Carcinogenicity of 2-Acetylaminofluorene and N-Hydroxy-2-acetylaminofluorene in the Rabbit

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SUMMARY

Rabbits fed either 70 mg of 2-acetylaminofluorene or 50 mg of N-hydroxy-2-acetylaminofluorene daily for 56 weeks gained less weight and showed a decrease in survival time as compared to controls. Only 8/19 rabbits fed 2-acetylaminofluorene and 3/6 animals fed N-hydroxy-2-acetylaminofluorene survived for 56 weeks, whereas 12/15 control rabbits lived. Hyperplasia (in 10/19 rabbits) and squamous metaplasia (in 2/19 rabbits) of the epithelium of the urinary tract were observed in rabbits fed 2-acetylaminofluorene and hyperplasia was seen in 2/6 animals fed N-hydroxy-2-acetylaminofluorene. Tumors were found only in the urinary tract of the rabbits fed 2-acetylaminofluorene (3/19 animals with tumors) or N-hydroxy-2-acetylaminofluorene (1/6 rabbits) except for 1 adenocarcinoma of the uterus in a rabbit fed 2-acetylaminofluorene. One of 7 female control rabbits had a leiomyosarcoma of the uterus. In a second group of experiments, either 2-acetylaminofluorene or N-hydroxy-2-acetylaminofluorene (30 mg/kg) was given to rabbits intraperitoneally 3 times weekly for 40 weeks. All control rabbits (12/12) and 2-acetylaminofluorene-injected rabbits (14/14) survived 40 weeks whereas only 9/17 of the rabbits receiving N-hydroxy-2-acetylaminofluorene lived 40 weeks. Average weight gains for the 40-week period were: controls, 2.06 kg; 2-acetylaminofluorene injected, 1.58 kg; and N-hydroxy-2-acetylaminofluorene injected, 0.93 kg. The only tumor in the control rabbits was a leiomyosarcoma of the uterus. Two of 14 rabbits injected with 2-acetylaminofluorene had undifferentiated tumors in the abdominal wall and 1 had carcinoma of the urinary bladder. The rabbits injected with N-hydroxy-2-acetylaminofluorene had an incidence (10/17 rabbits) of peritoneal sarcomas. In addition, 1 had a sarcoma arising in the wall of the cecum and 1 had an adenocarcinoma of the fallopian tube. N-Hydroxy-2-acetylaminofluorene was also locally carcinogenic when injected subcutaneously as the cupric chelate, tumors being produced in 3/14 rabbits. The results of these studies in the rabbit showed that N-hydroxy-2-acetylaminofluorene was more toxic than 2-acetylaminofluorene and more carcinogenic at the site of injection, but it was not more active than 2-acetylaminofluorene as a systemic carcinogen in this species.

INTRODUCTION

AAF has been tested for carcinogenicity in a number of animal species (15) and, in general, it is a potent systemic carcinogen. Although Bonser and Green (1) reported that AAF was carcinogenic in the rabbit, only urinary tract tumors were produced in this species. Because of our interests in the comparative carcinogenicity and metabolism of AAF in the rabbit (4, 5), we wanted to extend their work to see if we could produce tumors in other tissues by the oral administration of AAF at higher dose levels given over a shorter period of time. While these studies were in progress, a great deal of experimental data bearing on the role of the N-hydroxylation of AAF, and of aromatic amines and their amides in general, in the carcinogenic process was published. These data have recently been summarized in two review articles (10, 14). Because of the role of N-hydroxy-AAF as a proximate carcinogenic metabolite of AAF and of the high urinary excretion of this metabolite (as the glucuronide) by the rabbit (4), we then undertook studies on the comparative toxicity and carcinogenicity of AAF and N-hydroxy-AAF in this species. Preliminary reports of parts of this work have appeared (5, 16).

MATERIALS AND METHODS

New Zealand white rabbits were used throughout these studies. They were housed in individual cages in a constant temperature room, fed a commercial rabbit chow (Purina), and were observed for at least one week before experimental use to ensure that they were free of respiratory disease or diarrhea.

When given orally, either 70 mg of AAF or 50 mg of N-hydroxy-AAF were packed into No. 4 gelatin capsules and administered to the rabbits by the use of a miniature balling gun which was fabricated from stainless steel. The compounds were fed to the rabbits daily (7 days/week), and control rabbits were given empty gelatin capsules. All animals were weighed weekly.

