Mammary Carcinogenesis in the Rat by Topical Application of Fluorenylhydroxamic Acids

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SUMMARY

One topical application of 0.02 mmole of N-hydroxy-2-fluorenylacetamide to the left thoracic glands of adult female Sprague-Dawley rats gave a 70% tumor incidence at the site. The tumors appeared 3 to 6 months after the application and were mammary adenocarcinomas. In contrast, the tumor incidence after application of N-2-fluorenylacetamide was 30%, the latent period was 8 to 11 months, and the tumors were not confined to the site of application. N-Hydroxy-3-fluorenylacetamide, a strong mammary carcinogen by the i.p. route, and N-3-fluorenylacetamide, a marginally active mammary carcinogen, when applied locally showed a similar pattern of carcinogenicity as the 2-isomers. These data suggested that the fluorenylhydroxamic acids, rather than the arylamides, induce mammary tumors. Experiments comparing the microsomal N-hydroxylation of N-2- and N-3-fluorenylacetamide by mammary gland and liver showed that mammary gland microsomes, in contrast to those of liver, did not N-hydroxylate the arylamides. Experiments concerned with the sulfation of N-hydroxy-2- and -3-fluorenylacetamide by mammary gland gave no evidence that mammary gland formed the N-sulfates of N-hydroxy-2-fluorenylacetamide or of N-hydroxy-3-fluorenylacetamide. The data suggest that N-hydroxylation may be implicated in mammary carcinogenesis. However, the two-step activation mechanism believed to initiate hepatocarcinogenesis by N-2-fluorenylacetamide does not appear to be operative in mammary gland.

INTRODUCTION

2-FAA is a hepatocarcinogen for the male and a mammary carcinogen for the female Sprague-Dawley rat by systemic administration (31). The compound is N-hydroxylated by rat liver and the hydroxamic acid is then converted to an unstable ester. These reactions are believed to underlie the hepatocarcinogenic action of 2-FAA (32). It is not known whether 2-FAA induces mammary tumors by the same activation sequence that appears to be necessary for hepatocarcinogenesis. We have investigated this problem by the direct exposure of the mammary gland to 2-FAA, to N-hydroxy-2-FAA, and to the respective 3-isomers. In addition to the carcinogenicity tests, we studied the N-hydroxylation and N-sulfation of these compounds by the mammary gland and the liver of female Sprague-Dawley rats. The experiments suggested that N-hydroxylation, but not N-sulfation, may play a role in mammary carcinogenesis. The data leading to these conclusions form the basis of this report.

MATERIALS AND METHODS

Preparation of Unlabeled Compounds. N-Hydroxy-2-FAA, m.p. 150–151° (30); 2-FAA, m.p. 196–198° (35); 2-FA, m.p. 127–129° (25); N-hydroxy-3-FAA, m.p. 131–132°, and 3-FA, m.p. 150–152° (43); 3-FAA, m.p. 194–196° (39); and N-hydroxy-2-FA, m.p. 170–175° (with decomposition) (26) were prepared by the published procedures. The UV and infrared spectra of the compounds matched those of authentic samples. 3-Methylthio-2-FAA, m.p. 168–169°, was obtained from the reaction of N-acetoxy-2-FAA with D,L-methionine (27). The infrared spectrum of the compound was identical with that of authentic 3-methylthio-2-FAA. N-Hydroxy-3-FA, a new fluorenylhydroxylamine, was prepared by the procedure used for the 2-isomer (26); m.p. 118–119° (with decomposition); λmax methanol 314 (The methanol must be completely air free.), 244 nm; υmax KBr 3280 (OH) cm⁻¹; m/e 197 (M⁺). The mass spectrum showed no nitrofluorene [m/e 211 (M⁺)]. In order to obtain pure N-hydroxy-3-FA the starting material, 3-nitrofluorene, must be purified by preparative thin-layer chromatography.

C₁₃H₁₁NO
Calculated: C 79.16, H 5.62, N 7.10
Found: C 79.64, H 5.44, N 7.35

DMSO, (pharmaceutical grade, Lot 81205 C) was supplied by the Squibb Institute for Medical Research, New Brunswick, N.J.

