On the Sulfate Ester of N-Hydroxy-N-2-fluorenylacetamide as a Key Ultimate Hepatocarcinogen in the Rat

J. H. Weisburger, R. S. Yamamoto, G. M. Williams, P. H. Grantham, T. Matsushima, and E. K. Weisburger

Experimental Pathology Branch, National Cancer Institute, NIH, Bethesda, Maryland 20014

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 (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 secure 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.

Received August 31, 1971; accepted November 19, 1971.

1Presented in part at the 61st Annual Meeting of the American Association for Cancer Research, Philadelphia, Pa., April 9 to 11, 1970 (36).
2Visiting Scientist, NIH, 1968 to 1971, Permanent address: Division of Biochemistry, National Cancer Center Research Institute, Tokyo, Japan.
3The abbreviations used are: FAA, N-2-fluorenylacetamide; N-OH-FAA, N-hydroxy-N-2-fluorenylacetamide; TCA, trichloroacetic acid.
were recorded as hepatocellular carcinomas (40), and this were processed in a conventional manner and stained with accumulating evidence that the hyperplastic nodule is as the frank hepatocellular carcinoma occupying a portion of a hyperplastic nodule with malignant atypicality (9, 10), as well additional category of focal hepatoma in a preexisting hematoxylin and eosin. The classification of lesions follows practice is followed by others (4). However, because of the additional category of focal hepatoma in a preexisting hematoxylin and eosin. The classification of lesions follows practice is followed by others (4). However, because of the additional category of focal hepatoma in a preexisting hematoxylin and eosin. The classification of lesions follows practice is followed by others (4). However, because of the additional category of focal hepatoma in a preexisting hematoxylin and eosin. The classification of lesions follows practice is followed by others (4). However, because of the additional category of focal hepatoma in a preexisting hematoxylin and eosin. The classification of lesions follows practice is followed by others (4). However, because of the additional category of focal hepatoma in a preexisting hematoxylin and eosin. The classification of lesions follows practice is followed by others (4). However, because of the additional category of focal hepatoma in a preexisting hematoxylin and eosin. The classification of lesions follows practice is followed by others (4). However, because of the additional category of focal hepatoma in a preexisting hematoxylin and eosin. The classification of lesions follows practice is followed by others (4). However, because of the additional category of focal hepatoma in a preexisting hematoxylin and eosin. The classification of lesions follows practice is followed by others (4). However, because of the
One ml of the suspension of nuclei was incubated in a
shaking incubator at 37° 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
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 + acetanilide</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>

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

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.

Average weight ± S.E.

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>9</td>
<td>1</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>14</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>1</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>

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

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

Weight ± S.E.

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 dietsa</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></td>
<td></td>
<td>g</td>
<td>g%</td>
<td>None</td>
<td>Area</td>
<td>Nodular</td>
</tr>
<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>3</td>
<td>4</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 + SO4=(3-fold level)</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>N-OH-FAA + acetanilide + PO4E</td>
<td>12</td>
<td>314 ± 11</td>
<td>10.8 ± 0.4</td>
<td>3.4</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>N-OH-FAA + SO4=</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 + PO4E</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>SO4= (3-fold level)</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>PO4E</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></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%; SO4 = (3-fold level), (sodium sulfate), 2.52%; PO4E (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.

yields 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

MARCH 1972

495

Published Online: 1 Jan, 1972

Cancer Research

Downloaded from cancerres.aacrjournals.org on January 2, 2018, © 1972 American Association for Cancer Research.
Weisburger et al.

Table 4
Search for sulfotransferase activity in rat liver nuclei; binding of isotope from N-hydroxy-N-2-fluoren-9-"C-ylacetamide to nuclear DNA

Nuclei from 3 rat livers prepared in a hypertonic medium were incubated at 37° for 60 min in complete system, or minus specific factors, with labeled N-OH-FAA. The amount of radioactivity bound to DNA was measured after isolation of DNA by 2 procedures, banding in cesium chloride or phenol extraction, as described in text.

<table>
<thead>
<tr>
<th>Incubation systema</th>
<th>Isotope bound</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TCA precipitate</td>
</tr>
<tr>
<td></td>
<td>nmoles/ml</td>
</tr>
<tr>
<td>Complete, 0 time control</td>
<td>7.0</td>
</tr>
<tr>
<td>Complete, +F + soluble fraction</td>
<td>20.2</td>
</tr>
<tr>
<td>Complete, +F</td>
<td>11.2</td>
</tr>
<tr>
<td>Complete, −F</td>
<td>10.8</td>
</tr>
<tr>
<td>−SO4 + +F</td>
<td>10.1</td>
</tr>
<tr>
<td>−ATP, +F</td>
<td>8.5</td>
</tr>
<tr>
<td>−ATP, −SO4 + +F</td>
<td>8.4</td>
</tr>
<tr>
<td>−ATP, −SO4 + −F</td>
<td>9.4</td>
</tr>
</tbody>
</table>

a Some tests involved addition or omission of 100 mM fluoride (+F, −F), and omission of 10 mM MgSO4 (−SO4) or of 10 mM ATP (−ATP). The protein content of the hypertonic nuclei was 6.46 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 tumorigenesis 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,
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 1 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 acetotoluidides 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 \textit{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.”
N-hydroxylation and the lowest sulfotransferase capability and would therefore offer the least risk if he were to be exposed inadvertently.

ACKNOWLEDGMENTS

We are indebted to the following for excellent support: Miss L. Mohan and Mrs. A. Parker, biochemical studies; Mr. T. Benjamin, Mr. F. Hood, and Miss J. Torgersen, biological studies; Miss J. Zuefle and associates, histological services; and Mrs. F. Williams and Mrs. A. Maher, secretarial and editorial functions.

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) demonstrated 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

26. Reuber, M. D. Development of Preneoplastic and Neoplastic Lesions of the Liver in Male Rats Given 0.025 Percent

498

CANCER RESEARCH VOL. 32


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


<table>
<thead>
<tr>
<th>Updated version</th>
<th>Access the most recent version of this article at: <a href="http://cancerres.aacrjournals.org/content/32/3/491">http://cancerres.aacrjournals.org/content/32/3/491</a></th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>E-mail alerts</th>
<th>Sign up to receive free email-alerts related to this article or journal.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reprints and Subscriptions</td>
<td>To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at <a href="mailto:pubs@aacr.org">pubs@aacr.org</a>.</td>
</tr>
<tr>
<td>Permissions</td>
<td>To request permission to re-use all or part of this article, use this link <a href="http://cancerres.aacrjournals.org/content/32/3/491">http://cancerres.aacrjournals.org/content/32/3/491</a>. Click on &quot;Request Permissions&quot; which will take you to the Copyright Clearance Center's (CCC) Rightslink site.</td>
</tr>
</tbody>
</table>