For the i.p. injections, suspensions of AAF or N-hydroxy-AAF were prepared using sterile glassware and suspending

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1 The abbreviations and nomenclature used in this paper are given below, followed in each case by the Chemical Abstracts nomenclature in parentheses: AAF, 2-acetylaminofluorene (N-2-fluorenylacetic acid); N-hydroxy-AAF, N-hydroxy-2-acetylaminofluorene (N-2-fluorenylacetic acid); N-hydroxy-2-amino-2-fluorenylhydroxylamine. See Reference 10 for definitions of the terms proximate metabolite and ultimate proximate carcinogen.
In this series, all animals were given Achromycin hydrochloride as a suspending vehicle for the i.p. injections in this series. In Series I, the suspending medium was 7% gum acacia in 0.9% NaCl and the doses, which were given in a volume of 3 ml/kg body weight, were 50.0 mg/kg for AAF and 54.0 mg/kg for N-hydroxy-AAF. Control rabbits were injected with the suspending vehicle alone. Because of the high incidence of peritonitis and localized peritoneal abscesses which developed after several months in this series even in the control group, all surviving rabbits were given 50 mg of Achromycin hydrochloride in 1 ml of 0.9% NaCl i.m. every 4th week from the 27th through the 44th week of the experiment. In Series II, the suspending medium was 0.9% NaCl. The doses, given in a volume of 3 ml/kg body weight, were 30.0 mg/kg for AAF and 32.2 mg/kg for N-hydroxy-AAF. In this series, all animals were given Achromycin hydrochloride as above except the injections were begun during the 1st week of the experiment. In both Series I and II, the rabbits were weighed weekly and the individual dose for each rabbit for the subsequent week was calculated. The injections were given 3 times weekly for 40 weeks.

Suspensions of the cupric chelate of N-hydroxy-AAF, cupric acetate, and cupric oxide in tricaprylin were prepared as described by Miller et al. (13). One ml of suspension containing 40 mg of the cupric chelate of N-hydroxy-AAF was injected into each of 3 legs of a single rabbit. The 4th leg of the rabbit served as a control and was injected with either 1 ml of tricaprylin or with 1 ml of tricaprylin containing either 14.8 mg of cupric acetate monohydrate or 5.8 mg of cupric oxide. The cupric chelate of N-hydroxy-AAF was injected weekly for either 1 week (1st leg), 3 weeks (2nd leg), or 6 weeks (3rd leg). All control injections (4th leg) were given once a week for 6 weeks. Fourteen rabbits (8 male, 6 female) were injected in this manner. The sex of the animal and the particular leg used for the injection of a specific compound for the indicated number of times were randomized prior to the start of the experiment.

For all groups, most animals were killed because of rapid and marked weight loss; some animals were found dead in the cage. As indicated in Tables 1 and 2, some control animals were sacrificed occasionally and a few rabbits were killed in order to terminate an experiment (301 weeks in Table 1, 171 weeks in Table 2). Tissues were fixed in 10% formalin, sectioned at 6–8 μ and stained with hematoxylin and eosin for histologic examination.

### RESULTS

**General Observations.** The toxicity of AAF in the rabbit was manifested by an increased mortality at the higher dose levels (Table 1) and by reduced weight gain at all dose levels (Tables 1, 2). Using weight gain and survival as criteria, N-hydroxy-AAF was more toxic than AAF.

In the group which received AAF orally (Table 1), only 8/19 (42%) rabbits survived 56 weeks; these rabbits received a total dose of 28 gm of AAF. The 56-week survival of the control rabbits in this group was 80% (12/15). There was no difference in the 56-week survival between male and female rabbits fed AAF. With a smaller number of rabbits given N-hydroxy-AAF orally (Table 1), 3/6 rabbits survived 56 weeks. The total dose of N-hydroxy-AAF in the surviving rabbits of this group was 20 gm and thus, on a molar basis, was only two-thirds the dose that the AAF-fed rabbits received.

