometer, against an appropriate blank. The amount of sulfate ion was determined from the absorbance on a linear calibration coordinate and was expressed as free and total sulfate per 24-hr urine. The difference, total minus free, represents the “conjugated” sulfate liberated by the acid hydrolysis step.

Sulfotransferase Activity in Rat Liver Nuclei. Two slightly different experimental series, one with nuclei isolated in 0.9% sodium chloride solution, the other with nuclei obtained in hypertonie medium [methods of Sporn and Dingman and of Chauveau (see Refs. 21 and 28)], were done. Since the results obtained were similar, only the last procedure will be described. Livers from 3 male young adult Fischer rats perfused with 0.9% sodium chloride solution were pooled. A 17-g aliquot was homogenized in a Potter-Elvehjem instrument with a plastic pestle in 4 volumes of 0.32 M sucrose solution. The homogenate was filtered through 8 layers of gauze and sedimented 3 times at 700 X g in 0.32 M sucrose. Then the pellet was gently resuspended and sedimented through 2.1 M sucrose at 17,300 X g for 2 hr. This preparation was suspended in 17 ml of 0.15 M KCl solution.

In 1 series, the suspension of nuclei in 10 ml of 0.25 M sucrose-5 mM magnesium chloride was sonically disrupted at 50 watts 8 times for 30-sec periods in the rosette vessel of a Branson sonifier with a microtip. Complete disruption of nuclei was achieved. The results were similar to those seen with intact nuclei and will not be detailed.
One ml of the suspension of nuclei was incubated in a shaking incubator at 37ø for 60 min in a complete medium (4 ml) containing, in addition, 1.6 ml of 0.1 M Tris buffer (50 mM), pH 7.4; a sulfate-activating mixture of 0.4 ml of 0.1 M MgSO₄ solution (10 mM); 0.4 ml of 0.1 M ATP solution (10 mM); 0.4 ml of 0.1 M NaF solution (100 mM); and 0.2 ml of a solution of 1 mg of 9,14C-labeled N-OH-FAA per ml (0.05 μCi) in ethanol (7, 15, 27, 41). Each assay was done in duplicate. Furthermore, as controls, incubations were performed with omission of ingredients, as shown in Table 4.

After the incubation, the tubes were cooled in ice, the contents were extracted 3 times with peroxide-free ether, and the aqueous phase was freed of ether by a gentle stream of nitrogen.

One ml of the treated incubation mixture was layered on 6.8 ml of cesium chloride solution, after the addition of 500 μg of previously isolated rat liver DNA. Gradient centrifugation for 64 hr at 45,000 rpm in the Spinco centrifuge was followed by isolation of the DNA-containing fractions, which were pooled. After the addition of 2 mg of calf thymus DNA, the DNA was precipitated with 10% TCA solution.

One ml of the incubation mixture was precipitated with 1 ml of 10% TCA solution. The precipitate was carefully washed 3 times with 5% TCA solution.

In addition, 2 ml of the mixture were extracted with phenol, with the use of 0.2 ml of a 2% sodium dodecyl sulfate solution. The nucleic acid fraction was precipitated from aliquots of the phenol extract with TCA, as before. The radioactivity in another aliquot was determined so as to provide the total count rate prior to the DNA isolation steps.

Radioactivity was assayed in all fractions by solution in Hyamine or Soluene, followed by dilution with the toluene base containing the scintillation mixture and by counting in a liquid scintillation spectrometer. The concentration of DNA was determined by the diphenylamine method of Burton, RNA by the orcinol reaction, and protein by a modification of the method of Lowry (21).

RESULTS

Effect of Acetanilide on Liver Carcinogenesis by FAA and N-OH-FAA. As we reported previously, administration of a 44 M excess of acetanilide, together with FAA, inhibited liver tumor formation. The present experiment involving a small number of rats was conducted as a control series to the new tests with the N-hydroxy derivative. Interestingly, when acetanilide was fed together with N-OH-FAA, itself at the level equimolar to FAA, a like inhibition was observed (Table 1). Whereas 12 of 12 rats fed N-OH-FAA had malignant liver tumors, the addition of acetanilide reduced the carcinoma incidence to 1 of 12. Only 1 grossly observable nodule which appeared histologically as carcinoma was seen in the liver of the 1 positive rat. The relative liver weights of the rats in the acetanilide-supplemented group were less than one-half those of the rats on carcinogen alone which further documents the effective inhibition of the action of the carcinogen on the liver. In this relatively short-term experiment on male rats, no other lesions were seen.

