Evaluation of the Carcinogenicity of Aminofluorenols by Implantation into the Bladder of the Mouse*

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SUMMARY

To test the hypothesis that o-hydroxy amines are proximate agents in chemical carcinogenesis paraffin pellets containing the hydrochlorides of 2-amino-1-fluorenol, 2-amino-3-fluorenol, 2-amino-5-fluorenol, 2-amino-7-fluorenol, 2-fluorenamine, and 1-amino-2-naphthol were implanted into the urinary bladders of Swiss female mice. Paraffin pellets alone were implanted as controls. Histological examination of the bladders 40 weeks after implantation showed no evidence of carcinoma. Inflammatory changes, squamous metaplasia, and epithelial hyperplasia were noted with equal frequency in the control and experimental animals. The stability of implanted 2-amino-1-fluorenol-1-C14 hydrochloride and 1-amino-2-naphthol-H3 hydrochloride and the elution of these compounds from the pellets were examined by radioactive tracer methods. Implanted 2-amino-1-fluorenol-1-C14 hydrochloride was stable, whereas 1-amino-2-naphthol-H3 hydrochloride decomposed soon after implantation. The severe irritation of the mouse bladder by paraffin raises the question whether paraffin is a suitable vehicle in the bladder implantation test.

The hypothesis that o-hydroxy amines are proximate agents in carcinogenesis by aromatic amines (6) receives its support chiefly from the induction of bladder carcinoma after certain o-aminophenols, incorporated into paraffin or cholesterol, are introduced into the mouse bladder by the technic devised by Bonser and her associates (3, 12). An evaluation of the technic and a discussion of the histological criteria used for assessing tumor incidence have been presented (3, 4). The principal advantages of this technic seem to be that only small amounts of the test compound are required and that presumably a high local concentration of the compound at the test site is maintained. As a consequence of the above hypothesis (6), the o-aminophenols, 2-amino-1-fluorenol and 2-amino-3-fluorenol, which are urinary metabolites of the carcinogen N-(2-fluorenyl)-acetamide (24), ought to be carcinogenic for the mouse bladder. Accordingly, the hydrochlorides of these o-aminofluorenols were tested for their carcinogenicity by the implantation technic. The present report also includes an evaluation of the carcinogenicity of the hydrochlorides of 2-amino-5-fluorenol, 2-amino-7-fluorenol, and of 2-fluorenamine. 1-Amino-2-naphthyl hydrochloride was selected as a positive control, since this o-aminophenol had been reported to give a 28 per cent incidence of bladder carcinoma after implantation in paraffin (4).

MATERIALS AND METHODS

Animals, Maintenance of Animals, and Collection of Urine

Stock female albino mice weighing 25–30 gm. were purchased from the Dan Rolfsmeyer Company, Madison, Wisconsin. The animals were kept in an air-conditioned room at 26° C. Food (Purina Fox Chow Checkers) and water were allowed ad libitum before and after implantation of the compounds. For the collection of urine the mice were set up individually in all-glass metabolism cages which permitted the separation of urine and feces.1

1 These cages are manufactured by Delmar Scientific Laboratories, 317 Madison Street, Maywood, Illinois.
Urine (0.5–1 ml.) was collected for 24-hour periods in receivers cooled in dry ice-isopropyl alcohol. At the end of this period the cages were rinsed with a few ml. of water and an aliquot of the combined wash-liquid and urine was counted.

**Preparation of Paraffin Pellets Containing the Test Compounds**

The test compounds and paraffin² (Fisher, Paraffin [Hard], Lot #799846, m.p. 60°–62° C.) were weighed into a centrifuge tube. The tube was placed into an oil bath at 90 ± 5° C., and the substances were mixed with a glass rod. The tube was then allowed to cool. After the mixture had solidified, it was remelted with continuous stirring. Pellets were made by dropping the mixture onto a cold Petri dish. Pellets weighing from 12 to 22 mg. were selected for implantation. The concentration of the test compounds in the pellets was 14–15 per cent. It has been stated that an even distribution of the carcinogen in the pellet is difficult to achieve by the above method (12). This point was checked by calculating the quantities of 1-amino-2-naphthol hydrochloride in five different pellets from the percentage weight and comparing this value with the amount of material obtained in a spectrophotometric analysis.³ In each case the weights determined spectrophotometrically deviated from the weights calculated from the gravimetric data by 10 per cent or less. Since estimates based on the percentage weight assume homogeneous mixing, the agreement between the two methods indicated uniform distribution of the compound throughout the vehicle. The ultraviolet absorption spectrum of l-amino-2-naphthol hydrochloride was incorporated into the pellets. The fluorine derivatives were again recrystallized from dilute hydrochloric acid and dried in a vacuum over sodium hydroxide. 1-Amino-2-naphthol hydrochloride (Eastman, Red Label, Lot #2308) was purified by recrystallization (9). The product (colorless needles) had the same spectroscopic \((\lambda_{\text{max}} \% \text{Ethanol} 243 \text{ m/} \mu; \epsilon, 31,400; \lambda_{\text{min}} \% \text{Ethanol} 222 \text{ m/} \mu; \epsilon, 20,000)\) and chromatographic properties as analytically pure 1-amino-2-naphthol hydrochloride prepared from 1-nitroso-2-naphthol (8). 1-Amino-2-naphthol hydrochloride:

Calcd. for C₁₀H₈ONCl:
C, 61.4; H, 5.15; N, 7.16.

