**N-Hydroxy-2-acetylaminofluorene: A Metabolite of 2-Acetylaminofluorene with Increased Carcinogenic Activity in the Rat***

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**SUMMARY**

N-Hydroxy-2-acetylaminofluorene, a major metabolite of 2-acetylaminofluorene in the rat, was more active than the parent amide in producing tumors in the liver, mammary gland, small intestine, and the ear duct of the rat. In addition, the N-hydroxy metabolite induced many papillomas and squamous-cell carcinomas of the forestomach when given in the diet. When injected intraperitoneally it produced a variety of multiple sarcomas in the peritoneum. 2-Acetylaminofluorene was inactive at the latter two sites. Dietary 3-methylcholanthrene, which is a potent inhibitor of carcinogenesis by 2-acetylaminofluorene, did not inhibit the carcinogenicity of N-hydroxy-2-acetylaminofluorene. These data provide strong evidence that N-hydroxy-2-acetylaminofluorene is one of the proximate agents in carcinogenesis by 2-acetylaminofluorene in the rat.

Recent studies have identified a conjugate of N-hydroxy-2-acetylaminofluorene (N-hydroxy-AAF) as a major metabolite of 2-acetylaminofluorene in the rat (5, 18). This metabolite was excreted in the urine in only small amounts by normal adult rats fed the carcinogen for less than 1 week or by normal adult rats given single doses intraperitoneally. However, the urinary excretion of the N-hydroxy-AAF conjugate increased with continued administration of a diet containing the carcinogenic level of 0.03 per cent of AAF, and after 6 weeks it amounted to approximately 10–15 per cent of the AAF ingested (18). Rats which were strongly protected against the carcinogenic effects of AAF by the inclusion of 0.003 per cent of 3-methylcholanthrene in the diet (16) excreted only low levels of the N-hydroxy metabolite even by the 18th week (18). The rats fed AAF, alone or with methylcholanthrene, excreted conjugates of the 1-, 3-, 5-, and 7-hydroxy derivatives of AAF in amounts similar to those reported by the Weisburgers and Morris (32) for rats given single doses of AAF. The levels of these phenolic urinary metabolites were much less affected by dietary methylcholanthrene or by the time of administration of AAF than was the level of the N-hydroxy metabolite (18). The foregoing data, together with the lesser excretion of the N-hydroxy-AAF conjugate by two rodent species more resistant to tumor induction by AAF than the rat (18), suggested that N-hydroxy-AAF might be one of the proximate agents in carcinogenesis by AAF. Further support for this concept was obtained from comparisons of the carcinogenic activities of AAF and N-hydroxy-AAF in the rat under several conditions; these data form the basis of this report.

**MATERIALS AND METHODS**

The experiments utilized either young adult male or female rats which weighed approximately 200 gm. at the beginning of the experiments or immature female rats with starting weights of...
50–70 gm. The animals were maintained in screen-bottomed cages in groups of two to four. Food and water were available ad libitum. For studying the effect of methylcholanthrene on tumor induction a semi-purified diet was used. It contained crude casein, 180 gm.; corn oil, 50 gm.; salts mixture (14), 40 gm.; glucose monohydrate (Cerelose, Corn Products Refining Co.), 729 gm.; choline chloride, 1 gm.; percomorph liver oil (Mead), 300 mg.; calcium pantothenate, 7 mg.; thiamin hydrochloride, 3 mg.; pyridoxine hydrochloride, 2.5 mg.; and riboflavin, 2.5 mg. Twenty mg. of 2-methyl-1,4-naphthoquinone was added per kg. of diet at the 16th week of the first experiment and after the 12th week in the experiments with adult animals. The 3-methylcholanthrene (Eastman) was added in solution in acetone, and the solvent was allowed to evaporate at room temperature. The diets were mixed every 5-7 days and were stored at 5°C.

For the intraperitoneal injections the compounds were suspended with the aid of a glass homogenizer1 in a solution containing 0.9 per cent of sodium chloride and either 1.75 or 7 per cent of gum acacia (31); the 7 per cent gum acacia solution was used for all the experiments with adult rats, whereas the 1.75 per cent solution was used for the experiments with young female rats. The suspensions were prepared fresh on each day of use. It was not practical to use aseptic technic throughout, but precautions were taken to minimize possible contamination. The gum acacia-saline solution was boiled, filtered rapidly through glass wool into a sterile flask, and stored at 5 ~ for no more than 1 week. All the glassware, except the homogenizers, as well as the syringes and needles were sterilized in an autoclave. Each rat was swabbed with 70 per cent ethanol prior to each injection. The suspensions contained either 10 mg. of AAF or 10.7 mg. of N-hydroxy-AAF or 1-hydroxy-AAF per ml. The adult male or female rats received 0.25–0.55 ml. of these suspensions per 100 gm. body weight 3 times/week for 4 weeks. Since the suspensions settled on standing, the suspensions were always mixed just prior to each filling of the syringes, and each time just enough was drawn into the syringe for a single rat. In each experiment the control animals received the same amounts of the gum acacia-saline solution per 100 gm. body weight.

