The Carcinogenicity of the o-Methoxy Derivatives of N-2-Fluorenylacetamide and of Related Compounds in the Rat

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SUMMARY

In order to test the idea that the lack of carcinogenicity of the o-amidofluorenols, N-(1-hydroxy-2-fluorenyl)acetamide and N-(3-hydroxy-2-fluorenyl)acetamide, is due to the hydrophilic phenolic hydroxyl group, the methylated derivatives, N-(1-methoxy-2-fluorenyl)acetamide and N-(3-methoxy-2-fluorenyl)acetamide as well as the hydrochlorides of 1-methoxy-2-fluorenamine and 3-methoxy-2-fluorenamine, were prepared, and their carcinogenicity was evaluated in the rat. N-(1-Methoxy-2-fluorenyl)acetamide and 1-methoxy-2-fluorenamine hydrochloride were inactive. The low-to-moderate carcinogenicity of N-(1-methoxy-2-fluorenyl)acetamide and 3-methoxy-2-fluorenamine hydrochloride when administered orally to male rats for 5 months, gave a tumor incidence of 27 and 50%, respectively. Approximately one-half of the lesions produced by either compound were adenocarcinomas of the small intestine. N-(3-Methoxy-2-fluorenyl)acetamide and 3-methoxy-2-fluorenamine hydrochloride were inactive. The low-to-moderate carcinogenicity of N-(1-methoxy-2-fluorenyl)acetamide and of 1-methoxy-2-fluorenamine hydrochloride contrasted sharply with the high carcinogenicity of the isomeric N-(7-methoxy-2-fluorenyl)acetamide and of N-2-fluorenamine acetamide, which were run for comparison under identical conditions. N-(7-Methoxy-2-fluorenyl)acetamide appeared to be specifically active toward the mammary gland.

The intracellular removal of the O-methyl group of N-(1-methoxy-2-fluorenyl)acetamide was demonstrated by means of N-(1-methoxy-2-fluorenyl)acetamide labeled with 14C in the methyl group. The formation of N-(1-hydroxy-2-fluorenyl)acetamide, indicated by the radioactive tracer experiments, was confirmed by the isolation of N-(1-hydroxy-2-fluorenyl)acetamide from the urine of rats dosed with N-(1-methoxy-2-fluorenyl)acetamide. The metabolic O-demethylation of N-(1-methoxy-2-fluorenyl)acetamide was compatible with the view that N-(1-hydroxy-2-fluorenyl)acetamide, when liberated in situ, may be weakly carcinogenic. However, the striking differences in the carcinogenicities, as well as in the sites of action of the o-methoxy compounds and of N-2-fluorenyl acetamide, make it exceedingly improbable that the carcinogenic activity of N-2-fluorenylacetamide is mediated through N-(1-hydroxy-2-fluorenyl)acetamide by the sequential reactions of deacetylation and oxidation.

INTRODUCTION

Several model studies from this laboratory have shown that the o-quinone imines, 2-imino-1,2-fluorenoquinone and 2-imino-3,4-fluorenoquinone, which are derived from the carcinogen N-2-fluorenylacetamide by the sequential enzymatic reactions of hydroxylation, deacetylation, and oxidation (6, 10, 12, 27, 34, 35), form stable adducts with a variety of proteins (17, 18). However, the relevance of the binding of these o-quinone imines to chemical carcinogenesis has remained obscure largely because the o-amidofluorenols, 1-OH-AAF and 3-OH-AAF were not carcinogenic by oral and intraperitoneal administration or by bladder implantation. It might be argued that the lack of activity of these o-amidofluorenols is due to the presence of the free phenolic hydroxyl group in the molecule. Because a free hydroxyl group decreases lipid solubility by conferring hydrophilic properties on the molecule (2), the entry of these compounds into the cell may be retarded. In addition, the free hydroxyl group may facilitate the formation of water-soluble conjugates and, thus, the elimination of the compounds from the cell. These factors would tend to counteract the attainment of the minimum intracellular concentration which may be required for biologic activity. Observations concerning the enhancement of the carcinogenic activity of a number of hydroxylated compounds by methylation (5, 22, 23) appear to support this line of reasoning. Once the methylated compound has penetrated the cell membrane, the methyl group would presumably be removed by intracellular demethylases and the active compound (or its precursor) would be released. Based on these considerations, the methylated derivatives of 1-OH-AAF and of 3-OH-AAF, 1-MeO-AAF and 3-MeO-AAF, respectively, were prepared (28). The present report deals with the evaluation of the carcinogenicity of these compounds in the rat.


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1 This investigation was supported by USPHS Research Grant CA-02571 from the National Cancer Institute.
rat as compared to the activity of N-2-fluorenylacetamide and of 7-MeO-AAF, a position isomer of the \( \alpha \)-methoxy derivatives of N-2-fluorenylacetamide. In addition, the \( \alpha \)-demethylation in vivo of 1-MeO-AAF which displayed some carcinogenic activity has been investigated.