Although there was no difference in the rate of survival of the control rabbits and those receiving the lower doses of AAF i.p., there was an increased mortality in the animals given N-hydroxy-AAF i.p. (Table 2). The surviving rabbits in Series I of this group received an average of 16 gm of AAF or 15 gm of N-hydroxy-AAF (only 1 rabbit survived) over a period of 40 weeks. The surviving animals in Series II received an average of 11 gm of AAF or N-hydroxy-AAF. In Series I of this group, the survival of the control rabbits was poor (5/9, 55%). This was attributed to the toxicity of the gum acacia which was used as a suspending vehicle for the i.p. injections in this series. Rabbits injected with gum acacia showed peritonitis and localized peritoneal abscesses, fibrous adhesions, ascites, enlarge-

### TABLE 1

<table>
<thead>
<tr>
<th>Compound administered</th>
<th>Sex</th>
<th>No. of animals (alive/start)</th>
<th>Av. wt. gain (kg) at</th>
<th>Survival time (wk.)</th>
<th>Epithelial changes in the urinary tract</th>
<th>Other tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>20 wk.</td>
<td>56 wk.</td>
<td>20 wk.</td>
<td>56 wk.</td>
<td>Mean</td>
</tr>
<tr>
<td>None</td>
<td>M</td>
<td>8/8</td>
<td>6/6</td>
<td>1.83 ± 0.11*</td>
<td>2.31 ± 0.15</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>7/7</td>
<td>6/7</td>
<td>2.05 ± 0.19</td>
<td>2.21 ± 0.24</td>
<td>90</td>
</tr>
<tr>
<td>AAF</td>
<td>M</td>
<td>9/9</td>
<td>4/9</td>
<td>1.34 ± 0.13</td>
<td>1.82 ± 0.15</td>
<td>66</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>10/10</td>
<td>4/10</td>
<td>1.51 ± 0.15</td>
<td>1.53 ± 0.25</td>
<td>72</td>
</tr>
<tr>
<td>N-Hydroxy-AAF</td>
<td>M</td>
<td>6/6</td>
<td>3/6</td>
<td>1.63 ± 0.17</td>
<td>1.48 ± 0.54</td>
<td>84</td>
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</tbody>
</table>

* AAF, 2-acetylaminofluorene; N-hydroxy-AAF, N-hydroxy-2-acetylaminofluorene.

Standard error of the mean.

Low-grade leiomyosarcoma of the uterus.

The numbers in parentheses are the survival times, in weeks, of the animals with the tumors indicated.

Adenocarcinoma of the uterus.
Charles C. Irving, Ralph Wiseman, Jr., and Joseph M. Young

TABLE 2
Incidence of Tumors in Rabbits Which Were Given Intraperitoneal Injections of AAF or N-Hydroxy-AAF for 40 Weeks

<table>
<thead>
<tr>
<th>Compound administered</th>
<th>Sex</th>
<th>Initial body wt. (kg)</th>
<th>Wt. gain at 40 wk. (kg)</th>
<th>Number of animals (alive/start) at 40 wk.</th>
<th>Wt. gain at 40 wk. (kg)</th>
<th>No. of animals (alive/start) at 40 wk.</th>
<th>Peritoneum (sarcoma)</th>
<th>Bladder (carcinoma)</th>
<th>Other tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Series I</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>M</td>
<td>1.39 ± 0.07</td>
<td>1.43 ± 0.32</td>
<td>5/9</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>AAF</td>
<td>M</td>
<td>1.35 ± 0.08</td>
<td>1.70 ± 0.22</td>
<td>6/9</td>
<td>0</td>
<td>2 (30, 64)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>N-Hydroxy-AAF</td>
<td>M</td>
<td>1.33 ± 0.04</td>
<td>1.35</td>
<td>1/8</td>
<td>2 (29, 31)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Series II</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>None</td>
<td>M</td>
<td>2.02 ± 0.05</td>
<td>2.19 ± 0.08</td>
<td>6/6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>AAF</td>
<td>F</td>
<td>1.91 ± 0.09</td>
<td>1.93 ± 0.20</td>
<td>6/6</td>
<td>0</td>
<td>1* (60)</td>
<td>0</td>
<td>1* (107)</td>
<td></td>
</tr>
<tr>
<td>N-Hydroxy-AAF</td>
<td>M</td>
<td>2.05 ± 0.09</td>
<td>1.74 ± 0.19</td>
<td>7/7</td>
<td>0</td>
<td>1 (99)</td>
<td>1* (171)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-Hydroxy-AAF</td>
<td>F</td>
<td>1.90 ± 0.06</td>
<td>1.43 ± 0.18</td>
<td>7/7</td>
<td>0</td>
<td>1 (99)</td>
<td>1* (171)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* AAF, 2-acetylaminofluorene; N-hydroxy-AAF, N-hydroxy-2-acetylaminofluorene.