Throughout the injection period the rats were weighed weekly, and these weights were used to determine the dose for each rat for the subsequent week. After the injections were completed in these experiments and throughout the experiments in which the compounds were fed in the diet, the rats were weighed biweekly. The animals were palpated for tumors every 2d week after the 6th week in the experiments with young female rats and after the 12th week in the experiments with adult animals.

All the rats were given terramycin (75 mg/liter) in the drinking water for 6 consecutive days at 4- to 8-week intervals to control respiratory infection.

Most of the animals were killed when death appeared imminent or at the termination of the experiments, but a few were found dead. In all cases the animals were carefully examined, and representative pieces of each tumor were fixed in neutral 10 per cent formalin, sectioned at 6 µ, and stained with hematoxylin and eosin for histological studies.

The AAF (m.p., 191°–198° C., uncorr.) was prepared in this laboratory as previously described (20). The 1-hydroxy-AAF (m.p., 200°–205° C. uncorr.) was kindly prepared for us by Dr. T. Lloyd Fletcher of the University of Washington, Seattle. Initially the N-hydroxy-AAF used in these studies was prepared as previously described (5). Later an improved procedure was devised. The details of this method are as follows: In a 500-ml. hydrogenation bottle 6.3 gm. (0.03 mole) of 2-nitrofluorene (11) was dissolved in 250 ml. ethyl acetate with the aid of mild heat and by rubbing with a glass rod. Twenty ml. of acetic anhydride, 1 gm. of 5 per cent palladium on charcoal catalyst, and 0.25 ml. of triethylamine were added, and the mixture was hydrogenated at room temperature at a pressure of 10–20 lb. above atmospheric pressure until 0.06 moles of hydrogen had been absorbed. The catalyst was removed by filtration through two thicknesses of Whatman No. 1 paper and was reused for a second hydrogenation. The filtrate was placed in a round-bottomed flask fitted with a reflux condenser, and 100 ml. of water and 100 ml. of conc. ammonium hydroxide were added. This mixture was heated in a water bath and vigorously stirred with a magnetic bar so that

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1 Duall Tissue Grinder, Size C, Kontes Glass Co., Vineland, N.J.
good mixing and rapid refluxing occurred for 20 minutes. After cooling the aqueous layer was discarded and the ethyl acetate layer was taken almost to dryness in vacuo in a water bath held at 40°–50° C.; a stream of nitrogen was used to control the boiling. The residue was dissolved in 400 ml. of ethyl ether, and the solution was extracted immediately with 200-ml and 100-ml volumes of 0.5 N sodium hydroxide. The combined alkaline extract was extracted twice with 100-ml volumes of ethyl ether, and the alkaline layer was carefully acidified to approximately pH 6 with conc. hydrochloric acid. The white flocculent precipitate of N-hydroxy-AAF was centrifuged and washed 3 times with water by suspension and centrifugation. The wet precipitate of crude N-hydroxy-AAF was then washed into 3 liters of boiling water; practically all of it dissolved in the hot water, leaving behind some brown insoluble matter. The hot solution was quickly filtered through coarse filter paper and allowed to cool below room temperature in the refrigerator. The microcrystalline precipitate was collected on Whatman No. 1 paper in a Buchner suction funnel, washed with cold water, and the funnel with its contents was dried in vacuo in the dark over sulfuric acid. The dry cake of N-hydroxy-AAF separated readily from the filter paper. The yield of crystalline product amounted to ~1–3 gm. (29–32 per cent) and melted at 144°–146° C. uncorr. when the sample was inserted in the melting point apparatus at about 135° C. and when the heating rate was 1° C. per minute. A few milligrams of the product dissolved immediately in 1 ml. of 0.5 N sodium hydroxide to give a faintly yellow but optically clear solution; this test demonstrated the absence of AAF. The product was stored in the refrigerator away from light. The hot solution was quickly filtered through coarse filter paper and allowed to cool below room temperature in the refrigerator. The microcrystalline precipitate was collected on Whatman No. 1 paper in a Buchner suction funnel, washed with cold water, and the funnel with its contents was dried in vacuo in the dark over sulfuric acid. The dry cake of N-hydroxy-AAF separated readily from the filter paper. The yield of crystalline product amounted to ~1–3 gm. (29–32 per cent) and melted at 144°–146° C. uncorr. when the sample was inserted in the melting point apparatus at about 135° C. and when the heating rate was 1° C. per minute. A few milligrams of the product dissolved immediately in 1 ml. of 0.5 N sodium hydroxide to give a faintly yellow but optically clear solution; this test demonstrated the absence of AAF. The product was stored in the refrigerator away from light. The preparation of large amounts of N-hydroxy-AAF was facilitated by processing 5–10 combined hydrogenation charges. The hydrogenated charge of N-hydroxy-AAF was more toxic than an equimolar amount of AAF. The greater toxicity was manifested by a greater weight loss at 1 month, a lesser gain at 5 months, and a higher mortality (Table 1). Because of the early deaths of four of the fifteen male rats fed N-hydroxy-AAF, the male rats fed both compounds were transferred to the control grain diet at 3 months, whereas the female rats were fed the compounds for 4 months. The experiment was terminated at 8 months.