Preparation of Labeled Compounds. 2-FAA-9-¹⁴C and N-hydroxy-2-FAA-9-¹⁴C were obtained from New England Nuclear, Boston, Mass. 2-FAA-9-¹⁴C (2.97 mCi/mmole) and N-hydroxy-2-FAA-9-¹⁴C (2.97 mCi/mmole) were mixed with unlabeled compounds and recrystallized from ethanol:water. The specific radioactivities of the recrystallized compounds were 2.81 and 7.79 mCi/mmole.
respectively. As judged by thin-layer chromatography, the labeled compounds contained no radioactive impurities after recrystallization (2). N-Hydroxy-3-FAA-9-14C, m.p. 129-131° (1.05 mCi/mmole), was prepared as described previously (44). The UV absorption spectrum of the labeled compound was identical with that of an authentic sample (43). The compound showed no radioactive impurities when it was chromatographed on silica gel (44). 3-FAA-3H, m.p. 192-194° (7.05 mCi/mmole), was prepared by acetylation of 3 fluorenamine-3H (New England Nuclear) in pyridine with acetic anhydride. The amide was recrystallized from ethanol:water. The UV spectrum of 3-FAA-3H was identical with that of an authentic sample (39) and the compound was free of radioactive contaminants as indicated by thin-layer chromatography (2).

Determination of the Sulfation of N-Hydroxy-2-FAA-1-14C and of 3-FAA-3H by Microsomes from Liver or Mammary Gland. Each incubation system contained 12 mg of microsomal protein, 12 μmoles of NADPH (Calbiochem, Los Angeles, Calif.), 240 μmoles of nicotinamide, and 600 μmoles of NaF (18). The total volume was adjusted to 6 ml with 0.01 M phosphate buffer, pH 7.4, containing 1.1% KC1. 2-FAA-9-14C or 3-FAA-3H (0.5 μmole in 0.05 ml DMSO) were added, and the mixtures were incubated in flasks open to air at 37° for 30 min. At the completion of the incubations 1 ml of methanol containing 1.0 μmole of the appropriate unlabeled compound and 4 ml of cold acetate buffer, pH 6.0, were added. The mixtures were then extracted with ether (2 × 10 ml) and the combined extracts were washed once with water. The hydroxamic acids were then extracted with ether from the ether (2 × 2 ml) and precipitated with 1 M HCl (3 ml). The precipitate was dissolved in ether (2 × 10 ml). The ether was washed with water and dried (MgSO4), and the solvent was evaporated. The residues were dissolved in methanol and chromatographed on cellulose sheets (Polygram Cel 300, Macherey-Nagel and Company, Düren, West Germany) with the upper phase of cyclohexane:terti-butyl alcohol:glacial acetic acid:water (160:5:15:20, v/v) (29). The chromatography separated N-hydroxy-2-FAA or N-hydroxy-3-FAA (Rf 0.55) from C-hydroxylated metabolites and from any contaminating amide (Rf 0.45). The radioactive compounds were located with a Model LB 2721 scanner (Brinkmann Instruments, Inc., Westbury, N.Y.). To ensure complete separation of the hydroxamic acids from the amides, the hydroxamic acids were extracted from the cellulose with methanol and rechromatographed with the above solvent. The hydroxamic acids were eluted with methanol and their specific radioactivities were determined. The labeled hydroxamic acids were reduced routinely to the corresponding amides (16) with no change of the specific radioactivity.

Preparation of Mammary Gland Parenchymal Cells. All tissue preparations were carried out in a cold room at 4°. The whole mammary gland (thoracic and inguinal-abdominal) with the associated fat pad was excised from decapitated 12-week-old rats. Fat-free parenchymal cells were prepared by the method of Moon et al. (34) except that the tissue was not sonically disrupted during the incubation with collagenase (We wish to thank Dr. R. C. Moon for suggesting this modification of the original procedure.). The average yield of parenchymal cells from 1 rat was 0.17 g of wet tissue.

Cell Fractionation and Protein Determination. The livers of the rats from which the mammary gland had been obtained were perfused with cold 0.9% NaCl solution and excised. The livers and mammary parenchymal cells were homogenized in ice-cold 0.01 M phosphate buffer, pH 7.4, containing 1.1% KCl with the use of a glass homogenizer equipped with a Teflon pestle. The microsomal and soluble fractions were obtained from the 20% (w/w) homogenates by differential centrifugation (36). The microsomes were washed once with 0.25 M sucrose and recentrifuged at 105,000 × g for 30 min (18). The protein content of the homogenates and of the cell fractions was determined by the method of Lowry et al. (28) with crystalline bovine serum albumin as a standard. Mammary gland parenchymal cells from 24 to 30 rats yielded approximately 15 mg of microsomal protein and 60 mg of soluble protein.

Animals and Maintenance. Female Sprague-Dawley rats, 6 to 12 weeks old, were purchased from the Holtzman Company, Madison, Wis. The rats receiving the test compounds were caged individually in an air-conditioned room (26°). The rats were fed a standard diet (Purina chow pellets). After the application of the compounds had been completed, they were placed on the semisynthetic diet used routinely in this laboratory for the maintenance of animals undergoing carcinogenicity tests (20). The rats used for the enzymatic assays were kept on the standard diet. The enzyme assays reflect therefore the enzyme activities of normal tissues. All animals were allowed food and water ad libitum.