† Standard error of the mean.

‡ The numbers in parentheses are the survival times, in weeks, of the animals with the tumors indicated.

§ Low-grade leiomyosarcoma of the uterus.

¶ Undifferentiated tumor arising in the wall of the abdomen.

⁄ Sarcoma arising in the wall of the cecum.

There was no significant difference in either the survival or weight gain of control rabbits and the group of rabbits given the subcutaneous injections of the cupric chelate of N-hydroxy-AAF. Thus, 13/14 rabbits in this group survived 48 weeks and had an average body weight of 4.02 ± 0.14 kg at this time. At a comparable time, 12/15 control rabbits survived and had an average body weight of 3.96 ± 0.06 kg (males) or 3.92 ± 0.14 kg (females).

Hyperplasia and Neoplastic Changes in the Epithelium of the Urinary Tract. One of the most frequent pathologic changes observed histologically in rabbits given AAF or N-hydroxy-AAF was hyperplasia of the transitional cell lining of the renal pelvis, the ureters, and the urinary bladder. Hyperplasia of the epithelial lining of the urinary tract in the AAF-fed rabbits (Table 1) was observed in 10/19 animals and ranged from slight (5-8 cells in thickness) in 8 rabbits to moderate (greater than 9 cells in thickness) in 2 animals. In addition to an increased thickness of the transitional epithelium, papillary folds and some nuclear pleomorphism were present. Generally, the frequency of mitosis was not increased. An example of transitional cell hyperplasia of the epithelium of the transitional epithelium, papillary folds and some nuclear pleomorphism were present. Generally, the frequency of mitosis was not increased. An example of transitional cell hyperplasia of the epithelium of the urinary bladder, in a rabbit fed AAF for 35 weeks, is illustrated in Fig. 1. In 2 rabbits fed AAF (one dying at 22 weeks and the second at 41 weeks), there was squamous metaplasia of the epithelium of the renal pelvis. In one of these rabbits (22 weeks), an early transitional cell carcinoma of the renal pelvis, with invasion of the underlying wall, was observed (Fig. 2). Only 1/15 control rabbits in this group showed any hyperplasia of the epithelium of the urinary tract, and in this instance the degree of hyperplasia was minimal. Hyperplasia (slight) was observed in 2/6 rabbits fed N-hydroxy-AAF.

ment of the abdominal lymph nodes, the spleen, and the adrenals, and had an accumulation of fluid in the thoracic cavity with congestion of the lungs. The toxicity of gum acacia has not been observed in rats in which it has been used as a suspending medium for i.p. injections (12). Even though the toxicity of the gum acacia complicated the survival data in Series I of this group, N-hydroxy-AAF was definitely more toxic than AAF when given i.p. (Table 2). This was demonstrated more clearly in Series II in which a lower dose of AAF and JV-hydroxy-AAF was used and 0.9% NaCl was the suspending vehicle for the i.p. injections. All of the control and AAF-injected rabbits survived 40 weeks, whereas only 9/17 (53%) rabbits given N-hydroxy-AAF survived 40 weeks. There was not any difference in the 40-week survival between the male and female rabbits given N-hydroxy-AAF i.p. in Series II.

In all rabbits given AAF or N-hydroxy-AAF, the rate of weight gain decreased beginning with the first week and lasting throughout the period of administration of the compounds. Weight gains at 20 weeks (about the last weekly interval when all of the animals were alive) and at 56 weeks (termination of feeding of the compounds) for the rabbits given AAF or N-hydroxy-AAF orally are shown in Table 1. For the rabbits given AAF i.p. (Table 2), the weight gains at 40 weeks (termination of the injections) in Series I and II should be considered separately because of the toxicity of the gum acacia used as the suspending medium. In Series II of this group, the AAF-injected rabbits gained only about three-fourths (80% for males, 74% for females) as much weight as the controls, whereas the animals injected with N-hydroxy-AAF gained less than one-half (42% for males, 49% for females) as much weight as the controls during the 40-week interval.
Hyperplasia of the epithelium of the urinary tract was frequent even with a lower dose of AAF or N-hydroxy-AAF. In Series I of the group of rabbits given AAF or N-hydroxy-AAF i.p., only 1/9 control rabbits had hyperplasia (minimal), while 5/9 rabbits injected with AAF had hyperplasia (1 slight, 4 moderate) and 6/8 animals given N-hydroxy-AAF showed hyperplasia (3 slight, 2 moderate, 1 quite marked). As the dose of AAF or N-hydroxy-AAF was decreased even further in Series II of this group, the incidence and the severity of the hyperplasia was decreased also. Thus, in Series II, 2/12 control rabbits (both slight), 3/14 AAF-injected rabbits (all moderate), and 6/17 animals injected with N-hydroxy-AAF (all slight) showed hyperplasia of the urinary tract epithelium.