MATERIALS AND METHODS

Preparation of Compounds. 1-MeO-AAF, m.p. 148–150°C, \( \Delta^{\text{max}} \) 288 m\( \mu \) (\( \varepsilon \), 24,000), and 3-MeO-AFF, m.p. 169–171°C, were prepared as described previously (28).

1-MeO-AF・HCl. 1-Methoxy-2-nitrofluorene (0.241 gm, 1 mmole), m.p. 100–102°C (28), in ethanol (25 ml) and concentrated hydrochloric acid (1 ml), was hydrogenated at room temperature and atmospheric pressure with palladium black (0.075 gm) (Nutritional Biochemicals Corp., Cleveland 28, Ohio) as a catalyst. After hydrogen uptake had ceased, the reaction mixture was filtered and the solvent was evaporated at reduced pressure. The resulting solid was dissolved in hot distilled water and filtered through celite into concentrated hydrochloric acid (3 ml). The amine hydrochloride which crystallized on cooling in an ice-bath was purified by two recrystallizations from dilute hydrochloric acid. 1-MeO-AF・HCl (0.120 gm, 45% yield) decomposed over a range from 205 to 217°C on cooling in an ice-bath was purified by two recrystallizations from dilute hydrochloric acid. 1-MeO-AF・HCl (0.120 gm, 45% yield) decomposed over a range from 205 to 217°C. The compound was recrystallized from 95% ethanol.

Calculated for \( C_{14}H_{14}NOCl \): C, 67.89; H, 5.68; N, 5.59.

Found: C, 67.89; H, 5.68; N, 5.59.

The compound was chromatographed on Whatman No. 1 paper by the descending technic with 2.4 N hydrochloric acid as the solvent. The radioactive peak which coincided with the fluorescence-quenching spot (\( t_{\text{RF}} \) = 0.46) seen on exposure of the chromatogram to ultraviolet light distance from the origin, showed a single radioactive peak which coincided with the fluorescence-quenching spot (\( t_{\text{RF}} \) = 0.46) seen on exposure of the chromatogram to ultraviolet light (2537 A), Authentic 1-MeO-AAF, run concurrently, had the same \( t_{\text{RF}} \) value. Radioassay of 1-MeO-\( ^{14} \)C-AAF before and after thin-layer chromatography on silica gel GFs4 with chloroform:methanol (97:3) as a solvent. The radioactivity profile, obtained by plotting the radioactivity against the distance from the origin, showed a single radioactive peak which coincided with the fluorescence-quenching spot (\( t_{\text{RF}} \) = 0.46) seen on exposure of the chromatogram to ultraviolet light (2537 A), Authentic 1-MeO-AAF, run concurrently, had the same \( t_{\text{RF}} \) value. Radioassay of 1-MeO-\( ^{14} \)C-AAF before and after thin-layer chromatography on silica gel GFs4 gave practically identical values for the specific radioactivity (1.07 × 10\(^ {6} \)).

CONCLUSION

The carcinogenicity of fluorenylacetamide derivatives has been investigated, and it has been found that 1-MeO-AAF is more potent than 3-MeO-AFF in inducing tumors in the rat. The 1-MeO-AAF has been shown to be a potent carcinogen in rats, with a potency similar to that of N-2-fluorenylacetamide. The 3-MeO-AFF, on the other hand, was shown to be inactive in the same system.

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\begin{align*}
N-2-\text{Fluorenylacetamide}. & N-2-\text{Fluorenylacetamide, m.p. 196-198°C, was obtained by acetylation of 2-fluorenamine, m.p. 128-130°C (19), in the usual manner (30).} \\
\text{Preparation of 1-MeO-\( ^{14} \)C-AAF.} & \text{To a solution of 1-OH-AAF (100 mg, 0.42 mmole), m.p. 210-212°C (25), in 10 ml of 0.5 N potassium hydroxide was added 2.0 mmoles of dimethyl-\( ^{14} \)C sulfate (specific radioactivity = 1.25 me/mmole) (New England Nuclear Corp., 575 Albancy Street, Boston, Mass.). After the reaction mixture had been stirred for 1 hour, 2.1 mmoles of unlabeled dimethylsulfate were added, and stirring was continued for one additional hour. The reaction mixture was centrifuged and the precipitate was washed twice with 0.5 N potassium hydroxide. The residue was dissolved in 95% ethanol, and 20 mg of unlabeled 1-MeO-AAF and a small amount of Norite A were added. The mixture was heated briefly on the steam bath and was then filtered through a layer of celite. Water was added to the filtrate to incipient turbidity. After 12 hours at 4°C, the 1-MeO-\( ^{14} \)C-AAF was collected and dried over calcium chloride. There was obtained 27.0 mg of 1-MeO-\( ^{14} \)C-AAF, m.p. 150-151°C (with softening at 138-140°C). The labeled compound was chromatographed on silica gel GFs4 with chloroform:methanol (97:3) as a solvent. The radioactivity profile, obtained by plotting the radioactivity against the distance from the origin, showed a single radioactive peak which coincided with the fluorescence-quenching spot (\( t_{\text{RF}} \) = 0.46) seen on exposure of the chromatogram to ultraviolet light (2537 A), Authentic 1-MeO-AAF, run concurrently, had the same \( t_{\text{RF}} \) value. Radioassay of 1-MeO-\( ^{14} \)C-AAF before and after thin-layer chromatography on silica gel GFs4 gave practically identical values for the specific radioactivity (1.07 × 10\(^ {6} \)).}
\end{align*}
dpm/mg and 1.02 \times 10^6 \text{ dpm/mg}, respectively). The ultraviolet absorption spectrum of the chromatographed labeled compound was superimposable on that of authentic 1-MeO-AAF (Chart 1). Material of lower specific radioactivity (3.10 \times 10^4 \text{ dpm/mg}) was obtained from the combined mother liquor and wash liquids to which carrier 1-MeO-AAF had been added. The specific radioactivity of this material (29 \text{ mg}), m.p. 148-149.5 °C, remained virtually unchanged (3.21 \times 10^4 \text{ dpm/mg}) when the compound was subjected to thin-layer chromatography on silica gel GF254 with chloroform:methanol (97:3) as a solvent. The ultraviolet absorption spectra of this material before and after thin-layer chromatography were identical and indistinguishable from the spectrum of authentic 1-MeO-AAF. Prior to the administration of 1-MeO-14C-AAF, 4- to 10-mg samples were chromatographed routinely on silica gel GF254 (1 mm thickness) with chloroform:methanol (97:3) as a solvent.

