IV. Action of Related Compounds

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Studies of the physiological action of 2-acetaminofluorene (AAF) were started because the compound had been found to have insecticidal properties, and it was desirable to have some knowledge of possible toxicity to mammals. When its carcinogenic properties were discovered (12), AAF was no longer considered for use as an insecticide. However, there were several other fluorene compounds that showed insecticidal promise, and therefore investigations were initiated to determine whether some compounds related to AAF were carcinogenic. Furthermore, such a study would probably aid in understanding the action of AAF.

EXPERIMENTAL

For the most part the compounds under investigation were incorporated in the diet and fed for some time to albino rats. This was the method which was found to be effective for AAF. The rats ate these diets for 100 days or more. The colony of rats has been described previously (12, 13).

2-Aminofluorene.—In a previous paper (12) it was suggested that the effective carcinogenic substance was 2-amino fluorene (AF) rather than AAF. This was because the introduction of AAF by parenteral routes (thus avoiding possible hydrolysis in the alimentary tract) did not seem to lead to tumors. The idea has been modified (13) since some of the animals that received AAF parenterally finally developed tumors, although nodular hyperplasia characteristic of the AAF effect either did not appear or was minimal and appeared late. It was noticed also in the original work that the rats excreted a substance which colored the pine shavings of the cage orange; solutions of AF of the proper concentration did the same, whereas solutions of AAF did not. This was probably a lignin color reaction with an amino compound (8). If so, then animals treated with AF should develop cancer.

Five female rats were given a diet containing 0.031 per cent of AF, and 6 were fed half this concentration or 0.016 per cent. The compound was administered for 403 days to the survivors. Those still alive 578 days from the start of the feeding were killed and autopsied. The 5 animals receiving the 0.031 per cent diet developed tumors similar in appearance and distribution to those seen in the rats fed AAF. The average time from the beginning of feeding to death was 410 days. Three of the 6 rats which received 0.016 per cent AF developed tumors, and the average time until death was 516 days. Postmortem examination revealed tissue changes similar to early AAF toxicity (cystic liver, enlarged rough liver, and small translucent spots in the lungs). Histological examination of tissue sections was made on 4 of the animals receiving 0.031 and on 2 of those on the 0.016 per cent diet. These 6 rats were the first of the group to die. The remainder did not differ from them materially in gross appearance, and tissue sections of these rats were not prepared. All the rats examined histologically showed distinct nodules of hyperplastic hepatic cells (Fig. 1) and various numbers of small cysts in the liver. In 3 of the 5 bladders examined histologically there were foci of nodular epithelial hyperplasia forming distinct papillomas (Fig. 2). Another bladder showed minimal epithelial irregularity while the remaining 1 showed no abnormality. Two animals

*This compound and the chlorofluorene, fluorene, fluorenone and xanthone were furnished by the Insecticide Division of the Bureau of Entomology and Plant Quarantine, U. S. Department of Agriculture.

Melting points as reported in the literature for pure samples and as observed by us for the samples tested for carcinogenicity, are:

<table>
<thead>
<tr>
<th>Compound</th>
<th>Reported</th>
<th>Observed</th>
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<tbody>
<tr>
<td>2-Aminofluorene</td>
<td>129-130° C.</td>
<td>126° C. corr.</td>
</tr>
<tr>
<td>2-Chlorofluorene</td>
<td>97-98° C.</td>
<td>91° C. corr.</td>
</tr>
<tr>
<td>Fluorene</td>
<td>113-116° C.</td>
<td>108° C. uncorr.</td>
</tr>
<tr>
<td>Fluorenone</td>
<td>84° C.</td>
<td>81° C.</td>
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<tr>
<td>Xanthone</td>
<td>173-174° C.</td>
<td>162.5° C. corr.</td>
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Fortunately, the compound (AF) that proved to be carcinogenic was one of the purer compounds.
had squamous cell carcinomas of the head (Fig. 3), 2 showed nodular grouping of follicles in the thyroid, and there was one instance each of adenocarcinoma of the intestine (Fig. 4), bilateral adenoma of the adrenal medulla, focal epithelial hyperplasia of the renal pelvis, and mild irregularity of the pancreatic acini with pancreatic cysts. One lung showed irregular proliferation of mucous glands around a chronically inflamed bronchus and another showed two nodules of proliferated alveolar lining cells like those described in the AAF animals (3). The 2 ovaries which were examined had prominent clumps of large stromal cells; 1 contained several simple cysts, probably derived from follicles. One uterus showed extensive endometritis, while the other showed irregular grouping of small endometrial glands into indistinct nodules near the lining epithelium.

Concentrations of AF of from 0.016 to 0.25 per cent were fed to a series of C57 mice. On gross inspection, all 5 of the animals receiving the 0.25 per cent AF diet developed typical tumors. Histologically, 2 showed distinct adenoma formation in the liver, and 2 others had slight irregularity of hepatic cell arrangement in places, suggesting early nodular hyperplasia. Carcinoma of the bladder appeared in 3 and 1 showed slight thickening and metaplasia of the bladder epithelium. In 2 animals there was irregular thickening of the epithelium of the kidney pelvis, and 1 of these showed a squamous cell type of carcinoma in the kidney. A tiny nodule of tumor tissue in the lung of this animal was clearly a metastasis. No other hyperplasias or tumors were seen.