Found:
C, 61.3; H, 5.27; N, 6.90.

Solution of the compound in cold water or 95 per cent ethanol resulted in decomposition within a few hours, as evidenced by the appearance of a brown color. The instability of 1-amino-2-naphthol hydrochloride precluded its purification by column chromatography. Descending chromatography of the compound⁴ with 20 per cent hydrochloric acid gave a single pink spot \((R_f = 0.46-0.49)\) with 0.03 per cent diazotized p-nitroaniline in 0.1 N 6-micron intervals until either 200 serial sections had been taken or the bladder was completely sectioned, whichever occurred first. Every twentieth section was mounted and stained with hematoxylin and eosin (4). The bladders of fifteen mice without pellets⁴ and the bladders of fifteen mice with paraffin pellets containing no test compound⁴ were sectioned completely.

**Preparation of Compounds**

The hydrochlorides of 2-amino-1-fluorenol (21), 2-amino-3-fluorenol (21), 2-amino-5-fluorenol (22), and of 2-amino-7-fluorenol (23) were prepared by methods which have been described. The hydrochloride of 2-fluorenamine was obtained by recrystallizing 2-fluorenamine (13) from 10 per cent hydrochloric acid. Prior to incorporation into the pellets the fluorine derivatives were again recrystallized from dilute hydrochloric acid and dried in a vacuum over sodium hydroxide. 1-Amino-2-naphthol hydrochloride (Eastman, Red Label, Lot #2308) was purified by recrystallization (9). The product (colorless needles) had the same spectroscopic \((\lambda_{\text{max}} \% \text{Ethanol} 243 \text{ m/} \mu; \epsilon, 31,400; \lambda_{\text{min}} \% \text{Ethanol} 222 \text{ m/} \mu; \epsilon, 20,000)\) and chromatographic properties as analytically pure 1-amino-2-naphthol hydrochloride prepared from 1-nitroso-2-naphthol (8). 1-Amino-2-naphthol hydrochloride:

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hydrochloric acid. Alternatively, the compound was detected with the Folin-Ciocalteu reagent (10). The lower limit of detection was 5–6 μg.

2-Amino-1-fluorenol-1-C\textsubscript{14} hydrochloride. —2-Amino-1-fluorenol-1-C\textsubscript{14} hydrochloride (550 dpm/μg) was obtained from the acid hydrolysis of N-(1-hydroxy-2-fluorenyl-1-C\textsubscript{14}) acetamide (11, 17). Ascending chromatography with 5 per cent hydrochloric acid gave a single spot with the Folin-Ciocalteu reagent. This spot coincided with the single peak (R\textsubscript{F} = 0.21), which was detected when the chromatograms were scanned with a Vanguard Scanner (Chart 1, A). The chromatogram was developed as in A. The radioactivity of the chromatograms was scanned with a Vanguard Scanner (Chart 1, A).

Instrument Company, Model 880, Chromatogram Scanner (Chart 1, A).

1-Amino-2-naphthol-H\textsubscript{3} hydrochloride. —200 mg. of 1-amino-2-naphthol hydrochloride was dissolved in 35 ml. of 70 per cent sulfuric acid containing 2 c. of tritium, and the solution was kept at 50° C. overnight. The solution was then adjusted to pH 6 with 10 N potassium hydroxide, cooled, and extracted with three 50-ml. portions of ethyl acetate. The ethyl acetate was washed with 50 ml. distilled water and extracted with 40 ml. 10 per cent hydrochloric acid. Concentrated hydrochloric acid (50 ml.) was added to the extract, and the mixture was kept at 4° C. overnight. The precipitate was collected, washed with 40 per cent hydrochloric acid, and dried in a vacuum over potassium hydroxide. The crude product (87 mg.) was purified by two successive crystallizations from 0.4 per cent hydrochloric acid with charcoal and sodium bisulfite (8). Carrier 1-amino-2-naphthol hydrochloride (91 and 108 mg., respectively) was added in each crystallization. The purified material (68 mg., 10\textsuperscript{4} dpm/μg) was obtained after drying overnight in a vacuum over potassium hydroxide at 78° C. Descending chromatography of the labeled compound with 20 per cent hydrochloric acid gave a single pink spot with diazotized p-nitroaniline. Its R\textsubscript{F} value (0.46) was identical with that of an authentic sample run concurrently. Radioactivity measurements of the chromatograms (80) likewise gave a single peak whose R\textsubscript{F} value coincided with that of the authentic sample chromatographed simultaneously.\textsuperscript{8}