Carcinogenicity Tests. The carcinogenicity of the compounds for the mammary gland was tested by the technique of Dao et al. (7). Three thoracic mammary glands located on the left side of 6- to 8-week-old rats were exposed through an incision and 0.02 μmole of each compound was applied to the 3 glands. The rats were weighed and examined for the appearance of tumors once weekly. Animals with large tumors were sacrificed prior to the termination of the tests. All rats were decapitated 12 weeks after the application of the compounds had been completed. The livers and mammary parenchymal cells were excised. The livers and mammary parenchymal cells from 24 to 30 rats yielded approximately 15 mg of microsomal protein and 60 mg of soluble protein.

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sulfation of the hydroxamic acids by mammary parenchymal cells and rat liver was estimated in the presence of cofactors (21, 22). L-Methionine or tRNA (8, 19, 21, 44) were used as acceptors of the arylamidonium ion that results from the decomposition of the ester. Tissue preparations containing the enzymes sulfating N-hydroxy-2-FAA were obtained as follows. Tissue, 1 g, was suspended in 9 g of 0.01 M phosphate buffer, pH 7.4, containing 1.1% KCl and the suspension was sonically disrupted for 0.5 min at a frequency of 20,000 Hz and at an average output power of 60 to 70 watts (Sonifier Model S-110, Branson Instruments, Inc., Danbury, Conn.). Aliquots of the sonicate (4 mg of protein) or aliquots of the soluble fraction (2 to 4 mg of protein) were added to a solution containing the cofactors [ATP (5 µmoles), MgCl₂ (8 µmoles), and Na₂SO₄ (20 µmoles)] in 0.2 ml of water, 5 mg of tRNA in 1.0 ml phosphate buffer, and 0.1 to 0.2 µmole of the labeled substrate in 0.01 ml DMSO. The volume was adjusted to 2.3 ml with buffer. In several experiments, L-methionine (10 µmoles) was used as acceptor instead of tRNA. In control experiments, the tissue preparations were replaced by an equal volume of buffer. The incubations were carried out for 1 hr at 37°C in flasks open to air.

The incubation of the mixtures containing L-methionine was terminated by the addition of 6 ml of cold acetone and 1 ml of methanol which contained 0.7 µmole of unlabeled 3-methylthio-S-2-FAA. The precipitated proteins were removed by centrifugation and the supernatant liquid was evaporated with a stream of nitrogen. After the mixture had been basified to pH 9 to 10 with NaOH, the o-CH₃-S-2-FAA-l'-14C was extracted with benzene and purified to constant specific radioactivity by thin-layer chromatography (2). The o-CH₂-S-2-FAA-l'-14C in the incubation systems containing tissue preparations was corrected for the trace amounts of methylmercaptoamide in the controls. The isolation, purification, and determination of the specific radioactivity of tRNA adducts was carried out as described previously (22, 44).

Determination of the Deacetylation of N-Hydroxy-2-FAA-9-14C and N-Hydroxy-3-FAA-9-14C. Each incubation system consisted of 0.2 µmole of the acetyl-labeled hydroxamic acid in 0.01 ml DMSO and of an aliquot of a sonicate from liver or mammary parenchymal cells. Each aliquot contained 8 µg of protein. The volume of the mixture was adjusted to 2.0 ml with 0.01 M phosphate buffer, pH 7.4, containing 1.1% KCl. The incubations were carried out for 1 hr at 37°C in flasks open to air. Sodium acetate (1.5 µmoles) in 1.0 ml of water was added, the pH was adjusted to 5.0 with 1 M HCl, and the acetic acid was distilled (1, 38). The amounts of acetic acid-14C present in incubation mixtures containing sonically disrupted tissue extracts were corrected for the amounts of acetic acid-14C in controls. The small quantities of acetic acid-14C in the controls arose very probably from partial hydrolysis of the hydroxamic acid during distillation.

Measurement of the Conversion of N-Hydroxy-2-FAA-9-14C or of N-Hydroxy-3-FAA-9-14C to 2-FA-9-14C or 3-FA-9-14H. The composition of the incubation systems was the same as described above except that N-hydroxy-2-FAA-9-14C or N-hydroxy-3-FAA-9-14C were the substrates. The incubation was terminated by the addition of 1.0 ml of methanol containing unlabeled 2-FA or 3-FA (1.0 µmole). After the mixtures had been made alkaline, the amines were extracted with ether and purified by thin-layer chromatography (2). The compounds were eluted from the chromatograms with methanol and their specific radioactivities were determined.