In a total of 42 rabbits given AAF, either orally or i.p., 5 animals had carcinoma of the bladder. Although the total incidence was low (12%), there did not appear to be any relationship between the tumor incidence or the latent period and the dose of AAF within the groups of rabbits receiving AAF. Thus, in the group given the high dose of AAF orally (Table 1), 2/19 (10%) rabbits had carcinoma of the bladder. One of these rabbits died at 116 weeks and had an ulcerated transitional-cell carcinoma at the left ureterocystic junction with extensive invasion of the bladder wall but with no extension beyond the wall. The other animal died at 133 weeks and had an anaplastic carcinoma which had penetrated through the bladder wall to the serosa and involved the retroperitoneal lymph nodes. In the group of rabbits given AAF i.p. (Table 2), 3/23 (13%) animals had bladder tumors. Two of these were in Series I of the group. One, occurring in a rabbit dying at 30 weeks, was an early squamous-cell carcinoma arising from the transitional epithelium. The other, in a rabbit dying at 64 weeks, was a firm, nodular tumor (Fig. 3) classified as a poorly differentiated transitional-cell carcinoma (Fig. 4) which showed some squamous features. The 5th bladder tumor in the rabbits given AAF occurred in an animal dying at 99 weeks in Series II of the i.p. group and was an anaplastic carcinoma which had penetrated through the wall of the bladder to the serosa.

Only 1/31 (3%) rabbits given N-hydroxy-AAF, either orally or i.p., had a bladder tumor. This was a transitional-cell carcinoma, confined to the bladder, and occurred in an animal dying at 103 weeks in the group given N-hydroxy-AAF orally (Table 1).

Induction of Tumors at the Site of Injection of N-Hydroxy-AAF or Its Cupric Chelate. A moderately high incidence of sarcomas of the peri toneum resulted upon the i.p. injection of N-hydroxy-AAF into rabbits over a period of 40 weeks (Table 2). Even in Series I of this group, in which the survival was poor because of the combined toxicity of the gum acacia and the N-hydroxy-AAF, 2/8 rabbits had i.p. sarcomas. A higher incidence of sarcomas of the peritoneum was obtained in Series II of this group, in which the survival was better. In this case, 10/17 (59%) rabbits injected with N-hydroxy-AAF had i.p. sarcomas.

The peritoneal sarcomas were similar to those described by Miller et al. (12) following the i.p. injection of N-hydroxy-AAF in the rat. The sarcomas occurred as multiple nodules, up to 1.5 cm in diameter, covering almost all of the visceral organs (Fig. 5), and invaded the abdominal wall and the diaphragm (Fig. 10). There was an accumulation of up to 400 ml of clear straw-colored or bloody ascitic fluid in these animals. The presence of tumor cells in the ascitic fluid was demonstrated cytologically. Generally, the tumor was a relatively undifferentiated pleomorphic sarcoma, with large multinucleated cells, large cells with multilobulated nuclei, and with mitoses being commonly observed. Some of the tumors showed a spindle-cell pattern (Fig. 8) and a few contained myxoid features (Fig. 7) and liposarcomatous patterns (Fig. 9). Metastatic deposits of the tumor were found in the liver (Fig. 11), the lungs (Fig. 12), and in the heart.