Radioactivity measurements. —C\textsubscript{14} and H\textsubscript{3} were estimated by liquid scintillation counting in 22-m1. low-potassium vials. The radioactivity of urine or samples immiscible with organic solvents was measured after shaking the sample (0.5–1 ml.) with thixotropic gel powder (Cab-O-Sil, 0.7 gm.) and 15 ml. of the following scintillation solution: toluene (760 ml.), 1,4-dioxane (760 ml.), ethanol (480 ml.), naphthalene (160 gm.), 2,5-diphenyloxazole (10 gm.), 1,4-bis-(5-phenyloxazolyl)-benzene (0.1 gm.) (Counting System A). The radioactivity of H\textsubscript{3}-containing material eluted from paper chromatograms and the radioactivity of samples soluble in organic solvents was measured in 10 ml. of the following counting system: toluene (1000 ml.), 2,5-diphenyloxazole (4 gm.), and 1,4-bis-(5-phenyloxazolyl)-benzene (0.1 gm.) (Counting System B). All samples were counted in duplicate with a standard error of 5 per cent or less. Corrections for quenching were made with the use of internal standards.

Stability of the implanted labeled compounds. —At the times stated in Table 5 the pellets were removed from the bladders and placed into centrifuge tubes. In the case of pellets containing 2-amino-1-fluorenol-1-C\textsubscript{14} hydrochloride the paralysis of small amounts of 1-amino-2-naphthol hydrochloride by recrystallization the compound was not further recrystallized. It was felt that the chromatographic data provided adequate proof for the radiochemical purity of the labeled compound.\textsuperscript{8}

\textsuperscript{8} Because of considerable losses during the purification of small amounts of 1-amino-2-naphthol hydrochloride by recrystallization the compound was not further recrystallized. It was felt that the chromatographic data provided adequate proof for the radiochemical purity of the labeled compound.
fin was dissolved in toluene. The precipitate was removed by filtration and washed with toluene (total volume, 10 ml.). The toluene-insoluble residue was dissolved in ethanol (2 ml.). Aliquots of the toluene and ethanol solutions were counted. Another aliquot of the ethanol solution was placed on a 2 X 28-cm. filter paper strip and chromatographed by the ascending technic either with n-butyl alcohol:acetic acid:water (4:1:5) or 5 per cent hydrochloric acid. The chromatograms were sprayed with the Folin-Ciocalteu reagent (10) and scanned subsequently. The paraffin of pellets containing 1-amino-2-naphthol-HCl hydrochloride was dissolved in toluene (5 or 10 ml.) as above. The toluene-insoluble residue was separated by centrifugation and washed repeatedly with toluene. The precipitate was dissolved in 95 per cent ethanol (2 or 5 ml.). Aliquots of the toluene and 95 per cent ethanol solutions were assayed for radioactivity. Two aliquots of the 95 per cent ethanol fraction were placed on a 10 X 40-cm. filter paper strip divided into three sections. Each section was 2 cm. wide and separated from the adjacent section by 1 cm. A freshly prepared solution of 1-amino-2-naphthol hydrochloride (6 µg.) was applied to the third section. After the chromatogram had been developed with 20 per cent hydrochloric acid and dried in air, the section containing the standard and one of the sections containing the radioactive material were sprayed with diazotized p-nitroaniline. The remaining section was cut into 2 X 2-cm. consecutive segments (20). Each square was placed into a counting vial and extracted with 1 ml. of hyamine11 for 4 hours at 45°-50° C. (20).

Examination of the bladder for protein-bound radioactivity after implantation of 2-amino-1-fluorenol-3-C14 hydrochloride.—At sacrifice the bladders were placed into 5 per cent trichloroacetic acid for 18 hours and then extracted with ethanol in a Soxhlet apparatus for 24 hours. The extracted bladders were dissolved in 1 ml. of hyamine at 60° C. for 18 hours. The radioactivity of the solution was measured with the use of Counting System A. As a control, the bladder of a normal mouse was treated and assayed for radioactivity in the same way.