Estimation of 2-FA-9-l4C and o-CH₂S-2-FAA-l4C in Partial Hydrolysates of Homogenates of Mammary Tissue Incubated with N-Hydroxy-2-FAA-9-14C. The mammary tissue (glandular portion plus fat pad) from 16 rats was excised and minced with scissors. Ten % (w/w) homogenates were prepared by homogenization of the tissue in 0.01 M phosphate buffer, pH 7.4. The homogenization was carried out in an all-glass homogenizer equipped with a tight-fitting pestle. Duplicate or quadruplicate aliquots (10 ml) of the homogenate were added to 0.42 µmole of N-hydroxy-2-FAA-9-14C in 0.3 ml 95% ethanol, and the mixtures (10.3 ml) were incubated for 2 hr at 37°C in flasks open to air. At the completion of the incubation, the mixtures were frozen in Dry Ice-acetone and lyophilized. The dry solids were extracted with a mixture of absolute ethanol:ether (3:1) for 8 hr and with ether for 16 hr (17). The residues were transferred to 40-ml centrifuge tubes, moistened with 8 ml of water, and solubilized by autoclaving at 30 lb/sq in for 6 hr (17). The suspensions were adjusted to 25 ml with water and homogenized. After the protein content and the radioactivity of the homogenates had been determined, NaOH was added to a final concentration of 3 M and the mixtures remained at room temperature for 3 hr. Under these conditions, 2-FA and o-CH₂S-2-FAA are released from adducts formed by the interaction of proteins with 2-nitrosofluorene (26) and with esters of N-hydroxy-2-FAA (33), respectively. 2-FA and 3-methylthio-S-2-FAA (2 µmoles of each in 1 ml methanol) were added to the hydrolysates, and macromolecules were precipitated with absolute ethanol. The compounds were then extracted with benzene:hexane (15:85) (27) and purified by thin-layer chromatography to constant specific radioactivity (2).

Radioactivity Measurements. The radioactivity of all samples was determined in Scintisol-Complete (5 to 10 ml) (Isolab, Inc., Elkhart, Ind.) by liquid scintillation spectrometry. All samples were counted in duplicate with an error not exceeding 5%, and the counts were corrected for quenching. The counting efficiencies were 70 to 80% for 14C and 30% for 14H.

RESULTS

The carcinogenicities of N-hydroxy-2-FAA, N-hydroxy-3-FAA, and the corresponding arylamides after 1 application to the left thoracic mammary glands of female Sprague-Dawley rats are listed in Table 1. The incidence of tumors at the site of application appeared to be distinctly greater after exposure of the mammary gland to N-hydroxy-2-FAA than after exposure to 2-FAA. Thus, 5 of 7 rats receiving N-hydroxy-2-FAA developed mammary tumors all of which were confined to the site of ap-
in application. In contrast, only 1 of 6 rats receiving 2-FAA bore mammary adenocarcinomas at the site of application. One other rat exhibited a tumor arising from the inguinal-abdominal mammary glands. The location of this tumor suggested that, in this instance, 2-FAA had been absorbed into the circulation and transported to a site remote from the site of application. The differences in the carcinogenicities of the hydroxamic acid and amide were also reflected by differences in the latent period for the appearance of tumors. The latency for the induction of mammary neoplasms by N-hydroxy-2-FAA was 3 to 6 months. In the case of 2-FAA, mammary tumors were seen only 8 to 11 months after application of the compound. The tumor incidence after local application of 3-FAA and N-hydroxy-3-FAA was similar to that observed with the corresponding 2-isomers. Thus, 4 of 6 rats receiving N-hydroxy-3-FAA developed tumors at the site of application. In contrast, application of 3-FAA yielded no tumors. The high tumor yield after application of N-hydroxy-3-FAA confirmed the previous observation that N-hydroxy-3-FAA is a potent mammary carcinogen (15).

As indicated above, the low local carcinogenicity of 2-FAA in comparison to the strong carcinogenicity of N-hydroxy-2-FAA suggested that the carcinogenicity of topically applied 2-FAA was due to the conversion of the arylamide to the hydroxamic acid. In order to determine whether the weak local carcinogenicity of 2-FAA might be due to its N-hydroxylation by mammary gland, we investigated the N-hydroxylation of 2-FAA and of 3-FAA by mammary microsomes (Table 2). Since N-hydroxylation

### Table 1

<table>
<thead>
<tr>
<th>Compound applied</th>
<th>Rats with tumors/rats used</th>
<th>Tumor incidence</th>
<th>Latent perioda</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>At site of application</td>
<td>At distant sites</td>
</tr>
<tr>
<td>2-FAA</td>
<td>2/6</td>
<td>1/6 (2)*</td>
<td>1/6 (1)*</td>
</tr>
<tr>
<td>N-Hydroxy-2-FAA</td>
<td>5/7</td>
<td>5/7 (5')</td>
<td>0/7</td>
</tr>
<tr>
<td>3-FAA</td>
<td>0/6</td>
<td>0/6</td>
<td>0/6</td>
</tr>
<tr>
<td>N-Hydroxy-3-FAA</td>
<td>3/6</td>
<td>2/6 (2)*</td>
<td>1/6 (1)*</td>
</tr>
<tr>
<td>N-Hydroxy-3-FAA</td>
<td>4/6</td>
<td>4/6 (12')</td>
<td>1/6 (1')</td>
</tr>
</tbody>
</table>

a A single dose of 0.02 mmole of each compound was applied to the 3 left thoracic mammary glands as described in the text.