N-Hydroxy-AAF appeared to be slightly more carcinogenic for the liver than AAF. Thus, although the livers of the male rats fed either compound showed extensive liver damage of comparable severity when the animals were examined by laparotomy at 3 months, gross liver tumors were observed in three of the eleven surviving rats fed N-hydroxy-AAF, whereas none were found in the fourteen rats fed AAF. Five and two male rats fed N-hydroxy-AAF and AAF, respectively, had malignant liver tumors by 5 months; seven rats in each group had malignant hepatic tumors when the experiment was terminated at 8 months. Two of the fourteen female rats fed N-hydroxy-AAF had malignant liver tumors at 8 months, while none were found in the female rats fed AAF in this experiment, and we have rarely seen liver tumors in female rats of this stock fed AAF for periods up to 8 months.

N-Hydroxy-AAF also induced more keratinizing squamous-cell carcinomas of the ear duct and adenocarcinomas of the small intestine than AAF. Thus, eight of the 27 rats fed AAF which survived for 3 months developed carcinomas of the ear duct by 8 months, whereas eleven of 26 rats fed N-hydroxy-AAF had these tumors. Two of 27 rats fed AAF had adenocarcinomas of the small intestine as compared with thirteen of 26 rats fed N-hydroxy-AAF. The greater incidences of ear duct and intestinal tumors in the female rats as compared with the males were probably a consequence of the longer time for which these compounds were administered to the females. On the other hand, carcinomas of the mammary gland developed more rapidly in the female rats fed AAF. Nine rats fed AAF had this type of tumor at 6 months, as compared with two female rats fed N-hydroxy-AAF; by 8 months most of the female rats either compound had one or more mammary carcinomas.

The most striking difference between the rats fed the two compounds was in the lesions in the forestomach. Twenty-three of the 26 rats which were fed N-hydroxy-AAF and which survived

4 These tumors are apparently derived from the external auditory sebaceous gland (Zymbal's gland) (12, 26).
for at least 3 months had multiple papillomas of the forestomach; ten of these rats also had squamous-cell carcinomas in the forestomach. The extent of the involvement of the forestomach varied from rat to rat; as a minimum there were five or six papillomas 1–2 mm. in diameter, and in some cases the forestomach was practically covered with papillomas and/or carcinomas (Figs. 1–8). The higher incidence of carcinomas in the female rats was probably attributable to their greater intake of N-hydroxy-AAF. By contrast, no tumors were found in the forestomach of any of the rats fed AAF, and these tumors have not all of which were carefully examined. Special attention was given to the examination of the liver, ear ducts, small intestine, forestomach, and mammary tissue.

The effect of 3-methylcholanthrene on the carcinogenicity of AAF and N-hydroxy-AAF in adult rats. —The inclusion of methylcholanthrene in the diet had no effect on tumor induction by N-hydroxy-AAF, although, as in previous experiments (16), it strongly retarded the development of tumors due to AAF (Table 2). In two identical experiments a semi-purified diet which contained 0.030 per cent of AAF or 0.032 per cent of N-hydroxy-