### TABLE 1

**SUMMARY OF THE HISTOLOGICAL FINDINGS 40 WEEKS AFTER IMPLANTATION OF THE TEST COMPOUNDS**

<table>
<thead>
<tr>
<th>Compound implanted</th>
<th>No. bladders examined</th>
<th>Normal bladders</th>
<th>Calculi</th>
<th>Inflammation and/or squamous metaplasia</th>
<th>Hyperplasia of epithelium</th>
<th>Bladder carcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>None*</td>
<td>46</td>
<td>1</td>
<td>1</td>
<td>22</td>
<td>23</td>
<td>0</td>
</tr>
<tr>
<td>2-Amino-1-fluorenol-HCl</td>
<td>24</td>
<td>0</td>
<td>0</td>
<td>13</td>
<td>9</td>
<td>0†</td>
</tr>
<tr>
<td>2-Amino-3-fluorenol-HCl</td>
<td>29</td>
<td>3</td>
<td>0</td>
<td>15</td>
<td>9</td>
<td>0†</td>
</tr>
<tr>
<td>2-Amino-5-fluorenol-HCl</td>
<td>31</td>
<td>0</td>
<td>0</td>
<td>10</td>
<td>21</td>
<td>0</td>
</tr>
<tr>
<td>2-Amino-7-fluorenol-HCl</td>
<td>32</td>
<td>0</td>
<td>0</td>
<td>16</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>2-Fluorenamine-HCl</td>
<td>31</td>
<td>0</td>
<td>0</td>
<td>21</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>1-Amino-2-naphthol-HCl</td>
<td>31</td>
<td>0</td>
<td>1</td>
<td>11</td>
<td>20</td>
<td>0</td>
</tr>
</tbody>
</table>

* Paraffin pellets alone were implanted into these bladders.
† One mouse in this group had a tumor of the thigh (adenocarcinoma). The origin of the tumor was not determined.
‡ One mouse in this group had a tumor of the head (adenocarcinoma). The origin of the tumor was not determined.

RESULTS

**Histological findings after implantation of paraffin pellets with and without test compounds.—** The histological findings are summarized in Table 1. Of 234 bladders examined only four could be considered normal by the criteria of Bonser and Jull (5). One of the normal bladders was found in the control series. Three of the bladders given implants of 2-amino-3-fluorene hydrochloride also showed no changes. The pathological changes which were observed were inflammation and/or squamous metaplasia and hyperplasia of the bladder epithelium. No carcinoma was found in any of the bladders. A description of the pathological alterations is given below.

**Inflammation.—** The inflammatory changes were

11 Hyamine is the trade name for a 1 m solution of p-(diisobutylcresoxyethoxyethyl)dimethylbenzylammonium hydroxide.
observed in varying degree both in the paraffin controls and in the bladders exposed to pellets containing the test substances. Although these changes were occasionally mild and chronic, they were pronounced in the majority of the bladders. In mild forms only scattered lymphocytes and plasma cell aggregates were found in the subepithelial tissues, but in many cases these tissues showed residual changes of more severe inflammation which had subsided. These included fibrosis of the lamina propria and sometimes of the muscle layers. In other instances chronic inflammation was extensive and involved most of the lamina propria. The cellular infiltrate was usually a mixture of lymphocytes and plasma cells which indicated that it was actually an inflammatory infiltrate and not the leukemic infiltrate described by Bonser and Jull (5). In severe forms denudation of the mucosa occurred. This was possibly a secondary effect due to mechanical irritation by the pellets and, in two instances, by bladder calculi. The ligating silk suture sequestered nests of epithelium and often produced a cavity at the dome of the bladder, which was either a blind pouch or a false diverticulum with a constricted neck. Within this cavity exudative inflammation was common, and minute concretions were sometimes found. Foam cells and clefts with surrounding foreign-body giant cells were seen occasionally.

Squamous metaplasia.—This change consisted of keratinization of the mucosal surface and sometimes of sequestered nests of epithelium at the line of ligature or within the false diverticula. It was always associated with inflammation. Progression to benign or malignant tumors was not observed.

Epithelial hyperplasia.—This category included a series of progressive alterations which were almost always related to inflammation. The earliest changes consisted of a thickening and invagination of the mucosa which formed rounded nests, often with a central lumen. These nests resembled those of Brunn or those found in cystitis cystica (14, 19), and were usually accompanied by mild inflammation. In some cases the invaginating nests formed papillomatous projections which protruded into the lumen. These projections simulated neoplasms but were similar to the inflammatory polyps found in papillary cystitis of the human. Although certain alterations of the epithelial cells, such as slight enlargement, mild hyperchromaticity, slightly increased mitotic activity and occasionally keratinization were observed, these were not necessarily indicative of neoplastic transformation. In the more advanced lesions the degree of cellular alteration described above was greater, and the lower borders of the papillomatous pseudoneoplasms appeared slightly irregular. However, the muscular layer remained intact in every case except one. In this instance the epithelium was so well differentiated as to exclude neoplasia. It should be stressed that several histologic features were uniformly present in the pseudotumors. The most important of these was the prominent inflammatory infiltrate. The inflammatory cells were found throughout the “invading” epithelium as well as around it. The second feature was that this transitional epithelium assumed a constant pseudoglandular pattern. Nearly all the lumina were filled with inflammatory cells, the majority of which were neutrophiles. Desquamated and degenerating epithelial cells were also seen in some of these lumina. It is pertinent that in 99 of the 122 cases recorded as hyperplasia the epithelial lesions were traceable to suture material. Only in a minority of animals could such a relationship not be established, since the lesions occurred in the region of the urethra far removed from the ligature. Hyperplasia in this region was also encountered by Bonser and Jull (5). Hyperplasia associated with squamous metaplasia was found frequently in sequestered nests. In these instances the relationship to suture material was also readily established. The most profound epithelial alterations seen in the present study were those classified by Bonser and Jull (5) as “vegetative” and attributed by them to sequestration of epithelial nests. This interpretation was supported by the findings in this study. It should be emphasized that the dyskeratosis was marked in some cases. The epithelial cells were greatly enlarged and showed much nuclear variation including hyperchromaticity, coarsely clumped chromatin, prominent nucleoli and considerable mitotic activity. However, the lesions were either related to suture material or confined to the diverticulum at the dome of the bladder. They were always sharply confined and showed no invasive tendencies.