Effect of Dietary Sodium Sulfate on Liver Carcinogenesis by FAA and N-OH-FAA as Modified by Acetanilide. In the 2nd experimental series, patterned after the published protocols (38, 39), a slightly higher level, 0.03%, of FAA or the molar equivalent, 0.032%, of N-OH-FAA was used. Under these conditions, 8 of the group of 10 rats on FAA alone died early in the experiment, after 3 to 5 weeks, as noted earlier (Table 2). The 2 rats that survived for the entire experimental period had large hepatomas and sizable increases in liver weight. Again, as already reported, acetanilide sharply reduced the effect on the liver; only 1 of the 10 animals at risk had a malignant lesion. The liver weight was only slightly increased, and all animals survived.

When the same regimen of FAA plus acetanilide was supplemented with an equivalent amount, 0.84%, of sodium sulfate, no change in liver histology beyond that seen without sulfate was observed. All animals showed evidence of hyperplastic areas, and the liver weight was barely increased over the controls on acetanilide alone or sodium sulfate alone.

When the carcinogen used was N-OH-FAA, the relative gain in body weight was slightly less than that seen with FAA. Only 2 of 10 animals at risk died from toxicity, after 5 and 8 weeks on the diet, respectively. The liver weight was increased considerably, and all animals surviving the 26-week experimental period had nodular livers containing mainly large hepatomas. Acetanilide inhibited the development of liver cancers in this series also. Only 1 of 10 rats had a cancer. The liver weight was slightly increased over the control weight of approximately 3 g/100 g body weight, namely to 4 g/100 g.

The crucial finding was that the addition of sulfate ion, as dietary sodium sulfate equimolar to the amount of acetanilide, altered the reaction of the liver. As shown in Table 2, 6 of the 10 animals that received this treatment exhibited cancers. Thus, addition of dietary sodium sulfate restored the carcinogenicity to the liver of N-OH-FAA, which had been inhibited by addition of acetanilide to the diet of animals fed the carcinogen.

One point deserves comment. Animals fed carcinogen alone, whether FAA or N-OH-FAA, almost always showed a multinodular liver with many tumors, some of which were large. In contrast, tumors stemming from the joint administration of sulfate, acetanilide, and N-OH-FAA arose in a liver that grossly appeared relatively normal, and those tumors were large singular nodules. Corresponding to this morphological and histological analysis, the weight of the liver, while increased over that of controls, was not as large as it was when carcinogen alone was administered.

In view of these results, a 3rd experimental series was undertaken with only N-OH-FAA as carcinogen, inasmuch as the data in the 2nd series documented adequately the fact that sodium sulfate addition failed to relieve the inhibition by acetanilide of the toxicity and carcinogenicity of the parent FAA. The 3rd series with the N-hydroxy derivative, however, was expanded by the administration of 2 levels of sodium sulfate; one was equimolar to acetanilide and one was 3-fold higher. As a further control group, phosphate was given to 1 group of rats. After the 16-week feeding of the test diets, the period of observation on control diet was extended to 16 weeks, for a total of 32 weeks on treatment.
Table 1

Inhibition by dietary acetanilide of liver cancer induction in rats by FAA and N-OH-FAA

Groups of 6-week-old male Fischer F344 rats were fed the experimental diets containing carcinogens with or without acetanilide for 16 weeks, then continued on the control regimen of Wayne Lab Blox meal for 10 weeks longer. After necropsy of the rats, 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 weight (g)</th>
<th>Liver weight</th>
<th>Hyperplasia</th>
<th>Hepatoma</th>
<th>Rats with liver neoplasms (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAA</td>
<td>6</td>
<td>264 ± 7</td>
<td>15.5 ± 0.7</td>
<td>5.9</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>FAA + acetanilidet</td>
<td>5</td>
<td>293 ± 11</td>
<td>8.9 ± 0.4</td>
<td>3.0</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>N-OH-FAA</td>
<td>12</td>
<td>246 ± 5</td>
<td>16.4 ± 0.6</td>
<td>6.7</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>N-OH-FAA + acetanilide</td>
<td>12</td>
<td>290 ± 9</td>
<td>8.9 ± 0.4</td>
<td>3.1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

a Ingredients were added in the following concentrations: FAA, 0.020%; N-OH-FAA, 0.0213%; acetanilide, 0.8%.