**Animals, Diets, and Administration of Compounds.** The rats used in these studies were purchased from the Holtzman Co., Madison, Wisconsin. The average weights of the male rats employed in the carcinogenicity tests are listed in Table 1. The female rats which received 1-MeO-AAF intraperitoneally weighed 50 gm initially. The weights of the adult male rats used in the metabolic experiments ranged from 220 to 300 gm. For the carcinogenicity tests the rats were caged individually at 26°C, and food and water were allowed ad libitum. During the period of carcinogen ingestion the food consumption was recorded daily. The weight of the animals was determined at intervals of one week throughout the experiments. The diets

<table>
<thead>
<tr>
<th>Compound administered</th>
<th>Duration of administration (months)</th>
<th>Average intake (mmoles/rat)</th>
<th>No. of rats used</th>
<th>No. of rats surviving</th>
<th>Average initial weight (gm)</th>
<th>Average weight gain (gm)</th>
</tr>
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<tr>
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<td></td>
<td></td>
<td>16</td>
<td>14</td>
<td>14</td>
<td>8</td>
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<tr>
<td>1-MeO-AAF</td>
<td>5.3</td>
<td>3.3</td>
<td>15</td>
<td>14</td>
<td>14</td>
<td>9</td>
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<tr>
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<td>3.4</td>
<td>14</td>
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<td>10</td>
<td>10</td>
</tr>
<tr>
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<td>14</td>
<td>11</td>
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<tr>
<td>3-MeO-AF-HCl</td>
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<td>3.5</td>
<td>14</td>
<td>11</td>
<td>11</td>
<td>6</td>
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<tr>
<td>7-MeO-AAF</td>
<td>5.1</td>
<td>2.7</td>
<td>14</td>
<td>10</td>
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<td>2</td>
</tr>
<tr>
<td>AAF</td>
<td>5.3</td>
<td>2.9</td>
<td>15</td>
<td>10</td>
<td>8</td>
<td>0</td>
</tr>
</tbody>
</table>

The effect of the oral administration of methoxy derivatives of N-2-fluorenylacetamide, 2-fluorenamine hydrochloride, and of N-2-fluorenylacetamide on body weight and survival time of the Holtzman rat.

\* The experiment was terminated after 11 months.

\* Average weight gain = average final weight — average initial weight. The final weight refers to the weight of the rats at the end of the carcinogen intake. In the case of the controls, the final weight is the weight after 5.3 months.

\* 1-MeO-AAF, N-(1-methoxy-2-fluorenyl)acetamide; 1-MeO-AF-HCl, 1-methoxy-2-fluorenamine hydrochloride; 3-MeO-AAF, N-(3-methoxy-2-fluorenyl)acetamide; 3-MeO-AF-HCl, 3-methoxy-2-fluorenamine hydrochloride; 7-MeO-AAF, N-(7-methoxy-2-fluorenyl)-acetamide; AAF, N-2-fluorenylacetamide.