TABLE 1

<table>
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<tr>
<th>COMPOUND</th>
<th>SEX</th>
<th>Av. wt. change in gm.</th>
<th>Survivors</th>
<th>Liver</th>
<th>Mammary gland</th>
<th>Ear duct</th>
<th>Small int.</th>
<th>Forestomach</th>
<th>Other sites</th>
<th>Negative survivors</th>
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<td>1 0</td>
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<td>0 1 §</td>
<td>10 2</td>
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</tr>
<tr>
<td></td>
<td>F</td>
<td>190</td>
<td>5 mo. 30</td>
<td>14/15</td>
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<td>1 0</td>
<td>14</td>
<td>0 0</td>
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<td>3 mo. 41</td>
<td>11/15</td>
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<td>3 9</td>
<td>10</td>
<td>9 0 11</td>
<td>0 0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>188</td>
<td>5 mo. 22</td>
<td>15/15</td>
<td>0 0 2 0 10 9 10</td>
<td>0 0</td>
<td>15</td>
<td>0 0 11</td>
<td>0 0</td>
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<td>197</td>
<td>40 182 15/15</td>
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<td>51 15/15</td>
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<td>13</td>
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</table>

*The AAF and N-hydroxy-AAF were fed to the male rats for 3 months and to the female rats for 4 months; thereafter the rats were maintained on the control grain diet. Except for the papillomas of the forestomach, only malignant tumors are tabulated.

† Numerator equals no. of rats alive at 3 months; denominator equals no. of rats started.

‡ Liver tumor incidences at 3 and 5 months were obtained by laparotomy.

§ Leiomyosarcoma at junction between cecum and large intestine.

# A sarcoma associated with the vagina.

|| Rhabdomyosarcoma in body wall of lower abdomen.

** Two female control rats had fibroadenomas of the mammary gland.

been observed in rats fed AAF in other experiments (17, 20, 30). Histologically, the earliest change was a marked hyperkeratosis of the squamous epithelium. The papillomas (Fig. 4), which projected into the lumen of the stomach, were composed of squamous cells with a uniform appearance. In many cases the growth was directed into the submucosa (Fig. 5), whereas the tumor remained focal in type. Only when the squamous cells infiltrated the muscularis (Figs. 6 and 8) and showed atypical changes (Fig. 7) was the diagnosis of carcinoma made.

Two of the female control rats developed fibroadenomas of the mammary gland at 8 months. No other tumors were found in any of these rats, AAF with or without 0.003 per cent of methylcholanthrene was fed to adult rats for 12 weeks. The rats were subsequently maintained on the semi-purified diet without any carcinogen until the experiment was terminated at 30 weeks. Since the results for the two experiments were very similar, the data have been combined for presentation in Table 2. With this limited dose of the fluorene derivatives in the semi-purified diet tumor induction was relatively slow, and most of the tumors were not observed until close to the end of the experiment. As in the above experiment in which the rats were fed the compounds in the

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We are indebted to Miss Carole S. Wyatt for assistance with this experiment.
grain diet, the final liver tumor incidence was similar whether AAF or N-hydroxy-AAF was fed. However, whereas the addition of methylcholanthrene reduced the incidence of malignant liver tumors in rats fed AAF to one-third that obtained in the absence of methylcholanthrene, dietary methylcholanthrene did not alter the tumor incidence with N-hydroxy-AAF as the carcinogen. As in the experiment reported above, multiple papillomas of the forestomach were seen in a high proportion of the rats which were fed N-hydroxy-AAF and which survived for at least 25 weeks; papillomas were not found when AAF was fed. The incidence of papillomas was not altered by the administration of methylcholanthrene. The absence of squamous-cell carcinomas of the forestomach is apparently related to the short time of administration of N-hydroxy-AAF; the high incidence of squamous-cell carcinomas in the previous experiment (see Table 1) was found in the female rats which received the compound for 4 months.

Intraperitoneal administration of AAF and N-hydroxy-AAF to adult rats.—Preliminary trials demonstrated that N-hydroxy-AAF was much more toxic than AAF when both compounds were administered by intraperitoneal injection as an aqueous suspension. For instance, in one experiment 5 mg. of AAF per 100 gm. of body weight or an equimolar dose of N-hydroxy-AAF was injected 5 times at 48-hour intervals into male rats with initial weights of 190–205 gm. Four of sixteen rats died within 48 hours after the first injection of N-hydroxy-AAF, and five more died within the next week. None of the thirteen rats which received injections of AAF died during this time, and these rats showed an average weight gain of 8 gm. in 8 days. In experiments where the doses were equivalent to 3 mg. of AAF per 100 gm. of body weight, there was no mortality within the first 2–3 weeks, but only 60–85 per cent of the rats which received N-hydroxy-AAF survived for 3 months. Thrice weekly injections of 3 mg. of AAF per 100 gm. of body weight permitted survival of nearly all the rats in good health for longer than 3 months.