b The latent period (mo.) refers to the appearance of malignant lesions.

c Two mammary adenocarcinomas.

d The numbers in parentheses are the number of tumors.

e One mammary adenocarcinoma (in the left abdominal-inguinal region).

### Table 2

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>Substratea</th>
<th>Hydroxamic acid formedb (nmoles/0.5 hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>2-FAA-9-14C</td>
<td>2.01 ± 0.91 (5)*</td>
</tr>
<tr>
<td>Mammary gland</td>
<td>2-FAA-9-14C</td>
<td>&lt;0.01*</td>
</tr>
<tr>
<td>Liver</td>
<td>3-FAA-3H</td>
<td>&lt;0.06*</td>
</tr>
<tr>
<td>Mammary gland</td>
<td>3-FAA-3H</td>
<td>&lt;0.06*</td>
</tr>
</tbody>
</table>

a 0.5 μmole in 0.05 ml DMSO per 6 ml of incubation system containing 12 mg of microsomal protein.

b Determined by inverse isotope dilution.

c Specific radioactivity of 2-FAA-9-14C, 6.1 × 10⁴ dpm/μmole.

d The numbers in parentheses refer to the number of incubation systems used in each experiment.

The specific radioactivity of 2-FAA-9-14C (0.6 × 10² dpm/μmole) isolated from the incubation systems that contained mammary microsomes was of the same order of magnitude as the specific radioactivity of 2-FAA-9-14C isolated from controls that contained no microsomes.

The specific radioactivity of 3-FAA-3H, 9.4 × 10⁴ dpm/μmole.

The specific radioactivity of 2-FAA-9-14C (0.25 × 10⁴ dpm/μmole) isolated from the incubation systems that contained liver or mammary microsomes was of the same order of magnitude as the specific radioactivity of N-hydroxy-3-FAA-3H isolated from the controls that contained no microsomes.
of either compound was not detectable, it would appear that the mammary gland does not possess a microsomal enzyme that N-hydroxylates fluorenylamides. Since the female Sprague-Dawley rat converts systemically administered 2-FAA to N-hydroxy-2-FAA in the liver (16), it seemed possible that a fraction of the FAA that was applied topically might be absorbed and metabolized in the liver to N-hydroxy-2-FAA. This interpretation would be consistent with the occurrence of a tumor at a site remote from the site of application, the delayed latent period for the appearance of tumors, and the relatively low carcinogenicity of topically applied 2-FAA. Similarly, the lack of carcinogenicity of locally administered 3-FAA might be explained by the absence or the low rate of hepatic N-hydroxylation of this compound. To test this point, we studied the N-hydroxylation of 2-FAA and 3-FAA by microsomes from the livers of female rats. The N-hydroxylation of 2-FAA-9-14C by hepatic microsomes of female rats ranged from 1.3 to 3.4 nmoles per 12 mg of protein per 0.5 hr (Table 2). In contrast, 3-FAA was not N-hydroxylated under identical conditions. These experiments led us to infer that the weak activity of 2-FAA toward the mammary gland following topical application of the amide might be referable to its conversion to N-hydroxy-2-FAA in the liver and that the lack of carcinogenicity of 3-FAA might be due to the resistance of the compound to hepatic N-hydroxylation.

The neoplastic response of the mammary gland to a single application of N-hydroxy-2-FAA and N-hydroxy-3-FAA raised the question of whether the hydroxamic acids or metabolites thereof were the active carcinogens. Since N-hydroxy-2-FAA was in agreement with data published earlier by Irving et al. (19), in experiments dealing with the sulfation of N-hydroxy-3-FAA, tRNA was used as the acceptor of the hypothetical nucleophilic intermediate that would result from the decomposition of the sulfate of N-hydroxy-3-FAA. As judged by tRNA labeling, N-sulfoxy-3-FAA appeared not to be formed by the soluble fractions of mammary gland or female rat liver (Table 3). The lack of formation of N-sulfoxy-3-FAA by the soluble fraction of male rat liver has already been reported (44).