<table>
<thead>
<tr>
<th>Compound administered</th>
<th>No. of rats used</th>
<th>Sex</th>
<th>Total No. of tumors</th>
<th>Number of rats with tumors\a</th>
<th>Percent tumor incidence\b</th>
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</thead>
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<tr>
<td>None</td>
<td>16</td>
<td>M</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>None</td>
<td>12</td>
<td>F</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1-MeO-AAF</td>
<td>15</td>
<td>M</td>
<td>4</td>
<td>1 (1)</td>
<td>27</td>
</tr>
<tr>
<td>1-MeO-AF-HCl</td>
<td>12</td>
<td>F</td>
<td>2</td>
<td>2 (2)</td>
<td>50</td>
</tr>
<tr>
<td>1-MeO-ACF</td>
<td>14</td>
<td>M</td>
<td>7</td>
<td>2 (2)</td>
<td>4 (4)</td>
</tr>
<tr>
<td>3-MeO-AAF</td>
<td>14</td>
<td>M</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3-MeO-AF-HCl</td>
<td>14</td>
<td>M</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7-MeO-AAF</td>
<td>14</td>
<td>M</td>
<td>17</td>
<td>5 (5)</td>
<td>93</td>
</tr>
<tr>
<td>AAF</td>
<td>15</td>
<td>M</td>
<td>36</td>
<td>12 (16)</td>
<td>100</td>
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</table>

Comparison of the carcinogenic activities of methoxy derivatives of N-2-fluorenylacetamide, 2-fluorenamine hydrochloride, and of N-2-fluorenylacetamide in the Holtzman rat.

\* The numbers in parentheses are the number of tumors.

\* Percent tumor incidence = \# of tumor-bearing rats \div \# of rats used × 100.

\* These controls were fed the basal diet only.

\* These controls were given 10 intraperitoneal injections of 0.9% NaCl containing 1.75% gum acacia.

\* These rats received the compound in the amounts stated in Table 1.

\* This lesion was a malignant lymphoma involving the lymphnodes in the kidney.

\* These rats received 10 injections of 1-MeO-AF-HCl (4.5 mg/100 gm of body weight). The average amount of compound administered was 0.21 mmole/rat.

\* For abbreviation key see Table 1.
which contained the test compounds in a concentration of 0.030% for N-2-fluorenylacetamide, and of 0.033% for the other compounds, were prepared by adding the compounds, dissolved in the minimum amount of acetone, to the standard 20% casein diet used in this laboratory (11). After the acetone had evaporated, the diets were mixed in a mechanical food mixer for 1 hour. Individual batches of the diets were prepared fresh every 10 days and stored at 4°C.

For the intraperitoneal injection of 1-MeO-AAF, the compound (4.5 mg/100 gm of body weight) was dispersed in saline containing 1.75% gum acacia by homogenization in a Potter-Elvejehm type glass homogenizer (21). The suspensions were injected 3 times weekly until 10 injections had been given. 1-MeO-14C-AAF was dispersed similarly in 2.0 ml of the saline-gum acacia vehicle and administered either intragastrically by stomach tube or intraperitoneally through a disposable needle (gauge #23). Appropriate corrections were made for losses of radioactivity in the syringe and needle or stomach tube.

Autopsies were performed routinely on all animals immediately after death or at the termination of the experiments (11 months). The tumors, the liver, the lungs, and the kidneys were fixed in buffered formalin and the sections (5-6 µ thickness) were stained with hematoxylin-eosin.

Collection of Expired 14CO2 and of Urine. Following administration of 1-MeO-14C-AAF, the rats were placed into all-glass metabolism cages (manufactured by Delmar Scientific Laboratories, Maywood, Ill.) which permitted the separate collection of respiratory CO2, urine, and feces. The air, which was drawn through the cage by means of a water aspirator, was freed of CO2 and moisture by passage through a tower (22.5 × 6.5 cm) filled with a mixture of Asearite and Drierite. The expired CO2 was collected in 2 traps which were connected in series and which contained 60 and 120 ml of 2 N sodium hydroxide, respectively. The capacity of the system was considered to be more than adequate for the collection of the respiratory CO2 since 60 ml of 2 N sodium hydroxide were found sufficient to absorb the CO2 exhaled by an adult rat during 7 hours. The 2 N sodium hydroxide solution in both traps was replaced every 6 hours, and the radioactivity of the solution in each trap was determined with the use of scintillator solution A (15). The expired CO2 was collected in 2 traps which were connected in series and which contained 60 and 120 ml of 2 N sodium hydroxide, respectively. The capacity of the system was considered to be more than adequate for the collection of the respiratory CO2 since 60 ml of 2 N sodium hydroxide were found sufficient to absorb the CO2 exhaled by an adult rat during 7 hours. The 2 N sodium hydroxide solution in each trap was tested with the use of scintillator solution A (15). The expired CO2 was collected in 2 traps which were connected in series and which contained 60 and 120 ml of 2 N sodium hydroxide, respectively. 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Distillation of urine was subjected to steam-distillation. The radioactivity of the distillate was considered to be due to volatile urinary metabolites of the methyl group, such as formic acid (30). Another aliquot of the urine was extracted 3 times with an equal volume of ether. The radioactivity of the residue remaining after evapor