In the light of the pathological findings the following conclusions seem justifiable: (a) Since similar histological alterations occurred in the bladders exposed to paraffin pellets only, as well as in the bladders exposed to paraffin pellets containing a test compound, it would appear that the implantation of paraffin accounts for many of the changes and is not an innocuous procedure. (b) Since the most advanced epithelial proliferations were almost always found together with the more severe inflammatory changes, the implication is strong that these changes were related. (c) In the majority of bladders which showed epithelial hyperplasia or vegetative changes, a relationship of these changes to suture material could be estab-
lished. In some bladders epithelial proliferations associated with severe inflammation were found only in the false diverticulum produced by the ligature. Although it could not be entirely excluded that these lesions were caused by seepage of the chemical agent into these cavities, it seemed more reasonable that the closure of the bladder by a single silk suture (1) was responsible for the benign lesions. (d) The uniformity of the cellular elements, the orderly progression from invagination of the mucosa to more proliferative lesions, the prevalence of inflammation, which correlated with the extent of proliferation, and the absence of invasiveness supported the interpretation that the lesions were benign and were the result of inflammation secondary to irritation.

*Elution and stability of 2-amino-1-fluorenol-1-C\textsuperscript{14} hydrochloride.*—The lack of carcinogenicity of the \(o\)-aminofluorens and of \(l\)-amino-2-naphthol hydrochloride raised the question whether these \(o\)-hydroxy amines had been eluted from the pellets. However, the amount of material excreted in 24 hours possibly did not exceed 0.04 per cent of the implanted dose, which was the highest value recorded. The slow rate of elution was confirmed by determining the amount of radioactivity remaining in the pellets 25 weeks after implantation. The data (Table 3) indicated that only 25 per cent of the compound had diffused from the pellet during that time. It seems probable that the eluted material was 2-amino-1-fluorenol hydrochloride, since there was no evidence for the presence of other labeled compounds on chromatograms of the pellet contents (Chart 1, B). Since 2-amino-1-fluorenol hydrochloride was eluted only in trace amounts\textsuperscript{12}.

**Table 2**

**URINARY EXCRETION OF THE RADIOACTIVITY AFTER IMPLANTATION OF 2-AMINO-1-FLUORENOL-1-C\textsuperscript{14} HYDROCHLORIDE**

<table>
<thead>
<tr>
<th>Mouse no.</th>
<th>Estimated weight of implanted 1-OH-2-AF-HC\textsubscript{1}(mg.)</th>
<th>Days after implantation</th>
<th>dpm found in 24 hr.</th>
<th>mg. of 1-OH-2-AF-HC\textsubscript{1} equivalent to the radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.01</td>
<td>1</td>
<td>445</td>
<td>0.81</td>
</tr>
<tr>
<td>2</td>
<td>2.01</td>
<td>2</td>
<td>388</td>
<td>0.65</td>
</tr>
<tr>
<td>3</td>
<td>2.01</td>
<td>3</td>
<td>0</td>
<td>0.15</td>
</tr>
<tr>
<td>4</td>
<td>2.01</td>
<td>15</td>
<td>241</td>
<td>0.84</td>
</tr>
<tr>
<td>5</td>
<td>2.01</td>
<td>22</td>
<td>245</td>
<td>0.45</td>
</tr>
<tr>
<td>6</td>
<td>2.11</td>
<td>23</td>
<td>179</td>
<td>0.33</td>
</tr>
<tr>
<td>7</td>
<td>2.08</td>
<td>24</td>
<td>205</td>
<td>0.37</td>
</tr>
<tr>
<td>8</td>
<td>2.08</td>
<td>25</td>
<td>64</td>
<td>0.19</td>
</tr>
<tr>
<td>9</td>
<td>1.92</td>
<td>26</td>
<td>60</td>
<td>0.11</td>
</tr>
<tr>
<td>10</td>
<td>1.92</td>
<td>27</td>
<td>82</td>
<td>0.15</td>
</tr>
</tbody>
</table>