b In Tables 1 to 3, the final no. of rats were those alive at the end of the planned experimental period of 26 weeks (Tables 1 and 2) or 32 weeks (Table 3). Early deaths and related findings are described in appropriate footnotes.

c Average weight ± S.E.

d This experiment confirms results reported previously (38, 39).

Table 2

Effect of dietary sodium sulfate on inhibition of hepatocarcinogenesis in rats by FAA or N-OH-FAA

The protocols were like those described in Table 1, except for higher levels of carcinogens and addition of sodium sulfate to the diets in some groups.

<table>
<thead>
<tr>
<th>Experimental diets</th>
<th>Final no. of rats</th>
<th>Final body weight (g)</th>
<th>Liver weight</th>
<th>Hyperplasia</th>
<th>Hepatoma</th>
<th>Rats with liver neoplasms (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAA</td>
<td>2</td>
<td>260 ± 8</td>
<td>19.2 ± 0.6</td>
<td>7.3</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>FAA + acetanilide</td>
<td>10</td>
<td>315 ± 13</td>
<td>12.2 ± 0.8</td>
<td>3.8</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>FAA + acetanilide + SO₄⁻</td>
<td>10</td>
<td>298 ± 7</td>
<td>10.8 ± 0.4</td>
<td>3.6</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>N-OH-FAA</td>
<td>8</td>
<td>237 ± 4</td>
<td>18.6 ± 0.8</td>
<td>7.8</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>N-OH-FAA + acetanilide</td>
<td>10</td>
<td>287 ± 7</td>
<td>11.6 ± 0.4</td>
<td>4.1</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>N-OH-FAA + acetanilide + SO₄⁻</td>
<td>10</td>
<td>291 ± 5</td>
<td>12.1 ± 0.3</td>
<td>4.3</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Acetanilide</td>
<td>6</td>
<td>311 ± 17</td>
<td>9.2 ± 0.6</td>
<td>2.9</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>SO₄⁻</td>
<td>5</td>
<td>312 ± 9</td>
<td>9.4 ± 0.4</td>
<td>3.0</td>
<td>5</td>
<td>0</td>
</tr>
</tbody>
</table>

a Ingredients added in following concentrations: FAA, 0.03%; N-OH-FAA, 0.032%; acetanilide, 0.8%; SO₄⁻ (sodium sulfate), 0.84%.

b Eight rats on FAA died from toxicity within 3 to 5 weeks. Toxicity of 0.03% FAA in Fischer rats was noted previously.

c Weight ± S.E.

d Two rats on N-OH-FAA died from toxicity at 5 and 8 weeks.

In confirmation of many previous experiments with N-OH-FAA alone, all surviving animals exhibited hepatoma, most of the lesions being large and multiple (Table 3). Supplementation of the carcinogen-containing diets with either sulfate ion or phosphate ion did not change the outcome of the experiment and yielded a 100% incidence of extensive liver tumors.

Acetanilide again inhibited quite severely the effect of the carcinogen on the liver with regard to the histological classification of the lesions, as well as to the relative liver to body weight. Phosphate ion did not influence this effect. On the other hand, addition of one equivalent of sulfate ion increased the number of rats displaying the more advanced lesion in each category, although the total number of animals with malignant lesions was not significantly higher. However, 3 equivalents of sulfate ion definitely raised the percentage of rats displaying malignant lesions, reflecting an increased carcinogenic effect. Also, there was a shift to more animals with hyperplastic nodules. Thus, the higher level of sulfate was more effective than the equimolar amount. Control animals with carcinogen on sodium sulfate or phosphate had completely normal livers with respect to morphology or size.

Urinary Excretion of Free and Conjugated Sulfate Esters.