Table 3

<table>
<thead>
<tr>
<th>Compound</th>
<th>Percent of administered radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Expt. I</td>
</tr>
<tr>
<td>14C in expired CO2, cumulative % of dose:</td>
<td></td>
</tr>
<tr>
<td>6 hr</td>
<td>17.0</td>
</tr>
<tr>
<td>12 hr</td>
<td>25.6</td>
</tr>
<tr>
<td>18 hr</td>
<td>28.7</td>
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<tr>
<td>24 hr</td>
<td>31.1</td>
</tr>
<tr>
<td>36 hr</td>
<td>32.9</td>
</tr>
<tr>
<td>42 hr</td>
<td>22.6</td>
</tr>
<tr>
<td>14C in 42-hour urine:</td>
<td></td>
</tr>
<tr>
<td>Water-soluble</td>
<td>19.3</td>
</tr>
<tr>
<td>Steam-distillable</td>
<td>0.36</td>
</tr>
<tr>
<td>Ether-extractable</td>
<td>0.49</td>
</tr>
</tbody>
</table>

The elimination of 14C from 1-methoxy-14C-2-fluorenylacetamide in the respiratory air and in the urine after a single oral or intraperitoneal dose to the adult Holtzman rat.

The number of the experiment corresponds to the number shown in Chart 3. The weight of compound and the amount of radioactivity administered are given in Chart 3.

Radioactivity Measurements. The radioactivity of all samples was determined in duplicate by liquid scintillation spectrometry in 22-ml low-potassium vials. The radioactivity of samples immiscible with organic solvents was measured after shaking the sample (0.5 ml to 1.0 ml) with thixotropic gel powder (0.6 gm) (Cah-O-Sil, Packard Instrument Co., Inc., Downers Grove, Ill.) and 15 ml of scintillator solution A (15). The radioactivity of samples soluble in organic solvents was assayed in 10 ml of scintillator solution B (15). All samples were counted with a standard error not exceeding 5%. Corrections for quenching were made by means of the channel ratio procedure.

Identification of 1-OH-AAF in the Urine of Rats Dosed with 1-MeO-AAF. The male adult rats used in these metabolic experiments were placed into stainless steel metabolism cages which permitted the separation of the urine from the feces. 1-MeO-AAF was administered to these animals by gastric intubation of a suspension of the compound in the saline-gum acacia vehicle. The urine was collected for 48 hours in containers packed in Dry Ice. At the end of the collection period the urine was filtered through a layer of cotton, and the filtered urine was diluted with an equal volume of deionized water. The mixture was buffered with sodium acetate (1 M, pH 6) and incubated at 37°C for 18 hours with β-glucuronidase and takadiastase (Parke, Davis and Co., Ann Arbor, Mich.) in the presence of chloroform as described previously (9). After the incubation, the mixture was acidified to pH 2-3 with concentrated hydrochloric acid, and the metabolites were partitioned by solvent extraction as shown in Chart 2. The fraction contain

8 Bacterial β-glucuronidase, Type 1, Lot #23B-612, 40,000 Fishman units/gm, and bacterial β-glucuronidase, Type II, Lot. #132B-6430, 96,000 Fishman units/gm, were obtained from the Sigma Chemical Co., St. Louis, Missouri.
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RESULTS

Carcinogenicity Tests. The data on the toxicity and carcinogenicity of the compounds tested are summarized in Tables 1 and 2. As judged by weight gain and survival time, neither the o-methoxy derivatives of N-2-fluorenylacetamide (1-MeO-AAF and 3-MeO-AAF) nor those of 2-fluorenamine (1-MeO-AF·HCl and 3-MeO-AF·HCl) were toxic for the rat. Thus, rats consuming 1-MeO-AAF or 1-MeO-AF·HCl showed a slightly greater weight gain (~10%) than the controls, and the rats ingesting 3-MeO-AAF or 3-MeO-AF·HCl were about 20% heavier than the control rats. In contrast, a 40% growth inhibition was noted in animals ingesting N-2-fluorenylacetamide or 7-MeO-AAF (Table 1). A similar growth depression (38%) had been observed previously in rats fed a 20% casein diet containing 0.030% N-2-fluorenylacetamide for 75 days ad libitum (11).