Alternatively, the failure of these compounds to induce neoplasia might have been due to decomposition prior to elution. Because of the known instability of \(o\)-aminophenols the latter possibility must be seriously considered (10). An attempt was made to clarify these problems by implanting the hydrochlorides of 2-amino-1-fluorenol-1-C\textsuperscript{14} and 1-amino-2-naphthol-H. The detection of radioactivity in the urine after implantation of 2-amino-1-fluorenol-1-C\textsuperscript{14} hydrochloride proved that the compound was eluted (Table 2). The data are considered to be semiquantitative, because the quantitative collection of the minute amounts of urine excreted by the mouse in 24 hours is difficult. However, the amount of material excreted in 24 hours possibly did not exceed 0.04 per cent of the implanted dose, which was the highest value recorded. The slow rate of elution was confirmed by determining the amount of radioactivity remaining in the pellets 25 weeks after implantation. The data (Table 3) indicated that only 25 per cent of the compound had diffused from the pellet during that time. It seems probable that the eluted material was 2-amino-1-fluorenol hydrochloride, since there was no evidence for the presence of other labeled compounds on chromatograms of the pellet contents (Chart 1, B). Since 2-amino-1-fluorenol hydrochloride was eluted only in trace amounts\textsuperscript{12}.

**Table 3**

**DISAPPEARANCE OF THE RADIOACTIVITY OF 2-AMINO-1-FLUORENOL-1-C\textsuperscript{14} HYDROCHLORIDE FROM IMPLANTED PELLETS**

<table>
<thead>
<tr>
<th>Mouse no.</th>
<th>Radioactivity implanted (dpm X 10\textsuperscript{4})</th>
<th>Radioactivity remaining (dpm X 10\textsuperscript{4})</th>
<th>Radioactivity eluted (per cent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1*</td>
<td>11.0</td>
<td>8.04</td>
<td>27</td>
</tr>
<tr>
<td>2*</td>
<td>11.6</td>
<td>8.86</td>
<td>24</td>
</tr>
<tr>
<td>3*</td>
<td>11.4</td>
<td>8.48</td>
<td>26</td>
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<tr>
<td>4*</td>
<td>9.0</td>
<td>7.41</td>
<td>20</td>
</tr>
<tr>
<td>5†</td>
<td>10.4</td>
<td>8.69</td>
<td>17</td>
</tr>
</tbody>
</table>

\* These mice were sacrificed 25 weeks after implantation of the pellets.
† Mouse No. 5 was sacrificed 9 weeks after implantation of the pellet.

Alternatively, the failure of these compounds to induce neoplasia might have been due to decomposition prior to elution. Because of the known instability of \(o\)-aminophenols the latter possibility must be seriously considered (10). An attempt was made to clarify these problems by implanting the hydrochlorides of 2-amino-1-fluorenol-1-C\textsuperscript{14} and 1-amino-2-naphthol-H. The detection of radioactivity in the urine after implantation of 2-amino-1-fluorenol-1-C\textsuperscript{14} hydrochloride proved that the compound was eluted (Table 2). The data are considered to be semiquantitative, because the quantitative collection of the minute amounts of urine excreted by the mouse in 24 hours is difficult. However, the amount of material excreted in 24 hours possibly did not exceed 0.04 per cent of the implanted dose, which was the highest value recorded. The slow rate of elution was confirmed by determining the amount of radioactivity remaining in the pellets 25 weeks after implantation. The data (Table 3) indicated that only 25 per cent of the compound had diffused from the pellet during that time. It seems probable that the eluted material was 2-amino-1-fluorenol hydrochloride, since there was no evidence for the presence of other labeled compounds on chromatograms of the pellet contents (Chart 1, B). Since 2-amino-1-fluorenol hydrochloride was eluted only in trace amounts\textsuperscript{12}.

12 One month after implantation 25 ml. of pooled urine had been collected from six mice. The urine contained radioactivity (1,375 dpm) equivalent to 2.5 mg. of 2-amino-1-fluorenol-1-C\textsuperscript{14} hydrochloride.
instance (31 dpm/total bladder) was equivalent to 0.002 per cent of the implanted dose it was concluded that the binding of 2-amino-1-fluorenol hydrochloride to the bladder was negligible in these experiments.