In the group of rabbits given the cupric chelate of N-hydroxy-AAF s.c. in the leg, 3/14 animals developed tumors at the site of injection. In each case, the tumor developed in the leg which had received 6 weekly injections (240 mg total) of the cupric chelate of N-hydroxy-AAF. There were no tumors after 1 injection (40 mg) or 3 injections (120 mg) of the cupric chelate or after 6 injections of cupric oxide (35 mg total), 6 injections of cupric acetate (89 mg total) or 6 injections of tricaprylin (6 ml) alone. Green deposits of material, presumed to be the cupric chelate of N-hydroxy-AAF, were evident grossly or microscopically for as long as 176 weeks (termination of the experiment) in the legs receiving 6 injections of the cupric chelate. The first tumor in this group was palpated at 41 weeks after the injections were completed. This tumor ulcerated (Fig. 13) approximately 1 year later, at which time the animal was sacrificed. Squamous cell carcinoma was confirmed by histologic examination (Fig. 14). The second tumor was a firm, lobulated undifferentiated sarcoma which was palpated about 2 years after the injections of the cupric chelate of N-hydroxy-AAF. Many of the cells of this tumor were giant cells with multiple or bizarre nuclei. There was extensive pleomorphism and mitoses were very numerous. Some areas of the tumor were myxomatous and other areas showed spindle-shaped cells arranged in whorls (Fig. 15). The third tumor, found in a rabbit killed at 145 weeks, was a poorly differentiated squamous cell carcinoma which had metastasized to a regional lymph node (Fig. 16) and to the thymus, spleen, liver, lung, and small intestine.

Other Tumors. In Series II of the group of rabbits given AAF i.p., two rabbits had undifferentiated tumors arising in the wall of the abdomen (Table 2). One of these, occurring in an animal dying at 60 weeks, was a small (1.0 x 0.8 x 0.5 cm), poorly differentiated pleomorphic sarcoma invading the muscle. The other tumor, much larger, was an undifferentiated pleomorphic sarcoma found in a rabbit dying at 107 weeks (Fig. 6). In neither case was the peritoneum involved as it was after the i.p. injection of N-hydroxy-AAF. In both cases, the tumors occurred at some distance from the site of injection of the AAF.

There was no evidence of the origin of these two tumors.

One of the rabbits in Series II of the group given N-hydroxy-AAF i.p. which survived until the termination of the experiment (171 weeks) had a large spindle-cell sarcoma arising in the wall of the cecum (Fig. 17). This tumor had the appearance of a leiomyosarcoma in some areas and in other areas it had features similar to the pleomorphic sarcomas found in the peritoneum after i.p. injection of N-hydroxy-AAF.

Two female control rabbits had spindle-cell sarcomas arising in the muscle wall of the uterus. Both of these were found in animals killed at the termination of an experiment, one at 301 weeks (Table 1) and the other at 171 weeks (Table 2). Each tumor had features of a low-grade leiomyosarcoma (Fig. 18).
Several rabbits showed early leiomyomatous changes in the smooth muscle of the uterus or fallopian tube. An adenocarcinoma of the uterus in one rabbit of the AAF-fed group (Table I) of animals and an adenocarcinoma of the fallopian tube (Fig. 19) in a rabbit injected with N-hydroxy-AAF (Table 2, Series II) were also observed. Since some of the tumors in the female genital system were seen in control rabbits, they have been accepted as spontaneous tumors, although the role of AAF or N-hydroxy-AAF in the induction or promotion of these tumors in those animals receiving AAF or N-hydroxy-AAF cannot be excluded.

**DISCUSSION**

The studies on the carcinogenicity of AAF in the rabbit have confirmed the results of Bonser and Green (1) who reported that only the urinary tract was affected by AAF feeding. Although the total incidence of urinary tract tumors in rabbits fed or receiving injections of AAF was low, hyperplasia of the epithelium of the urinary tract was frequently observed and, in a few cases, squamous metaplasia was seen. Aside from a low incidence of tumors in the female genital system, which may have arisen spontaneously, and the tumors in the abdominal wall of two rabbits, tumors in other tissues of the rabbit, such as the liver or mammary gland, were not seen. This is in marked contrast to the systemic carcinogenicity of AAF exhibited in other susceptible rodents (15). Even though the rabbit has a life span approximately twice that of other rodents commonly used in the laboratory, the latent period for tumor induction after AAF administration in the rabbit was not markedly long. Thus, an early transitional-cell carcinoma of the renal pelvis was observed as soon as 22 weeks after beginning AAF administration and a squamous-cell carcinoma of the urinary bladder was seen at 30 weeks. A grossly detectable transitional-cell carcinoma of the bladder was found after only 64 weeks.