1-MeO-AAF and 1-MeO-AF·HCl proved to be carcinogens of low to moderate potency as shown by the tumor incidence (Table 2). In contrast, 3-MeO-AAF and 3-MeO-AF·HCl were inactive. The lesions produced by 1-MeO-AAF or 1-MeO-AF·HCl on oral feeding were slow-growing, single tumors which, on account of their small size, were detected only by careful inspection at autopsy. Since the oral administration of N-2-fluorenylacetamide or of derivatives thereof does not usually give rise to tumors of the small intestine, the relatively high frequency of intestinal neoplasms produced by 1-MeO-AAF or 1-MeO-AF·HCl seems noteworthy. These tumors were well-defined nodular lesions maximally 1.0-1.5 cm in diameter. In no instance was the intestinal lumen obliterated by these neoplasms (Fig. 1). Histologically, the tumors were moderately well-differentiated adenocarcinomas. There was usually an abrupt transition from normal intestinal mucosa to atypical gland shapes with other areas showing a more solidly cellular tumor (Fig. 2). Tumor glands and nests penetrated the muscularis mucosa in all instances, and in some cases the muscularis propria as well. Mitoses including abnormal mitotic figures were also readily observed (Fig. 3). The liver tumors found after ingestion of 1-MeO-AF·HCl were hepatocellular and cholangiolar carcinomas. The hepatocellular carcinoma compressed the adjacent normal liver and was characterized by lack of bile ducts and absence of a lobular pattern. The cells were arranged in cords several cell layers thick (Fig. 4). The tumor cells were larger than normal hepatocytes, and the cytoplasm appeared less structured than the cytoplasm of normal liver cells. Nuclear pleomorphism was prominent and mitotic figures were frequent (Fig. 5). The cholangiolar type of carcinoma expanded the liver lobe in which it was situated. Only a few hepatocytes were seen between solid cords and ducts of tumor cells in a fibrous stroma with polymorphonuclear leucocytes in the lumina of the ducts (Fig. 6). The shape of the cells was irregular in solid areas and columnar in areas containing duct elements. The cytoplasm of the tumor cells was paler than that of normal liver cells, and the nuclei were large and vesicular with prominent crenophilic nucleoli. The size of the nuclei varied markedly and mitotic figures were prominent (Fig. 7). The mammary tumors observed in the animals injected intraperitoneally with 1-MeO-AAF were typical adenocarcinomas which had the same histologic appearance as the mammary tumors elicited with 7-MeO-AAF (Fig. 8) or N-2-fluorenylacetamide. Some of the tumors...
contained areas of acinar or duct structure. Others were totally devoid of these structures and presented only solid masses of pleomorphic cells. The tumors were subcutaneous and fixed to the overlying skin in all cases. Areas of squamatization were noted in some of the tumors. In most of the lesions, single tumor cells as well as cords could be seen to extend into the surrounding fat. The lobular pattern of normal mammary gland tissue was replaced, at least partially, in each of the tumors (Fig. 9). The shape of the cells varied from cuboidal cells to small, irregular cells without definite cytoplasmic boundaries. Mitotic figures were numerous in all tumors. The malignant lymphoma seen in one of the rats ingesting 1-MeO-AAF was of the large cell type and consisted probably of reticulum cells. They presented a rather monotonous cell pattern with frequent mitoses and destruction of normal lymph node architecture. Tumor cells were found perivascularly in the kidney. These cells were identical with those observed in the lymph nodes.

7-MeO-AAF, a position isomer of 1-MeO-AAF or 3-MeO-AAF, in which the methoxy group occupies the extended para position, was considerably more carcinogenic for the rat than either 1-MeO-AAF or 1-MeO-AF·HCl and, on the basis of the tumor incidence, was almost as active as N-2-fluorenylacetamide. This compound had previously been shown to be particularly active in this respect. It is of interest that the majority of the tumors (72%) were of the large cell type and consisted probably of reticulum cells. They presented a rather monotonous cell pattern with frequent mitoses and destruction of normal lymph node architecture. Tumor cells were found perivascularly in the kidney. These cells were identical with those observed in the lymph nodes.

Metabolic Experiments. The purpose of the metabolic experiments was to determine whether and to what extent 1-MeO-AAF would be demethylated in vivo. If 1-MeO-AAF were demethylated, a part of the resulting 1-OH-AAF would likely be metabolized to 2-amino-1,2-fluorenoquinone by enzymatic deacetylation and oxidation in succession. This reaction sequence and the interaction of the o-quinone imine with proteins has been investigated in previous studies.

O-Demethylation of 1-MeO-14C-AAF. O-Demethylation of 1-MeO-AAF was demonstrated by the appearance of 14CO2 in the expired air after the oral or intraperitoneal administration of 1-MeO-14C-AAF. The rate of evolution of 14CO2 is shown in Chart 3 and appeared to be independent of the route of administration of the compound. Essentially all of the 14CO2 was exhaled by the rat within 36 hours after the administration of the labeled compound. The oxidation of the methyl group of 1-MeO-AAF appeared, therefore, to be completed within 32 to 36 hours following the oral or intraperitoneal administration of 1-MeO-AAF. From 20 to 33% (average, 25.1 + 5.2%) of the administered labeled methyl groups were oxidized to and eliminated as 14CO2. This value is likely to be a low estimate of the total O-demethylation since there may be other pathways for the disposition of the methyl group of 1-MeO-AAF besides oxidation to CO2. The amount of the administered radioactivity excreted in the 42-hour urine (23.1 + 3.9%) was comparable to that eliminated in the expired air (Table 3).

Most of the urinary radioactivity (95%) was water soluble and, in part, was presumably referable to conjugated metabolites of 1-MeO-AAF not extractable by ether. Only 3% of the urinary radioactivity, corresponding to 0.7% of the administered radioactivity, was distillable by steam. These data indicated that the elimination of the methyl group of 1-MeO-AAF in the form of volatile urinary compounds represented only a minor metabolic pathway. The ether-extractable radioactivity was examined for the presence of 1-MeO-14C-AAF by isotope dilution. Carrier 1-MeO-AAF was added to the ether extract of the urine and purified repeatedly by thin-layer chromatography on silica gel GF254 with chloroform:methanol (97:3) and n-hexane:acetone (6:4) or n-heptane:acetone (6:4) as solvents. Since only trace amounts of the urinary radioactivity (0.064 and 0.056% in Expts. I and II, Table 3, respectively) were due to unchanged 1-MeO-14C-AAF, 1-MeO-AAF was evidently extensively metabolized by the rat.