Stability of 1-amino-2-naphthol-H\textsuperscript{3} hydrochloride. —The chromatograms of 1-amino-2-naphthol-H\textsuperscript{3} hydrochloride and of the ethanol-soluble fraction\textsuperscript{13} from a pellet into which 1-amino-2-naphthol-H\textsuperscript{3} hydrochloride had been incorporated, but which had not been implanted, were virtually identical (Chart 2, A). The single radioactive peak (R\textsubscript{F} = 0.48) coincided with the pink spot obtained by chromatographing another aliquot of this fraction and spraying with diazotized p-nitroaniline. The RF value of the standard 1-amino-2-naphthol hydrochloride, run concurrently, was almost identical (0.49) with that of the pellet material. A different pattern was obtained when the ethanol-soluble fraction of pellets which had been implanted for 18 weeks was chromatographed (Chart 2, B). The major portion of the radioactivity remained at the origin which indicated that, unlike 1-amino-2-naphthol hydrochloride, the labeled material was insoluble in 20 per cent hydrochloric acid. There were two minor radioactive components whose RF values (0.30 and 0.41) differed measurably from that of 1-amino-2-naphthol hydrochloride. Furthermore, there was no color reaction when the chromatogram was sprayed with diazotized p-nitroaniline. An identical chromatographic pattern was observed in a pellet processed 4 weeks after implantation. In contrast, pellets containing 1-amino-2-naphthol hydrochloride which were kept at 4\textdegree C. for 5 weeks gave chromatograms typical of the pure compound. Further evidence for the instability of implanted 1-amino-2-naphthol hydrochloride came from radioactivity measurements of the distribution of the pellet contents between toluene and 95 per cent ethanol. Pure 1-amino-2-naphthol is soluble in 95 per cent ethanol and nearly insoluble in toluene. This solubility behavior was reflected in the distribution of the radioactivity of unimplanted pellets; 99 per cent of the radioactivity was soluble in 95 per cent ethanol, and 1 per cent was found in the toluene fraction. However, a shift in the distribution of the radioactivity was noted 2 weeks after implantation (Table 4). Five weeks after implantation the radioactivity was distributed approximately equally between 95 per cent ethanol and toluene, and at 16 weeks the major portion of the radioactivity was soluble in toluene and insoluble in 95 per cent ethanol.\textsuperscript{14} These data confirmed the instability of implanted 1-amino-2-naphthol hydrochloride which had already been apparent from the chromatograms. It seems clear that the implanted compound underwent extensive decomposition soon after insertion into the bladder.

An attempt was made to estimate the elution

\textsuperscript{13} The ethanol fraction contained 99 per cent of the total radioactivity of the pellet.

\textsuperscript{14} The ethanol as well as the toluene fraction of implanted pellets containing 1-amino-2-naphthol hydrochloride were, without exception, highly colored. No color was observed in these fractions with freshly prepared pellets.

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**Table 4**

The stability of 2-amino-1-fluorenol-1-C\textsuperscript{4} hydrochloride and 1-amino-2-naphthol-H\textsuperscript{3} hydrochloride after implantation

<table>
<thead>
<tr>
<th>Compound implanted</th>
<th>Moore no.</th>
<th>Time after implantation (weeks)</th>
<th>Per cent radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Toluene-insoluble</td>
</tr>
<tr>
<td>1-Amino-2-fluorenol-1-C\textsuperscript{4} hydrochloride</td>
<td>1</td>
<td>9</td>
<td>99.6*</td>
</tr>
<tr>
<td></td>
<td>2-5 (4)†</td>
<td>25</td>
<td>99.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>99.0‡</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(98.8–99.3)</td>
</tr>
<tr>
<td>1-Amino-2-naphthol-H\textsuperscript{3} hydrochloride</td>
<td>1</td>
<td>2</td>
<td>98.6*</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>4</td>
<td>87.2</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>13</td>
<td>46.0</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>16</td>
<td>31.7</td>
</tr>
<tr>
<td></td>
<td>5-12 (8)†</td>
<td>18</td>
<td>12.9</td>
</tr>
</tbody>
</table>

* These pellets were assayed, but not implanted.
† The number in parentheses is the number of pellets assayed.
‡ These values represent the averages of the toluene-insoluble and toluene-soluble radioactivity; the range of the radioactivities in this group is shown in parentheses below.
of l-amino-2-naphthol-H\textsuperscript{3} hydrochloride by measuring the urinary radioactivity. However, no conclusions could be drawn from these measurements, since 85 per cent of the radioactivity could be distilled.\textsuperscript{15} The tritium exchange, which may have taken place within or without the pellet, also obviated any measurements of the binding of l-amino-2-naphthol hydrochloride to the bladder.

DISCUSSION

The severe irritant action of paraffin for the mouse bladder which emerges as one of the relevant points of this study appears to have received little attention. Although Bonser and her associates have repeatedly recommended the use of paraffin in the implantation test (2, 4) it seems to us that the evaluation of the carcinogenicity of a compound becomes hazardous when the vehicle provokes a severe tissue response. The possibility that the irritation set up by the paraffin might promote tumor induction by a chemical which per se is not carcinogenic further complicates the interpretation of the histological data. The lack of carcinogenicity of 2-amino-1-fluorenol hydrochloride observed here agrees with the recent report of Bonser et al. (2). The inactivity of 2-amino-3-fluorenol hydrochloride noted here also seems to be in line with the findings of Bonser et al. (2). In contrast, 2-amino-7-fluorenol appeared to be relatively active (seven carcinomas/31 mice) in the hands of Bonser et al. (2). However, a strict comparison of the data is not possible since, with the exception of 2-amino-1-fluorenol hydrochloride, the aminofluorenols were implanted as the free bases and cholesterol was used as the vehicle. Moreover, Bonser et al. have demonstrated in the case of bis(2-amino-1-naphthyl) sodium phosphate that the choice of the vehicle has a marked influence upon the carcinogenicity (2). Under the circumstances, it seems safe to conclude that the hydrochlorides of the aminofluorenols are not carcinogenic for the mouse bladder. It also seems doubtful whether any of the histological changes seen can be attributed to the implanted compounds, and no conclusions can be drawn whether any of the benign lesions observed were premalignant.