Control animals fed Wayne Lab Blox excreted approximately 20 to 23 mg of inorganic sulfate per 24-hr period (Chart 1). Groups of rats on N-OH-FAA exhibited no change from this normal level. No significant increase in sulfate was detected when samples were hydrolyzed to liberate sulfate from sulfuric acid esters. Thus, the total and free sulfate levels were identical in the range of 20 to 23 mg/day, when sampled on Weeks 1, 2, 4, and 8, on the diet with carcinogen alone.

Addition of acetanilide to the diet containing carcinogen...
Sulfate Esters as Liver Carcinogens

Table 3

Restoration by dietary sulfate of liver carcinogenesis in rats in the inhibited system acetanilide + N-OH-FAA

The protocols were like those described in Table 1, except for higher levels of carcinogen, additions of sodium sulfate or sodium phosphate in some groups, 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 weight</th>
<th>Liver weight</th>
<th>Hyperplasia</th>
<th>Hepatoma</th>
<th>Rats with liver neoplasms (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-OH-FAA</td>
<td>7b</td>
<td>262 ± 14c</td>
<td>26.2 ± 4.8</td>
<td>9.7</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>N-OH-FAA + acetanilide</td>
<td>12</td>
<td>336 ± 9</td>
<td>11.4 ± 0.5</td>
<td>3.4</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>N-OH-FAA + acetanilide + SO₄</td>
<td>13</td>
<td>337 ± 6</td>
<td>12.1 ± 0.4</td>
<td>3.6</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>N-OH-FAA + acetanilide + SO₄²⁻</td>
<td>14</td>
<td>334 ± 6</td>
<td>12.5 ± 0.4</td>
<td>3.7</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>(3-fold level)</td>
<td>N-OH-FAA + acetanilide + PO₄³⁻</td>
<td>12</td>
<td>314 ± 11</td>
<td>10.8 ± 0.4</td>
<td>3.4</td>
<td>4</td>
</tr>
<tr>
<td>N-OH-FAA + SO₄²⁻</td>
<td>5</td>
<td>247 ± 6</td>
<td>22.4 ± 2.0</td>
<td>9.1</td>
<td>5</td>
<td>100</td>
</tr>
<tr>
<td>N-OH-FAA + PO₄³⁻</td>
<td>5</td>
<td>287 ± 9</td>
<td>23.8 ± 0.8</td>
<td>8.3</td>
<td>5</td>
<td>100</td>
</tr>
<tr>
<td>SO₄²⁻</td>
<td>6</td>
<td>361 ± 13</td>
<td>10.4 ± 0.4</td>
<td>2.9</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>SO₄²⁻ (3-fold level)</td>
<td>5</td>
<td>379 ± 8</td>
<td>10.7 ± 0.2</td>
<td>2.8</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>PO₄³⁻</td>
<td>6</td>
<td>363 ± 12</td>
<td>10.2 ± 0.4</td>
<td>2.8</td>
<td>6</td>
<td>0</td>
</tr>
</tbody>
</table>

a Ingredients added in following concentrations: N-OH-FAA, 0.032%; acetanilide, 0.8%; SO₄ = (sodium sulfate), 0.84%; SO₄²⁻ = (3-fold level), (sodium sulfate), 2.52%; PO₄³⁻ (disodium hydrogen phosphate), 2.25%.
b Four rats died after 4 weeks on diet; 5 others died after 24 weeks on experiment, with enlarged livers and large hepatocellular carcinomas.
c Weights ± S.E.

yielded the same levels, of the order of 20 mg/day, of total sulfate, i.e., free sulfate plus the amount liberated upon the hydrolysis of conjugates. However, significantly, this group showed very low levels of free sulfate in the urine over the entire time period. The difference between total and free sulfate measures the amount conjugated, in this instance with p-hydroxyacetanilide, which we could demonstrate by extracting this compound and establishing its presence by thin-layer chromatography.

Upon analysis of the urine of rats fed diets supplemented not only with carcinogen and acetanilide but also with an equimolar amount of sodium sulfate, higher levels of both free and total sulfate were observed. Over a period of time, this amount increased to a peak at about 4 weeks, then progressively declined. Interestingly, and as was expected, the difference in levels between total and free, mainly the conjugated sulfate, was approximately the same as in the previous group. Thus, it can be concluded that the amount of p-hydroxyacetanilide excreted as sulfate is nearly the same as that without supplementary sulfate. The picture remained the same except at a higher plateau of total and free sulfate when the dietary input was 3 equivalents of sodium sulfate.