The formation of 1-OH-AAF in the O-demethylation of 1-MeO-AAF was confirmed by the isolation of this o-amido-fluorenol, identified as shown in Chart 4, from the appropriate urinary fraction of rats dosed with 1-MeO-AAF. After further purification of the compound by thin-layer chromatography, the identity of the isolated product was established by the ultraviolet absorption spectrum which, in all cases, was indistinguishable from that of authentic 1-OH-AAF.

Besides 1-OH-AAF, the phenolic fraction consistently contained 2 metabolites which reacted intensely with the Folin-Ciocalteu reagent or with diazotized 7-nitro-2-fluorenamine (Chart 4). As judged by the degree of quenching of the fluorescence on exposure to ultraviolet light, as well as by the size of the spots, these compounds were excreted in larger amounts than was 1-OH-AAF. Although the structural identification of
these metabolites was not further pursued, the solubility characteristics, the chromatographic behavior, and the color reaction of these compounds suggested that they were mono- or polyhydroxylated phenols derived from 1-MeO-AAF. Since these metabolites migrated at a slower rate than 1-OH-AAF, and since 5-OH-AAF as well as 7-OH-AAF in the solvent systems used here showed a similar chromatographic mobility relative to that of 1-OH-AAF (34), it is possible that these compounds were products of the ring-hydroxylation of 1-MeO-AAF involving the 5 and 7 positions of the fluorene system.

In two separate experiments in which 11.1 and 20.0 mg of 1-MeO-AAF were administered by gastric intubation, 0.079 and 0.062 mg of 1-OH-AAF, respectively, were isolated from the 48-hour urine. The calculated values are based on the urinary excretion of 5.5 ± 0.7% of intraperitoneally administered 1-OH-AAF, ranging from 1.9 to 8.2 mg. Thus, in Expt. I, 0.079 mg = 1.4 mg.

The formation of N-(1-hydroxy-2-fluorenyl)acetamide (1-OH-AAF) after ingestion of 1-methoxy-2-fluorenylacetamide (1-MeO-AAF) estimated from the demethylation of 1-methoxy-14C-2-fluorenylacetamide (1-MeO-14C-AAF) and from the urinary excretion of N-(1-hydroxy-2-fluorenyl)acetamide.

The values are based on an average demethylation of 25% as determined from the 14CO2 expired after the administration of 1-MeO-14C-AAF (Table 3). quantities of 1-MeO-AAF. The discrepancy between the calculated and the isolated quantities of 1-OH-AAF may, in large part, be attributed to the metabolism of the o-amidofluorenol by the rat (12, 31). In order to test this point experimentally, 6 rats were injected intraperitoneally with 1-OH-AAF in doses ranging from 1.9 to 8.2 mg. Only 5.5 ± 0.7% of the injected compound was recovered unchanged from the 48-hour urine. These data suggested that the major portion of the 1-OH-AAF arising in the demethylation of 1-MeO-AAF likewise was not eliminated as such by the rat. On the assumption that 5.5% of 1-OH-AAF formed in vivo was eliminated in the urine, the excretion of 0.079 and 0.062 mg of the compound would be equivalent to the formation of 1.4 and 1.1 mg of 1-OH-AAF, respectively. As shown in Table 4, these quantities of 1-OH-AAF were considerably less than would be expected from a 25% demethylation. The calculations suggested that the demethylation of 1-MeO-AAF yielded, in addition to 1-OH-AAF, relatively large amounts of other metabolites.

DISCUSSION

The O-demethylation of 1-MeO-AAF in vivo, demonstrated here, supports the view that the carcinogenicity of 1-MeO-AAF for the rat may be ascribed to the liberation of 1-OH-AAF in situ, even though this α-amidofluorenol when fed or injected is inactive (21, 26). The carcinogenic activity of 1-MeO-AF·HCl may similarly be the result of the demethylation of this α-methoxy compound to 2-amino-1-fluorenol. Since 1-OH-AAF is rapidly metabolized, as shown here in vivo and previously in vitro (12, 31), and since the formation of 2-imino-1,2-fluorenoquinone from 1-OH-AAF or from 2-amino-1-fluorenol has been reasonably well documented, it is possible that the o-quinone imine is the agent ultimately responsible for the carcinogenicity of 1-MeO-AAF or of 1-MeO-AF·HCl. The lack of carcinogenicity of 3-MeO-AAF and of 3-MeO-AF·HCl would appear to be inconsistent with the o-quinone imine hypothesis considered above as an explanation for the carcinogenicity of...