A surprising result of the present work was the inactivity of 1-amino-2-naphthol hydrochloride. With the possible exception of 20-methylecholanthrene (8) only 1-methoxy-2-naphthylamine hydrochloride had shown greater carcinogenicity (7).

\textsuperscript{15} Five ml. of pooled mouse urine (44, 670 dpm) were cooled in ice and subjected to distillation in a vacuum. The distillate, which was collected on a cold finger immersed in a dry ice-isopropyl alcohol bath, contained 38.170 dpm.

CHART 1.—A: Paper chromatogram of an aliquot (100 \textmu l.) of the ethanol-soluble material (in 5 ml. 95 per cent ethanol) from a pellet (23.7 mg.) made by incorporating l-amino-2-naphthol-H\textsuperscript{3} hydrochloride (3.6 mg.) into paraffin. The pellet was processed as described in the text without being implanted. The 95 per cent ethanol solution was applied to Whatman No. 1 paper, and the chromatogram was developed by the descending technic with 20 per cent hydrochloric acid as the solvent. The radioactive peak, RF = 0.48, coincided with the pink spot obtained by spraying a separate section of the chromatogram with diazotized p-nitroaniline as described in the text.

B: Paper chromatogram of an aliquot (100 \textmu l.) of the ethanol-soluble material (in 2 ml. 95 per cent ethanol) from a pellet (21.9 mg.) made by incorporating l-amino-2-naphthol-H\textsuperscript{3} hydrochloride (3.0 mg.) into paraffin. Eighteen weeks after implantation the pellet was removed from the bladder and processed as described in the text. Neither the labeled material at the origin nor the labeled components with RF values of 0.30 and 0.41, respectively, reacted with diazotized p-nitroaniline when a separate section of the chromatogram was sprayed with the reagent as described in the text.
Since the published directions of the implantation technic and the criteria for interpretation of the histological data (1, 3, 12) were observed scrupulously, the negative outcome can hardly be attributed to minor technical problems, but raises doubts concerning the reproducibility of the method. These doubts have been reinforced by the report that the same preparation of 20-methylcholangrene which was inactive at Yale gave a 58 per cent tumor incidence at Leeds (2). Until these divergent results have been explained satisfactorily, the view of Bonser et al. (2) that the validity of the bladder implantation technic is firmly established cannot be accepted without reservation.

The lack of carcinogenicity of the hydrochlorides of 2-amino-1-fluorenol, 2-amino-3-fluorenol, and 1-amino-2-naphthol for the mouse bladder weakens the o-hydroxy amine hypothesis of cancer induction in its present form (6). A similar view has been expressed by Bonser et al. (2). Nevertheless, these workers are taking the position that 2-amino-1-naphthol may be the proximate agent in the induction of bladder cancer by 2-naphthylamine (2). The instability of implanted 1-amino-2-naphthol hydrochloride demonstrated here throws considerable doubt on this view. If the induction of bladder cancer is to be attributed specifically to an implanted o-hydroxy amine it is essential that the stability of the compound in the bladder be established. However, no such checks have been carried out either with 2-amino-1-naphthol hydrochloride or with any of the o-aminophenols which have been implanted to support the o-hydroxy amine hypothesis. It seems to us that the data reported here, as well as experiments which contradict the o-hydroxy amine hypothesis on other grounds (15, 16, 18), argue against the acceptance of 2-amino-1-naphthol as a proximate agent in the induction of cancer of the mouse bladder.

REFERENCES


FIG. 1, 2.—Invagination of mucosal epithelium, forming subepithelial nests with central cavities. Note inflammatory cells in Fig. 2. Fig. 1, ×100; Fig. 2, ×200.

Fig. 3.—Progression of mucosal invagination. This lesion was related to suture. ×100.

Fig. 4.—Papillary pseudotumor, again showing invaginated epithelial nests. Note inflammation and central lumina. This occurred in a control animal. ×100.
FIG. 5.—Epithelial hyperplasia and inflammation (base of lesion). Note the pseudoglands and the inflammatory cells. There was a sharp line of demarcation from the muscle. X200.

FIGS. 6, 7.—Examples of more advanced epithelial hyperplasia, showing eroded surfaces and inflammation. Fig. 6, X100; Fig. 7, X200.

FIG. 8.—Vegetative change, secondary to suture. X200.


Evaluation of the Carcinogenicity of Aminofluorenols by Implantation into the Bladder of the Mouse

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