From all these measurements it follows that the dietary levels of sulfur compounds are related to the levels of sulfate available for conjugation and excretion of conjugated and free urinary sulfates. When materials such as acetanilide, which are metabolized to hydroxylated derivatives conjugated in large measure with sulfate, are given in large doses, the endogenous sulfate pool is depleted, as evidenced by the minimal excretion of free sulfate in the urine.

Sulfotransferase in Rat Liver Nuclei. In 2 distinct experiments, rat liver nuclei isolated in an isotonic medium (data not shown) and in a hypertonic medium, and incubated with isotopic N-OH-FAA, carried label when the reaction mixture was precipitated with TCA (Table 4). The activity was...
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Table 4

<table>
<thead>
<tr>
<th>Incubation system</th>
<th>TCA precipitate</th>
<th>DNA (CsCl)</th>
<th>DNA (phenol extract)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmoles/ml</td>
<td>%</td>
<td>nmoles/ml</td>
</tr>
<tr>
<td>Complete, 0 time</td>
<td>7.0</td>
<td>3.8</td>
<td>1.8</td>
</tr>
<tr>
<td>control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complete, +F +</td>
<td>20.2</td>
<td>10.9</td>
<td>2.6</td>
</tr>
<tr>
<td>soluble fraction</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complete, +F</td>
<td>11.2</td>
<td>6.0</td>
<td>2.4</td>
</tr>
<tr>
<td>Complete, −F</td>
<td>10.8</td>
<td>5.8</td>
<td>2.6</td>
</tr>
<tr>
<td>−SO₄²⁻ +F</td>
<td>10.1</td>
<td>5.4</td>
<td>3.1</td>
</tr>
<tr>
<td>−ATP, +F</td>
<td>8.5</td>
<td>4.6</td>
<td>3.0</td>
</tr>
<tr>
<td>−ATP, −SO₄²⁻ +F</td>
<td>8.4</td>
<td>5.1</td>
<td>2.9</td>
</tr>
<tr>
<td>−ATP, −SO₄²⁻ −F</td>
<td>9.4</td>
<td>4.5</td>
<td>2.2</td>
</tr>
</tbody>
</table>

a Some tests involved addition or omission of 100 mM fluoride (+F, −F), and omission of 10 mM MgSO₄ (−SO₄²⁻) or of 10 mM ATP (−ATP). The protein content of the hypertonic nuclei was 6.64 mg/g liver, that of the soluble fraction (cell sap) was 16.6 mg/g liver.
b The data are expressed as nmoles of compound, measured as radioactivity, bound to fraction per ml reaction mixture, or as % of added isotope bound.
c Soluble fraction or cell sap was added as 0.4 ml of solution equivalent to 80 mg of liver in a reaction mixture with a total volume of 4.5 ml.

higher when fluoride ion, designed to inhibit ATPase activity and the possible deacetylation of substrate, was present. The highest labeling in this comparative series arose when a soluble fraction from rat liver containing sulfotransferase activity was added to the nuclear fraction. However, DNA as isolated on a cesium chloride gradient or by phenol extraction exhibited only very small amounts of label. The activity present was not highly sensitive to the presence or absence of additional sulfate ion or ATP, although fluoride seemed to give slightly higher activity. Even the presence of the soluble fraction affording sulfotransferase activity failed to make itself felt appreciably at the level of DNA labeling. It seemed therefore that the label observed in the TCA precipitate of the reaction mixture was chiefly membrane, rather than DNA bound. The combined experiments suggest that, if present at all under our experimental conditions, sulfotransferase occurs to a very small extent in nuclei. Of course, relatively little activity may be required to obtain significant biological effects. Furthermore, perhaps isolation of nuclei in nonaqueous media would serve to evaluate better the presence of this key enzyme with respect to hepatocarcinogenicity of N-OH-FAA.