Table 4

<table>
<thead>
<tr>
<th>Expt. No.</th>
<th>1-MeO-AAF administered (mg)</th>
<th>From demethylation of 1-MeO-14C-AAF (mg)</th>
<th>From urinary excretion of 1-OH-AAF (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>11.1</td>
<td>2.6</td>
<td>1.4</td>
</tr>
<tr>
<td>II</td>
<td>20.0</td>
<td>4.7</td>
<td>1.1</td>
</tr>
</tbody>
</table>

Table 4. Formation of 1-OH-AAF from the demethylation of 1-MeO-14C-AAF and from the urinary excretion of 1-OH-AAF.
REFERENCES

N-2-fluorenylacetamide, it would be anticipated that N-hydroxylation in the interpretation of the histologic sections, and Mrs. E. Fok has been demonstrated (13, 14). The same mechanism would presumably apply to the addition of 2-imino-1,2-fluorenone to proteins and/or other tissue constituents. However, in the case of 2-imino-2,3-fluorenone which would arise from the O-demethylation of 3-MeO-AAF and from the further metabolism of the resulting o-aminofluorenel, a similar 1,4-addition mechanism cannot be written. Moreover, besides the o-quinoxaline imine hypothesis, other mechanisms should be borne in mind as underlying the action of 1-MeO-AAF and of 1-MeO-AF·HCl. It has been recognized that metabolic or synthetic N-hydroxylation converts a large number of carcinogenic and noncarcinogenic aromatic amines to highly potent arylhydroxamic acids (3, 8, 9, 21, 24) which are currently looked upon as proximate agents in carcinogenesis by aromatic amines (20). The possibility cannot be disregarded that the carcinogenicity of 1-MeO-AAF and of 1-MeO-AF·HCl was the result of the N-hydroxylation of these compounds. However, examination of paper chromatograms of the appropriate urinary fraction from rats treated with 1-MeO-AAF showed no compounds which migrated at a faster rate and, consequently, were less polar than 1-OH-AAF and which reacted either with the Folin-Coeulaet reagent or with acidic p-dimethylaminobenzaldehyde. In analogy with the chromatographic behavior of N-hydroxy-2-fluorenyl acetamide and the ring-hydroxylated derivatives of N-2-fluorenylacetamide, it would be anticipated that N-hydroxylated derivatives of 1-MeO-AAF would likewise move at a faster rate than 1-OH-AAF or any ring-hydroxylated metabolites of 1-MeO-AAF. Nevertheless, it is felt that the evidence is insufficient to discount N-hydroxylation as playing a part in the mechanism of action of these compounds.

Even if the activity of 1-MeO-AAF were largely or entirely attributable to 1-OH-AAF, the striking differences in the carcinogenicities and in the sites of action of 1-MeO-AAF and of 1-MeO-AF·HCl versus N-2-fluorenylacetamide make it exceedingly improbable that the action of N-2-fluorenylacetamide is mediated through 1-OH-AAF. As a consequence, it appears unlikely that the binding of 1-OH-AAF (or of the 2-imino-1,2-fluorenoneouine derivative therefrom) to tissue proteins in vitro (29) or in vivo (16) is causally related to the carcinogenic process initiated by N-2-fluorenylacetamide.

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Carcinogenicity of Fluorenylacacetamide Derivatives

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Fig. 1. Adenocarcinoma of small intestine from a rat treated with 1-methoxy-2-fluorenamine hydrochloride. This overall view shows normal small intestine at left and pancreas in lower left. Bizarre tumor glands are on extreme right. H & E, enlarged from x 11.

Fig. 2. Same specimen as Fig. 1. This shows zone of transition from normal intestine (left) to tumor (right). H & E, x 60.

Fig. 3. Detail of area from extreme right of Fig. 1. Note bizarre gland shapes and mitoses. Poorly differentiated tumor cells are invading serosal connective tissue. H & E, x 130.

Fig. 4. Hepatocellular carcinoma from rat treated with 1-methoxy-2-fluorenamine hydrochloride. Tumor above compresses normal liver below. H & E, reduced from x 100.

Fig. 5. Detail of tumor from Fig. 4. Note mitoses, pleomorphic nuclei, abundant cytoplasm, and solid cellular pattern. H & E, reduced from x 400.

Fig. 6. Cholangiocarcinoma from rat treated with 1-methoxy-2-fluorenamine hydrochloride. Duct-like structures are filled with neutrophils in some areas and by proliferating tumor cells in others. There is an abundant fibrous stroma. H & E, reduced from x 100.

Fig. 7. Detail of tumor from Fig. 6. Note neutrophils, fibrosis, and mitotic figures. H & E, reduced from x 400.

Fig. 8. Breast carcinoma from rat treated with N-(7-methoxy-2-fluorenyl)acetamide. Note acinar pattern with irregular duct-like structures in connective tissue. Epithelium lining ducts is very pleomorphic. H & E, reduced from x 100.

Fig. 9. Same specimen as Fig. 8 (lower right) showing two contingent lobules with extreme cellular proliferation. H & E, reduced from x 400.
The Carcinogenicity of the \( o \)-Methoxy Derivatives of \( N \)-2-Fluorenylacacetamide and of Related Compounds in the Rat

H. R. Gutmann, S. B. Galitski and W. A. Foley

*Cancer Res* 1968;28:234-244.

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