The tumor incidences for adult rats which received thrice weekly injections of AAF or N-hydroxy-AAF for 3 months are shown in Table 3. The data for the male rats are a summation of three experiments, each of which was composed of six rats treated with AAF, seven rats treated with N-hydroxy-AAF, and five control rats which received only the gum acacia-sodium chloride solution. One experiment with six to eight female rats per group was also carried out. The individual experiments were carried out with the small groups of male rats injected with either compound, except that the survival and health of the N-hydroxy-AAF-treated rats of experiment 3, which received the lowest dose of compound, was somewhat better than for experiments 1 and 2.

As shown in Table 3, when the compounds were
administered by intraperitoneal injection to adult rats, \( N \)-hydroxy-AAF was much more active at three sites than was AAF. The differences in tumor incidences are especially striking, since all the rats treated with \( N \)-hydroxy-AAF were dead by 7 months, whereas eleven of the eighteen male rats and two of the female rats treated with AAF lived until the experiment was terminated at 10 months. Squamous-cell carcinomas of the ear duct and adenocarcinomas of the small intestinal epithelium (Fig. 17) were observed in eight and six, respectively, of the rats treated with \( N \)-hydroxy-AAF as compared with two and one, respectively, of the rats which received AAF. Furthermore, fibrous reaction was evident along the peritoneal surfaces of various organs (Figs. 11, 18). The predominant cells had large spindle-shaped nuclei characteristics of fibroblasts (Fig. 10). Many of these tumors were classified as mixed mesenchymal tumors, since they contained lipo-, myxo-, rhabdo-, and chondrosarcomatous elements (Figs. 14, 15). Areas of osteoid metaplasia in fibrosarcomas (Fig. 12) and metastatic sarcomas in the lungs (Fig. 16) were also observed.

Liver damage was more extensive in the rats which received injections of \( N \)-hydroxy-AAF than in those treated with AAF and was greater in male than in female rats. These hepatic changes were

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**TABLE 3**

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>SEX INIT. WT. (Gm.)</th>
<th>AV. WT. CHANGE IN Gm.</th>
<th>SURVIVAL (3 Mo.)</th>
<th>1 Mo.</th>
<th>3 Mo.</th>
<th>7 Mo.</th>
<th>10 Mo.</th>
<th>10 Mo.</th>
<th>10 Mo.</th>
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<tr>
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<td></td>
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<td>M 181 38 66 17/21</td>
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<td>11</td>
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<td></td>
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</table>

* The data for the males are the summation of three small experiments, each of which was composed of six rats treated with AAF, seven rats treated with \( N \)-hydroxy-AAF, and five control rats which received only the gum acacia-sodium chloride solution. Since there were no significant differences among the three groups of male rats injected with either compound, except that the survival and health of the \( N \)-hydroxy-AAF-treated rats of experiment 3 were somewhat better than for the rats of experiments 1 and 2, the data for the three experiments have been summated. All the rats which received injections of equimolar amounts of the compounds three weekly according to the following schedules (doses are expressed as mg AAF/100 gm body weight/injection). See "Materials and Methods" for the preparation of the suspensions.

Males—Exp. 1: 3.5 mg. for 2 weeks, 3.5 mg. for 2 weeks, and 3.5 mg. for 9 weeks. Exp. 2: 3.5 mg. for 4 weeks, 3.5 mg. for 1 week, and 3.5 mg. for 8 weeks. Exp. 2: 0 mg. for 15 weeks.

Females—3.5 mg. for 2 weeks, 3.5 mg. for 2 weeks, and 3.5 mg. for 9 weeks.

† All the rats which received injections of \( N \)-hydroxy-AAF were dead by 7 months. Only malignant tumors are tabulated.

‡ Adenomacarcinoma of the colon found at 7 mo.

Peritoneal sarcomas were found in eighteen of the 22 rats which were injected with \( N \)-hydroxy-AAF and which survived for at least 3 months, whereas none were found in the rats similarly treated with AAF. These sarcomas were nearly always multiple, and individual nodules varied from 1–2 mm. to 1 cm. in diameter (Fig. 9). The tumors invaded the diaphragm, liver, spleen, mesentry, and body wall and were usually associated with extensive accumulations (up to 100 ml.) of clear or bloody ascitic fluid. The association between the accumulation of ascitic fluid and the sarcomas, if any, is not clear, since occasional rats died with large amounts of ascitic fluid but with no grossly detectable sarcoma. Ascitic fluid did not accumulate in the rats given injections of only the gum acacia-saline solution or of this solution containing AAF. Histologically, a marked generally similar to, but less extensive than, those seen when the compounds were fed in the diet for the same period. At early times the damage consisted of severe hydropic degeneration which involved all the zones of the liver lobules. Electron microscopic examination revealed changes in the endoplasmic reticulum and in the mitochondria. At 2–3 weeks proliferation of the bile duct-like cells was evident, and this feature became more prominent within the next 2 weeks. Malignant hepatomas were seen in two male rats which received injections of \( N \)-hydroxy-AAF and which died during the 6th and 8th months, whereas the single malignant hepatoma among the male rats which received AAF was not found until the 9th month.