Since N-2-fluorenylhydroxylamine has been considered as an active agent initiating neoplasia (12, 21, 24), we tested the carcinogenicity of N-2-fluorenylhydroxylamine and of N-3-fluorenylhydroxylamine for the mammary gland by topical application. After exposure of the mammary gland to N-hydroxy-3-FAA, 2 of 6 rats developed tumors at the site of application while N-hydroxy-2-FAA was inactive (Table 1). The relatively low local tumor incidence, the low tumor yield, and the longer latent period (8 months) indicated that the fluorenylhydroxylamines were weaker mammary carcinogens than the respective hydroxamic acids. These data appeared to exclude fluorenylhydroxylamines as "ultimate" agents in mammary carcinogenesis by fluorenylhydroxamic acids. The possibility remained, nevertheless, that N-hydroxy-2-FAA and N-hydroxy-3-FAA were deacetylated to the respective hydroxylamines in situ and that the hydroxylamines were, in fact, the "ultimate" carcinogens, although this could not be demonstrated by local application because of limited penetration of the hydroxylamines into the mammary gland or because of other complicating factors. In the

### Table 3

<table>
<thead>
<tr>
<th>Substrate*</th>
<th>o-CH₂S-2-FAA-1-14C isolated† (nmoles/mg protein/hr)</th>
<th>14C bound (nmoles/100 mg tRNA/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>After incubation with mammary gland</td>
<td>After incubation with liver</td>
</tr>
<tr>
<td>N-Hydroxy-2-FAA-1-14C</td>
<td>&lt;0.01 ± 0.005†</td>
<td>6.14 ± 0.15†</td>
</tr>
<tr>
<td>N-Hydroxy-3-FAA-1-14C</td>
<td>0.24 ± 0.031†</td>
<td>0.30 ± 0.014†</td>
</tr>
</tbody>
</table>

*The amounts of substrate and the composition of the incubation systems are described under "Materials and Methods."

† Determined by inverse isotope dilution of 4 experiments.

‡ Mean ± S.D.

§ Means ± S.D. from 3 to 4 experiments.

If N-hydroxy-3-FAA were sulfated and the resulting ester would react with methionine in proteins in a similar fashion as N-sulfoxy-2-FAA, the alkaline degradation of the adduct would yield 2-methylthio-3-FAA or 4-methylthio-3-FAA. Since these compounds have not been synthesized, the method using tRNA as acceptor is the only one currently available to test the esterification of N-hydroxy-3-FAA.
light of these considerations, we evaluated the capacity of the mammary gland to deacetylate the carcinogenic hydroxamic acids in vitro. This was done by determining the release of $^{14}$C-labeled acetyl groups from the hydroxamic acids in unfortified sonicates. Under these conditions, the liberation of $^{14}$C-labeled acetyl groups would be a direct measure of the formation of the respective hydroxylamine. In these experiments, sonicates of mammary gland deacetylated N-hydroxy-2-FAA and N-hydroxy-3-FAA at approximately equal rates (Table 4). The deacetylation of the hydroxamic acids by sonicates of liver, carried out concurrently, was 5 to 10 times greater than the deacetylation by mammary gland sonicates indicating that hepatic deacetylase is more active than the deacetylace of mammary gland. Additional evidence for the formation of the hydroxylamines was provided by the isolation and estimation of 2-FA and 3-FA from sonicates of mammary gland and of liver. The amines arose very probably from the metabolic reduction of the hydroxylamines. The estimation of the deacetylation of the hydroxamic acids by the determination of the release of $^{14}$C-labeled acetyl groups gave 2- to 5-fold higher values than those derived from the isolation of the corresponding amines. This suggested that a substantial fraction of the hydroxylamines was metabolized to compounds other than the amines and that the determination of acetic acid-$^{14}$C in the incubation systems was a more reliable measure of deacetylation. The deacetylation of N-hydroxy-2-FAA and N-hydroxy-3-FAA demonstrated in these experiments does not permit us completely to dismiss fluorenylhydroxylamines as “ultimate” mammary carcinogens. We feel, however, that the low rate of formation of the hydroxylamines and the carcinogenicity tests argue against the view that N-hydroxy-2-FAA and N-hydroxy-3-FAA are the active species in mammary carcinogenesis by N-hydroxy-2-FAA and N-hydroxy-3-FAA.

**DISCUSSION**

Previous experiments demonstrating the carcinogenicities of 2-FAA, N-hydroxy-2-FAA, and related compounds involved prolonged administration of the compounds to the rat (9, 1, 15, 30, 33, 42). The present experiments show that a single application of a fluorenylhydroxamic acid to the mammary gland is sufficient for induction of mammary neoplasia. Heretofore, mammary carcinogenesis after a single topical application appeared to be a property of polycyclic aromatic hydrocarbons (6). The present evidence shows that the malignant transformation of the mammary gland can also be accomplished by a single topical application of fluorenylhydroxamic acids. Presumably because carcinogenesis by arylamines and arylhydroxamic acids appeared to require prolonged contact of the cell with the carcinogen, studies on the mechanism of action of N-hydroxy-2-FAA have stressed permanent or semipermanent changes observable after repeated dosages of the carcinogen (37). The induction of mammary neoplasia by a single exposure to an arylhydroxamic acid suggests that the biochemical changes specific for carcinogenesis may occur at an early time. Therefore, the study of early changes that take place in the mammary gland after the administration of these compounds may give valid clues regarding the biochemical alterations associated with carcinogenesis.