DISCUSSION

The combined evaluation of the 3 distinct experiments indicates that N-OH-FAA hepatocarcinogenesis in rats involves the O-sulfate ester of N-OH-FAA. This interpretation is based on the following findings: (a) acetanilide inhibited N-OH-FAA carcinogenesis and was shown to reduce the urinary level of free sulfate ion; (b) addition of excess sulfate to the test diet significantly restored N-OH-FAA tumorogenesis and of course elevated urinary sulfate levels. The synthetic sulfate ester of N-OH-FAA, unlike the nonesterified compound, actively reacts with biological macromolecules (7, 24) and is also highly mutagenic (20). In addition, in rat liver there is a sulfotransferase that catalyzes the transfer of a sulfate group from 3'-phosphoadenosine-5'-phosphosulfate to N-OH-FAA (7). The sulfate ester cannot be adequately tested for carcinogenicity because of its short half-life (24). The present experiments, therefore, represent valid indirect proof of the involvement of sulfate esters in N-OH-FAA hepatocarcinogenesis which we obtained by manipulating the availability of sulfate (Chart 2).

It was found in this study that sulfate supplementation did not restore FAA carcinogenicity. The main effect of chronic acetanilide was previously shown by this laboratory to be reduction in excretion of the N-hydroxylated and other metabolites (13). Since sulfate O-esterification takes place on the hydroxyl oxygen, reduction of N-hydroxylation would diminish the substrate for esterification. Therefore, sulfate supplementation would not be expected to be effective in this situation.

The question of why additional sulfate did not completely restore liver tumor formation by N-OH-FAA in the presence of acetanilide is difficult to explain. Our results show that the liver weight was not increased much. The liver tumors, while large, were singular rather than multiple. Only a moderate number of the animals at risk exhibited malignant neoplasms, although a larger number did show more advanced lesions,
Sulfate Esters as Liver Carcinogens

Chart 2. Two steps required for the activation of FAA. First, biochemical hydroxylation leading to N-OH-FAA is inhibited by acetanilide (bottom line) because of interference at that level through the formation of hydroxylated derivatives. Second, conversion of N-OH-FAA to sulfate ester, lowered since p-hydroxyacetanilide depletes the available free sulfate pool, accounts for inhibition of carcinogenicity. Tumor induction is restored by increasing dietary sulfate level, which in turn affects 3'-phosphoadenosine-5'-phosphosulfate (PAPS; top line) required for sulfotransferase action.

such as hyperplastic nodules, as compared with the livers of animals without supplements of sulfate. While we determined that urinary sulfate levels were restored by dietary sulfate, we have no information on the intracellular situation in the liver. In connection with an unpublished study dealing with the metabolism of FAA, in sizable series of tests we found that a single i.p. dose of labeled sodium sulfate failed to yield much incorporation of isotope into a typical sulfate ester metabolite, that of N-(7-hydroxy-2-fluorenyl)acetamide, isolated by the DEAE-cellulose chromatographic procedure of Grantham (12). Repeated i.p. injections of labeled sulfate eventually gave the labeled sulfate ester of the 7-hydroxy derivative. In contrast, Büch et al. (6), in a study of metabolism of p-hydroxyacetanilide, administered sulfate i.v. and appeared to demonstrate utilization of inorganic sulfate thus administered for conjugate production and urinary excretion. In our chronic feeding series, we also found conjugated sulfate ester of p-hydroxyacetanilide. Nonetheless, the relative competitive situation inside the liver cell between sulfate conjugation of the excess p-hydroxyacetanilide and that of N-OH-FAA, even in the face of an adequate intracellular sulfate supply, may be such that less of the required carcinogen sulfate ester is made than in the uninhibited situation in which carcinogen alone is administered.

Related to the overall concept are preliminary data that p-hydroxyacetanilide, while yielding low urinary levels of free sulfate, was not as good an inhibitor of the liver cancer induction by N-OH-FAA as was acetanilide. This compound did relieve the toxicity of N-OH-FAA (8) but, as was found in connection with an examination of the capability of the isomeric acetololuidides and aminobenzoic acids of inhibiting toxicity and carcinogenicity, it was concluded that these 2 responses were probably independent at the molecular and cellular level (38). Also relevant to the overall site of action of the ultimate carcinogen is the low sulfotransferase activity found in the nuclei of liver cells, which seem to be the key locus for the expression of carcinogenicity. Pores in the nuclear membrane may permit the quick transfer of the highly reactive sulfate ester formed outside of the nucleus. Other mechanisms may operate. In any case, this problem deserves further study, perhaps under in vitro conditions.