6 Unpublished data by H. A. Hartmann, E. C. Miller, and J. A. Miller.
The incidences of mammary carcinomas in the female rats given injections of N-hydroxy-AAF and AAF were similar at 4 months; further comparisons were not possible, since all the N-hydroxy-AAF-injected female rats died by the 6th month. Multiple mammary carcinomas were common.

**Intraperitoneal administration of AAF and N-hydroxy-AAF to young female rats.**—Thrice weekly injections of N-hydroxy-AAF for 4 weeks into 50- to 70-gm. female rats caused the rapid appearance of a high incidence of carcinomas of the mammary gland (Table 4) (Fig. 18). These tumors developed in lower incidence and much more slowly in young rats given injections of AAF. Thus, at 15 weeks

![Image](https://cancerres.aacjournals.org/content/25/4/821)

**TABLE 4**
THE INCIDENCES OF CARCINOMA OF THE MAMMARY GLAND IN IMMATURE FEMALE RATS GIVEN INJECTIONS INTRAPERITONEALLY OF AAF OR ITS N- OR 1-HYDROXY DERIVATIVES

<table>
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<tr>
<th>Exp. no.</th>
<th>Compound</th>
<th>Initial wt. (gm.)</th>
<th>Wt. gain at 4 wk. (gm.)</th>
<th>Incidence of mammary carcinoma* at</th>
<th>Negative survivors at 37 wk.</th>
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<td>10 wk.</td>
<td>15 wk.</td>
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<td>90</td>
<td>16</td>
<td>0</td>
</tr>
</tbody>
</table>

* Each rat received thrice-weekly injections for 4 weeks. Four mg. of AAF or equimolar amounts of the N- or 1-hydroxy derivatives were injected per 100 gm. body weight in 0.4 ml. of a medium containing 1.75 per cent of gum acacia and 0.9 per cent of sodium chloride.

† The first number in each column refers to the number of rats with one or more carcinomas at the times indicated. The number in parentheses is the total number of carcinomas in the entire group of rats.

‡ Two rats from each of these groups died from respiratory infection between the 12th and 27th weeks.

§ The second experiment was terminated at 33 weeks.

in the first experiment one of ten rats treated with AAF bore a single mammary carcinoma, whereas seven of twelve rats given injections of N-hydroxy-AAF bore a total of thirteen tumors. At this same time in the second experiment all sixteen rats injected with N-hydroxy-AAF had mammary tumors (a total of 37), whereas none of the rats which had received AAF had yet developed a gross tumor. On the other hand, 1-hydroxy-AAF had no apparent toxicity and induced no mammary tumors at the dose used. The carcinogenicity of this compound was examined, since it has been suggested as a possible carcinogenic intermediate in the induction of tumors by AAF (30) and since it appears to be a direct metabolite of N-hydroxy-AAF (15).

**DISCUSSION**

Several types of data support the view that the N-hydroxylation of AAF is one of the initial steps in the carcinogenic process induced by this agent in the rat. The data presented in this paper show that N-hydroxy-AAF is more carcinogenic than AAF at four sites in the rat where AAF usually induces tumors. Furthermore, N-hydroxy-AAF is highly carcinogenic at two other sites, the peritoneum and the forestomach, where AAF does not induce tumors. Similarly, the inability of dietary 3-methylcholanthrene to inhibit the carcinogenicity of N-hydroxy-AAF contrasts strongly with the ease by which this hydrocarbon retards the carcinogenic action of AAF. Dietary methylcholanthrene also greatly lowers the urinary excretion of the N-hydroxy metabolite when it is fed with AAF to rats (18). Similarly, the guinea pig, which has proved completely resistant to the carcinogenic action of AAF (30), does not excrete detectable amounts of N-hydroxy-AAF in the urine when AAF is fed (18). Finally, the high carcinogenic activity of the N-hydroxy metabolite of AAF contrasts strikingly with the very low carcinogenicity or lack of activity of the 1-, 3-, 5-, and 7-hydroxy metabolites of AAF (23). Taken together these data provide strong evidence that metabolically derived N-hydroxy-AAF is one of the proximate agents in carcinogenesis by AAF in the rat.