The response of the mammary gland to a single application might be useful in determining whether or not a compound is an “ultimate” carcinogen. Thus, this method of testing indicated that fluorenylhydroxylamines are probably not the active species that account for mammary carcinogenesis by fluorenylhydroxamic acids.

Although N-hydroxylation appears to be obligatory for hepatocarcinogenesis by arylamines (32), there has been no direct evidence that N-hydroxylation is also necessary for mammary carcinogenesis. The low carcinogenicities of 2-FAA and 3-FAA in comparison to the strong activities of N-hydroxy-2-FAA and N-hydroxy-3-FAA in mammary tumor induction suggested that N-hydroxylation may also be required for the initiation of neoplasia in the mammary gland. Since microsomes from mammary gland did not N-hydroxylate 2-FAA or 3-FAA, it seems likely that the liver, by virtue of its high capacity for N-hydroxylation (40), might be the tissue that performs the A'-hydroxylation necessary for mammary carcinogenesis. The low carcinogenicities of 2-FAA and 3-FAA in comparison to the strong activities of N-hydroxy-2-FAA and N-hydroxy-3-FAA in mammary tumor induction suggested that N-hydroxylation may also be required for the initiation of neoplasia in the mammary gland. Since microsomes from mammary gland did not N-hydroxylate 2-FAA or 3-FAA, it seems likely that the liver, by virtue of its high capacity for N-hydroxylation (40), might be the tissue that performs the N-hydroxylation necessary for mammary neoplasia by arylamines. This view is supported by the observation that only arylamines that are substrates for hepatic N-hydroxylation are mammary carcinogens. Thus, 2-FAA which undergoes hepatic N-hydroxylation by the female rat in vivo

<table>
<thead>
<tr>
<th>Substrate*</th>
<th>CH$_3$COOH formed* (nmoles/hr)</th>
<th>Fluorenamine formed* (nmoles/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>By mammary gland</td>
<td>By liver</td>
<td>By mammary gland</td>
</tr>
<tr>
<td>N-Hydroxy-2-FAA</td>
<td>14.9 ± 3.04</td>
<td>80.0 ± 1.76</td>
</tr>
<tr>
<td>N-Hydroxy-3-FAA</td>
<td>17.9 ± 2.48</td>
<td>187.0 ± 0.32</td>
</tr>
</tbody>
</table>

* Each incubation system contained 8.0 mg of protein and 0.2 μmole of substrate.

* N-Hydroxy-2-FAA-1-$^{14}$C (specific radioactivity, 8.42 x 10$^4$ dpm/μmole) and N-hydroxy-3-FAA-1-$^{14}$C (specific radioactivity, 1.47 x 10$^4$ dpm/μmole) were used as the substrates in these experiments. The CH$_3$COOH was determined as described in the text. The values are the means ± S.D. from 2 experiments and are corrected for the CH$_3$COOH in the controls.

* N-Hydroxy-2-FAA-9-$^3$H (specific radioactivity, 9.62 x 10$^4$ dpm/μmole) and N-hydroxy-3-FAA-9-$^3$H (specific radioactivity, 2.33 x 10$^4$ dpm/μmole) were used as the substrates in these experiments. The amines were estimated by inverse isotope dilution. The values are the means ± S.D. from 3 experiments and are corrected for the radioactivity of the controls.
Mammary Carcinogenesis by Fluorenylhydroxamic Acids

The amounts of 2-FA-9-\(^{14}\)C and \(\text{o-CH}_2\text{S-2-FAA-9-}^{14}\)C released by alkaline hydrolysis from macromolecular adducts after incubation of mammary tissue homogenates with N-hydroxy-2-FAA-9-\(^{14}\)C

<table>
<thead>
<tr>
<th>Compound isolated</th>
<th>Compound isolated(^a) (nmoles/100 mg protein)</th>
<th>Fraction of bound radioactivity released (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-FA-9-(^{14})C</td>
<td>11.3 ± 0.95</td>
<td>17.3</td>
</tr>
<tr>
<td>(\text{o-CH}_2\text{S-2-FAA-9-}^{14})C</td>
<td>0.201 ± 0.004</td>
<td>0.31</td>
</tr>
<tr>
<td>2-FA-9-(^{14})C</td>
<td>12.2 ± 1.89</td>
<td>9.02</td>
</tr>
<tr>
<td>(\text{o-CH}_2\text{S-2-FAA-9-}^{14})C</td>
<td>0.170 ± 0.055</td>
<td>0.13</td>
</tr>
</tbody>
</table>

\(^a\) Determined by inverse isotope dilution.
\(^b\) Means ± S.D. from 2 to 4 experiments.
\(^c\) These amounts were recovered from a tissue homogenate in which the macromolecular adducts had been separated from low-molecular-weight compounds by chromatography on Sephadex G-25 before hydrolysis.