In addition, it is by no means clear to what extent liver carcinogenesis is dependent upon the sulfate ester. The low levels of sulfotransferase in the rat mammary gland and Zymbal's gland (15), organs sensitive to N-OH-FAA, indicate that in these tissues there are probably other pathways of further activation (2, 37). Also, the lack of reactivity with nucleophilic reagents of esters of N-hydroxy-3-fluorenylacetamide, a powerful mammary but not liver carcinogen in the rat (41) suggests that the stereospecificity of the interaction of electrophilic reactants with cellular receptors is a severe controlling element in hepatocarcinogenesis.

Nonetheless, while our data offer the best possible proof of the involvement of sulfate ester in liver carcinogenesis, there are collateral data pointing in the same direction. We and others showed a number of years ago that the endocrine situation that so strongly influences liver carcinogenesis in the rat had relatively little bearing on levels of N-hydroxy derivatives. In fact, measured by urinary excretion, female rats had higher amounts compared with males (cf. Ref. 32). Modification of the endocrine balance by hypophysectomy, resulting in a striking abolition of carcinogenicity to the liver, did not correspondingly decrease the N-hydroxy derivative under acute (18) or subacute conditions (P. H. Grantham, R. S. Yamamoto, E. K. Weisburger, and J. H. Weisburger, unpublished observations). Hamsters, rabbits, and mice, clearly less susceptible to FAA liver tumor induction, elaborate substantial amounts of the N-hydroxy derivative from FAA (16, 23, 34). However, liver sulfotransferase levels parallel most remarkably the biological picture. Female rats possess much lower levels than males. Mice, hamsters, and rabbits also have low levels, denoting their lesser sensitivity to liver cancer formation, as compared with male rats (7).

Thus, as regards carcinogenicity to the liver of arylamides, like FAA, at least 2 regulating steps dictate the ultimate carcinogenic response: (a) N-hydroxylation and (b) production of the sulfate ester. Thus, 2 control points are available to modify or deflect a carcinogenic situation. We reported some years ago that, in man, N-hydroxylation appeared to be quite variable, and we recommended that where exposure to carcinogenic aromatic amines was an unavoidable hazard individuals should be selected with the lowest capability of N-hydroxylation (33). The additional suggestion can now be made that the as yet unknown variability of man with respect to sulfotransferase levels requires study, so that the least sensitive individual would have genotypically both the lowest

*See additional recent comments in "Addendum."
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*N*-hydroxylation and the lowest sulfotransferase capability and would therefore offer the least risk if he were to be exposed inadvertently.

**ACKNOWLEDGMENTS**

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**ADDENDUM**

Recently, it was reported that mild oxidation by a 1-electron oxidant, ferricyanide, converted N-OH-FAA into an intermediary radical which disproportionated into 2-nitrosofluorene and N-acetoxy-N-2-fluorenylacetamide [Bartsch et al. (3)]. Both of these products are reactive and are capable of attaching to certain cellular macromolecules. In a companion paper, Bartsch and Hecker (1) demonstrate that the same oxidation steps can be performed by hydrogen peroxide and horseradish peroxidase, although efforts to detect similar events in oxidation by liver-mixed-function oxidase were thus far negative.

**REFERENCES**


Fig. 1. Large hepatic hyperplastic nodule with focal malignant atypicality [darker staining basophilic area on (right)]. Rat was fed 0.032% N-OH-FAA. H & E, X 40.

Fig. 2. Area of malignant atypicality (right) shown in Fig. 1. Compared with hyperplastic cells on the left, the atypical cells are smaller, more basophilic, and their nuclei are more pleomorphic and hyperchromatic. H & E, X 63.

Fig. 3. Small hepatoma. Greatest diameter of tumor was 3 mm, including extension at left. Rat was fed 0.032% N-OH-FAA. H & E, X 40.

Fig. 4. Right side of small hepatoma shown in Fig. 3. Small basophilic cells with crowded, pleomorphic, hyperchromatic nuclei. H & E, X 160.
On the Sulfate Ester of \( N \)-Hydroxy-\( N\)-2-fluorenylacetamide as a Key Ultimate Hepatocarcinogen in the Rat