It is noteworthy that N-hydroxy-AAF can induce spongy-cell carcinomas of the forestomach when fed N-hydroxy-AAF, but not AAF, in the diet. Peritoneal sarcomas developed in some hamsters after repeated intraperitoneal injections of the N-hydroxy metabolites, but none were found in hamsters which received injections of the parent amide (unpublished data by E. C. Miller and J. A. Miller).
duce a high incidence of peritoneal sarcomas, since the other tumors induced by this compound and most of the tumors induced by AAF (30) are of epithelial origin. The induction of papillomas and carcinomas in the rat forestomach by N-hydroxy-AAF was anticipated, since these tumors had been observed after the prolonged feeding of 2-nitrofluorene to rats (20). However, these tumors have not been induced by AAF. 4-Nitrostilbene and 4-aminostilbene act similarly in the rat. The nitro derivative induced tumors in the forestomach, but the amine did not, although both compounds were carcinogenic at other sites (7, 10, 28). A possible explanation is that the forestomach can reduce these nitro compounds to the N-hydroxy stage; but, when the corresponding amide or amine is given, this tissue does not form or receive enough N-hydroxy derivative for tumor induction.

While the above findings delineate the nature of an initial step in carcinogenesis by AAF, the subsequent mechanism of action remains obscure. However, new experimental approaches to the mechanism of action may arise from knowledge of the structure of a carcinogenic metabolite. Thus, the metabolism of N-hydroxy-AAF might lead rather directly to protein and nucleic acid-bound metabolites via the quinolimide of this metabolite (cf. Charts 3 and 4 in [18]). In such cases N-hydroxy-AAF would function as an arylating agent. N-Hydroxy-AAF could also be deacetylated to form N-hydroxy-2-aminofluorene; this highly reactive hydroxylamine might form a variety of protein and nucleic acid-bound derivatives (5, 18). Several other possibilities can be derived from the hydroxyamic acid nature of this carcinogenic metabolite of AAF. One of these involves the possible lossen rearrangement (27) of N-hydroxy-AAF \textit{in vivo} to yield 2-hydroxyfluorene, which is presumably noncarcinogenic (9), and methyl isocyanate. The latter substance is very reactive chemically and is highly poisonous in the rat. Its carcinogenicity has not been determined. \textit{In vivo} methyl isocyanate would be expected to acylate -OH, -NH₂, -NH, and -SH groups with the methylcarbamyl, CH₃NCHO, group and thus alter the structure of proteins and nucleic acids and their precursors. According to this concept methyl isocyanate could be formed \textit{in vivo} from any N-acetyl, N-hydroxy amine. Another possibility is that N-hydroxy-AAF chelates iron, copper, or other metals \textit{in vivo} and removes these elements from important sites (e.g., cofactors, proteins, nucleic acids) or otherwise alters their normal functions. N-hydroxy-AAF forms strong chelates with cupric and ferric ions which are stable in the presence of ethylene diaminetetraacetic acid. Concepts such as these have the attraction of suggesting a common mechanism of action for a variety of carcinogenic amines and amides.

It seems likely that N-hydroxylation is involved in the mechanism of action of many carcinogenic amines, amides, and aminoazo dyes. Recent work (33) in this laboratory has shown that in the rat N-hydroxy-4-acetylaminobiphenyl is a metabolite of the mammary carcinogen 4-acetylanilinobiphenyl (17, 20, 22) and that it is more carcinogenic than the parent amine. The corresponding free amine 4-acetylaminobiphenyl is a bladder carcinogen in the human (13) and in the dog (6, 29); the possible involvement of N-hydroxylation in these situations deserves investigation. Similarly, it is possible that N-hydroxylation is part of either the normal or abnormal metabolism of the aromatic amines derived metabolically from tryptophan; in this way it could play a role in "spontaneous" tumor formation, e.g., in the bladder (2-4, 25).

Only a few examples are known in which the metabolites of carcinogens have exhibited strong carcinogenic activities approaching or exceeding those of the parent compounds. The N,N-di-methyl- and N-monomethylaminoazo dyes are interconvertible \textit{in vivo} and have equal carcinogenic activities in the rat (19). The 1-hydroxy metabolite of 2-naphthylamine appears to be one of the proximate agents in bladder carcinogenesis by 2-naphthylamine, for upon implantation in the mouse bladder it is more carcinogenic than the parent amine (1). Prior to the present work 2-aminofluorene and 2-acetylaminofluorene were

\[ \text{Unpublished data by J. A. Miller and E. C. Miller.} \]