Because the sulfation of N-hydroxy-2-FAA appears to be a 2nd step in the activation of arylamides (8, 16, 41), the question was raised whether carcinogenic fluorenylhydroxamic acids are activated in mammary gland by the same reaction. The experiments of Irving et al. (19) as well as the present data showed that mammary gland did not form the sulfate of N-hydroxy-2-FAA. There was also no evidence for the sulfation of N-hydroxy-3-FAA by mammary gland with methods used for the detection of N-sulfoxy-2-FAA. The formation of N-sulfoxy-3-FAA cannot be ruled out entirely, since synthetic N-sulfoxy-3-FAA is not available and the stability of the compound is unknown. The question remains whether N-sulfoxy-3-FAA would react with methionine or tRNA in the same way as N-sulfoxy-2-FAA. On the basis of the available evidence it seems implausible, however, that sulfation plays a major role in mammary carcinogenesis by fluorenylhydroxamic acids.

A mechanism that has been proposed for the initiation of neoplasia by N-hydroxy-2-FAA involves the oxidation of the hydroxamic acid to a free radical (3-5). The free radical would undergo dismutation to equimolar amounts of 2-nitrosoflourene and N-acetoxy-2-FAA, and the acetate would react with nucleophiles (33). It seems unlikely to us that this mechanism was operative in mammary carcinogenesis by N-hydroxy-2-FAA and N-hydroxy-3-FAA for the following reasons. The dismutation has been demonstrated only following oxidation of arylhydroxamic acids with oxidants and peroxidases (3-5). However, attempts to duplicate the reaction with tissue preparations were unsuccessful (3). The experiments summarized in Table 5 also argue against the operation of this mechanism in mammary tissue incubated with N-hydroxy-2-FAA-9-\(^{14}\)C. Partial hydrolysates of the protein adducts of mammary tissue contained a 60- to 70-fold excess of 2-FA over \(\text{o-CH}_2\text{S-2-FAA}\). The 2-FA was presumably derived from the interaction of 2-nitrosofluorene with sulphydryl groups of proteins (2, 26), while the methylmercaptide would come from the interaction of N-acetoxy-2-FAA with methionine in proteins (8, 33). It would appear that these data are not compatible with a mechanism according to which approximately equal amounts of the 2 compounds should be recovered.

Since we have been unable to produce any evidence that mammary tissue metabolizes N-hydroxy-2-FAA and N-hydroxy-3-FAA to derivatives identical with or similar to the ultimate agents in hepatocarcinogenesis, our data lead us to infer that the arylhydroxamic acids themselves, whether applied locally or formed metabolically, may be the active species in mammary neoplasia. Alternatively, the arylhydroxamic acids may be conjugated to O-glucuronides in the liver and transported to the mammary gland. In the case where mammary neoplasia resulted from the topical application of the arylhydroxamic acids, the O-glucuronides might be formed at the site (10) by the \(\beta\)-glucuronidase present in mammary gland (23). The \(\beta\)-glucuronide of N-hydroxy-2-FAA has already been considered as a proximate metabolite in mammary carcinogenesis by 2-FAA and N-hydroxy-2-FAA (19). Finally, there is the possibility that the arylhydroxamic acids are transformed to free radicals by a 1-electron oxidation (3, 4), and that the free radicals react directly with tissue constituents. Further studies are needed to show whether rat tissues oxidize carcinogenic arylhydroxamic acids to free radicals.

**ACKNOWLEDGMENTS**

The authors thank Peter Bell for the preparation of 3-FAA-\(^{14}\)H and Shirley Cadman and David Bronder for assistance.

*Note Added in Proof. Since this manuscript was submitted, a synthesis of N-hydroxy-3-aminofluorene by the procedure used here has been reported (Bartsch, H., Dworkin, M., Miller, J. A., and Miller, E. C. Biochim. Biophys. Acta, 286: 272-298, 1972). The compound gave essentially the same fragmentation pattern as ours, but melted at 99°.*
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Mammary Carcinogenesis by Fluorenylhydroxamic Acids

1941.


Mammary Carcinogenesis in the Rat by Topical Application of Fluorenylhydroxamic Acids

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