When fed to rabbits, N-hydroxy-AAF was not more carcinogenic than AAF, nor were lesions of the stomach (papillomas or squamous-cell carcinomas) or the small intestine (adenocarcinomas) observed as have been reported in the rat, mouse, hamster, and guinea pig (11). However, the lesions of the stomach in the rat, mouse, and hamster were confined to the esophageal forestomach; the rabbit does not have such a forestomach. Similar lesions of the stomach were not produced after oral administration of N-hydroxy-AAF in the guinea pig, which likewise does not have an esophageal forestomach, although adenocarcinommas of the small intestine were observed in this species (11).

On the other hand, N-hydroxy-AAF was more carcinogenic than AAF in the rabbit when injected i.p. A high incidence of peritoneal sarcomas resulted, thus confirming the local carcinogenic activity of N-hydroxy-AAF observed in other species. Similar peritoneal sarcomas have followed the i.p. injection of N-hydroxy-AAF in the rat (12), and in the mouse, hamster, and guinea pig (11). Peritoneal sarcomas were not seen in rabbits which received i.p. injections of AAF. Undifferentiated tumors in the abdominal wall, but not directly involving the peritoneum, were observed in two rabbits receiving AAF i.p. These tumors were located at least 12–15 cm from the site of injection and their origin was undetermined. N-Hydroxy-AAF was also active in the induction of tumors at the site of administration when injected s.c. in rabbits, although the incidence of tumors was not as great and the latent period was appreciably longer than that required for the induction of the peritoneal sarcomas. However, the dose of N-hydroxy-AAF given s.c. was less than one-fiftieth as much as that given i.p. The data presented here add another species of rodent to the list of those in which N-hydroxy-AAF has been shown to be more carcinogenic than AAF at sites of local application (11, 12) and thus strengthen the hypothesis (10, 14) that N-hydroxy-AAF is a proximate carcinogenic metabolite\(^3\) of AAF in susceptible species.

Since N-hydroxy-AAF was locally active as a carcinogen at 2 of 3 sites of administration in the rabbit, and since N-hydroxy-AAF (excreted as the glucuronide) is the major metabolite of AAF in the rabbit (4), some explanation is required for the marked differences in the site of action of AAF in the rabbit and other rodents susceptible to AAF carcinogenesis. Such an explanation might give us a clue to the mechanism of action of AAF in much the same manner as the observation relating the long-known fact that the guinea pig was not susceptible to AAF carcinogenesis to the failure of this species to form detectable amounts of N-hydroxy-AAF from AAF (10, 14).

In AAF carcinogenesis in the rabbit, two facts must be considered: (a) the lack of carcinogenicity of AAF for those tissues usually most susceptible in other rodents and (b) the susceptibility of the epithelium of the urinary tract, which in most rodents (with a few exceptions) is not highly prone to AAF carcinogenesis. With regard to the first point, since the carcinogenicity of AAF seems to depend on the maintenance of adequate levels of N-hydroxy-AAF or of further proximate carcinogenic metabolites\(^4\) in tissues, it would seem likely that the differences in the susceptibility of rabbit tissues would be due to differences in the further metabolism of N-hydroxy-AAF in the rabbit. Comparisons of some reactions affecting levels of N-hydroxy-AAF in tissue homogenates and cell free systems of various species have been reported (2, 6, 8, 9), and some deductions relating the rates of these reactions in vitro to the susceptibility of tissue to carcinogenesis by AAF or its metabolites have been summarized by Lotlikar *et al.* (9).

With regard to the reactivity of the epithelium of the urinary tract of the rabbit to administered AAF, this could be due to either an increased sensitivity of this tissue in the rabbit to some proximate carcinogenic metabolite\(^5\) of AAF or, more likely, an increased concentration of a potentially active metabolite in rabbit urine. The major urinary metabolite of AAF in the rabbit is the glucuronide of N-hydroxy-AAF, accounting for up to 30% of the dose of AAF given (4); this is a much higher urinary level of the metabolite than is found in other rodents after AAF administration. Little attention has been given to the carcinogenic potential of the glucuronide of N-hydroxy-AAF. Recent studies (3, 7) have shown that the glucuronide of N-hydroxy-AAF is alkaline labile, decomposing in 0.01 N NaOH to yield initially N-hydroxy-AF and, as a final product, azoxyfluorene. Since the rabbit excretes a fairly alkaline urine (average pH 8.5, frequently running as high as pH 8.9–9.0), it is possible that the alkaline lability of the glucuronide of N-hydroxy-AAF plays some role in the susceptibility of the epithelium of the urinary tract of the rabbit to AAF. The poten-
tial role of the chemical reactivity of C—O—N glucuronides of N-hydroxy metabolites of other carcinogenic aromatic amines or their amides in the mechanism of carcinogenesis, particularly in the urinary tract, should be considered.