\[ \text{Note added in proof: In recent studies with a dog approximately 10 per cent of an oral dose of 150 mg. of 4-acetylaminobiphenyl was excreted in the urine as a conjugate of the N-hydroxy metabolite. After treatment with \beta-glucuronidase and Takadiastase the metabolite was isolated in crystalline form and characterized as N-hydroxy-4-acetylanilinobiphenyl (unpublished data by C. S. Wyatt, J. A. Miller, and E. C. Miller).} \]

\[ \text{Note added in proof: It is of considerable interest that recently W. Troll and N. Nelson (Fed. Proc., 20:41, 1961) have detected N-hydroxy-2-naphthylamine, mainly in conjugated form, in the urines of dogs and humans after oral administration of 2-naphthylamine and that E. Boyland and D. Masson (private communication from Dr. Boyland) have isolated the sulfate of N-hydroxy-2-naphthylamine from the urine of dogs given 2-naphthylamine.} \]
the only known carcinogenic metabolites of AAF. 2-Aminofluorene is interconvertible in vivo with AAF (30) and is less carcinogenic than AAF in the rat (20, 21). As yet 2-diacetylaminofluorene has been detected as a metabolite of AAF only in rat liver slices (24); it is approximately equal to AAF in its carcinogenic potency (21). N'-Hydroxy-AAF and AAF are also interconvertible in vivo in the rat (18). It is probable that N'-hydroxy-AAF is inherently more carcinogenic than it appears to be, since a considerable amount is reduced in vivo to AAF (18). Some of this AAF is then metabolized directly and irreversibly to the 3-, 5-, and 7-hydroxy derivatives (15) which are essentially inactive carcinogenically (23). N'-hydroxy-AAF also appears to be the direct precursor of the minor metabolite 1-hydroxy-AAF (15) which similarly has little if any carcinogenic activity (23) and Table 4).13

The conjugate of N'-hydroxy-AAF which is excreted in the urine by the rat may have a role in the induction of extrahaepatic tumors when AAF is administered. Thus, it seems likely that the formation of N'-hydroxy-AAF from AAF and its conversion to the urinary conjugate (possibly a glucuronide [5]) occur only in one or a few sites such as the liver. Tumor induction at distant sites could then be dependent on the release of N'-hydroxy-AAF "in situ" (possibly by a β-glucuronidase) from the conjugate arriving via the blood. Analogous mechanisms involving β-glucuronidase have been proposed by Boyland (2) and by Elson (8) to explain the selective activity of other carcinogenic aromatic amines on certain tissues.

REFERENCES


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13 In a recent experiment 1-hydroxy-AAF also failed to induce any tumors in sixteen adult male rats which received thrice-weekly intraperitoneal injections of 3.5 mg. of the compound per 100 gm. body weight for 3 months. The experiment was terminated at 8 months. All of the rats given the same dose of N'-hydroxy-AAF had died by 7 months; the incidence of peritoneal sarcomas was high among the rats which survived for at least 4 months (unpublished data by E. C. Miller and J. A. Miller).


Figs. 9-12.—Mixed sarcomas of the peritoneum from rats which received multiple intraperitoneal injections of N-hydroxy-AAF.

Fig. 9.—Multiple sarcomas on the peritoneal surface of the viscera and on the abdominal wall. Arrows point to nodules on the body wall (upper left), on the liver (upper center), on the stomach (upper right), and on the mesentery (lower right and left).

Fig. 10.—Fibrosarcoma from the peritoneal cavity. Note the large hyperchromatic nuclei (700X).

Fig. 11.—Fibrosarcoma involving the wall of the small intestine (30X).

Fig. 12.—Area of metaplastic bone in a fibrosarcoma (150X).
FIGS. 13–16.—Mixed sarcomas of the peritoneum from rats which received multiple intraperitoneal injections of N-hydroxy-AAF.

Fig. 13.—Fibrosarcoma involving the capsule of the spleen (30X).

Fig. 14.—Rhabdomyosarcoma. Note the cross-striation and contraction lines (1300X, oil).

Fig. 15.—Liposarcoma. Note the large fat cells and the mitotic figures (700X).

Fig. 16.—Pulmonary metastasis from sarcoma in the peritoneal cavity (40X).
Fig. 17.—Adenocarcinoma of the small intestine. The lesion is localized but contains mucin-producing acini which are infiltrating the muscularis (30X).

Fig. 18.—Adenocarcinoma of the breast. These tumors are generally quite well differentiated (150X).
N-Hydroxy-2-acetylaminofluorene: A Metabolite of 2-Acetylaminofluorene with Increased Carcinogenic Activity in the Rat

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