REFERENCES


Fig. 1. Epithelium of urinary bladder after 35 weeks of 2-acetylaminofluorene feeding, showing slight hyperplasia, papillary fronds, and minimal nuclear pleomorphism. H & E, × 100.

Fig. 2. Carcinoma of the renal pelvis after 22 weeks of 2-acetylaminofluorene feeding. Nests of transitional tumor cells are infiltrating the submucosa. H & E, × 60.

Fig. 3. Carcinoma, dome of the bladder, at 24 weeks after i.p. injection of 2-acetylaminofluorene for 40 weeks was stopped.

Fig. 4. Poorly differentiated transitional-cell carcinoma seen in Fig. 3, showing invasion of the bladder wall. H & E, × 100.
FIG. 5. Intraperitoneal sarcoma in a rabbit after i.p. injection of N-hydroxy-2-acetylaminofluorene for 35 weeks. Tumor nodules cover the diaphragm, abdominal wall, mesentery, and all of the abdominal viscera.

FIG. 6. Undifferentiated tumor arising in the abdominal wall (reflected upward) at 107 weeks after i.p. injections of 2-acetylaminofluorene for 40 weeks were started. Note the absence of tumor nodules on the abdominal viscera (compare with Fig. 5).

FIG. 7. Undifferentiated sarcoma seen in Fig. 5, showing myxoid features. H & E, X 100.

FIG. 8. Intraperitoneal sarcoma after i.p. injection of N-hydroxy-2-acetylaminofluorene for 33 weeks, showing a spindle-cell pattern and numerous mitoses. H & E, X 100.
Fig. 9. Intraperitoneal sarcoma after i.p. injection of N-hydroxy-2-acetylaminofluorene for 31 weeks, showing features of a liposarcoma. H & E, X 60.

Figs. 10-12. Intraperitoneal sarcoma after i.p. injection of N-hydroxy-2-acetylaminofluorene, showing infiltration of the diaphragm (Fig. 10) and metastatic deposits of tumor in the liver (Fig. 11) and the lungs (Fig. 12). Each figure, H & E, X 100.
FIG. 13. Squamous cell carcinoma at the site of injection of the cupric chelate of N-hydroxy-2-acetylaminofluorene.

FIG. 14. Tumor seen in Fig. 13, showing nests of squamous carcinoma cells invading the subcutaneous tissue. H & E, × 30.

FIG. 15. Pleomorphic spindle-cell carcinoma occurring in another rabbit at the site of injection of the cupric chelate of N-hydroxy-2-acetylaminofluorene. H & E, × 100.

FIG. 16. Metastatic squamous cell carcinoma replacing a lymph node. The primary tumor was at the site of injection of the cupric chelate of N-hydroxy-2-acetylaminofluorene (see text). H & E, × 100.
Fig. 17. Sarcoma arising in the wall of the cecum. The tumor, showing in some areas features of a leiomyosarcoma, was found in a rabbit 131 weeks after i.p. injection of N-hydroxy-2-acetylaminofluorene for 40 weeks was stopped. H & E, X 200.

Fig. 18. Spindle-cell tumor arising in the smooth muscle of the uterine wall of a control rabbit at 171 weeks. The tumor has cellular features of a low-grade leiomyosarcoma. H & E, X 100.

Fig. 19. Infiltrative adenocarcinoma of the fallopian tube in a rabbit at 171 weeks after starting i.p. injections of N-hydroxy-2-acetylaminofluorene for 40 weeks. The tumor extended through the entire thickness of the wall of the fallopian tube. H & E, X 100.
Carcinogenicity of 2-Acetylamino-9-fluorene and N-Hydroxy-2-acetylamino-9fluorene in the Rabbit

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