None of the mice on the lower concentrations had grossly recognizable tumors, although a number of the organs were not entirely normal. Two mice which were fed 0.125 per cent of AF in the diet showed irregular thickening of the bladder epithelium, and 1 had slightly thickened renal pelvic epithelium. Liver irregularity was slight in these animals and no distinct adenomas were seen. The mice on the lower dosages showed decreasing effects with decreasing concentrations. No distinct changes were found in any of the organs studied from the 5 animals which received 0.016 per cent of AF for 326 days.

This experiment was stopped earlier than the similar one with AAF (13), so that comparative effectiveness of the two compounds cannot be judged from a consideration of incidence of lesions. That AF is possibly more active than AAF in mice is suggested by what seems to be a slightly shorter time of incubation.

Crystalline AF (100 to 150 mgm.) was placed in a subcutaneous pouch of each of 5 rats, and 250 mgm. in the same way 96 days later. Considerable coloration of the shavings occurred for a few days following the implantations, then disappeared, indicating an initial rapid absorption and excretion. After the second implantation the wounds healed satisfactorily, but the animals refused food and water. Three of them died on the 112th day and were eaten so that autopsy was impossible. A fourth died on the 118th day. Gross inspection revealed no lesions; crystalline material was found at the site of implantation. Postmortem changes were considerable so that tissues were not saved for microscopic examination. The last animal was autopsied 477 days after the initial implantation. In this rat there were small liver nodules and cysts of the kind seen in animals fed AF, but the other organs were normal.

Five rats were given subcutaneous injections of AF dissolved in propylene glycol, 4 times in 6 months. Each injection contained 20 mgm. of AF. The experiment ran for 202 to 582 days. Two of the 4 animals studied histologically developed leukemia; leukemia developed in 1 of the 3 animals injected with propylene glycol alone. There were no distinct liver nodules or cysts although one animal showed slight irregularity in size of the liver cells. The 3 bladders examined were normal. One animal had a subcutaneous sarcoma and a bony proliferation of a leg suggesting osteoma; another had an adenoma of the adrenal medulla together with a nodule in the mesentery suggesting a chromaffin tumor. A third had a small papillary adenoma of the lung. These are not types of tumors which have been seen frequently in AF- or

**DESCRIPTION OF FIGURES 1 TO 4**

**Fig. 1.**—Liver of Rat AF 1086. Margin of nodule of hyperplastic hepatic cells (above). Abnormal cells are larger and uniform. They do not penetrate among the preexisting liver cells, and there is no evidence of malignancy. Mag. X 100.

**Fig. 2.**—Bladder of Rat AF 1215. Carcinoma of bladder, showing infiltration of muscularis by groups of neoplastic epithelial cells. Mag. X 100.

**Fig. 3.**—Head of Rat AF 1109. Squamous cell carcinoma which arose adjacent to the external auditory canal, showing penetration of skeletal muscle by groups of tumor cells. The tumor is well differentiated and there is advanced keratinization in center of some of cell masses. Mag. X 100.

**Fig. 4.**—Intestine of Rat AF 1207. Hyperplastic nodule in mucosa of small intestine bordering a segment of normal mucosa. In this field there is little penetration of the muscularis, but in another place it is completely penetrated, indicating malignancy. Mag. X 100.
2-Chlorofluorene.—This compound differs from AF in having a chlorine in place of the amino group. Groups of female rats, five animals to a group, were fed chlorofluorene1 in concentrations from 0.016 to 0.25 per cent of the diet. The highest concentration decreased the rate of growth to some extent. All rats except those on the diet containing 0.125 per cent were autopsied after 100 days. The organs were normal microscopically, and organ weights were normal with the exception of the heart, which averaged 13 per cent less than the average of control animals of the same body weight. This difference was statistically significant. The rats on the 0.125 per cent diet were continued until they had been on the diet for 337 to 602 days. Of the four animals autopsied, one had leukemia, and another had cysts of a mammary gland and an adenoma of the hypophysis. There was no evidence of the type of abnormal cell growth seen in the animals fed AAF or AF.

Fluorene.—Fluorene has not been found to be carcinogenic when implanted subcutaneously or painted on the skin (for references, see [12]). However, it was considered advisable to administer it gastrically in the manner known to be effective for AAF. One series of rats was kept for 104 days on diets containing from 0.062 to 1.0 per cent fluorene.1 A yellow staining of the fur around the urethral orifice was a common observation. Concentrations of 0.5 and 1.0 per cent led to decreases in the rate of growth which were statistically significant, but the general condition of the animals seemed good. Grossly and histologically the organs appeared normal, although weights of the organs were not. The livers of rats on diets of 0.25 per cent or more were significantly heavier than normal for animals of the same weight, the spleens of all groups of fluorene-treated animals were lighter than normal, and the testes of the rats on the highest dose were significantly small. Three groups receiving fluorene in the diet in concentrations of 0.125, 0.25 and 0.5 per cent were observed for 453 days. At autopsy they were fat, healthy and showed no gross signs of tissue damage. The yellow fur was the only indication that these animals were not on a normal diet. Histologically, several animals showed pulmonary inflammation which was unrelated to the dose of fluorene. In three of these there was metaplasia of bronchial epithelium to squamous type. One animal on the 0.125 per cent diet showed a small benign tubular adenoma of the kidney of a type not seen before in this colony. There was no other metaplasia or hyperplasia of the types seen in animals fed AAF. Four of these animals had moderate testicular atrophy, 2 had pericarditis, and two showed bladder worms.

Fluorenone.—Fluorenone1 is the compound obtained by replacing the two hydrogens of the 9-carbon of fluorenone with an atom of oxygen. It was incorporated into the diet in concentrations of 0.031 to 0.5 per cent. Concentrations of 0.125 per cent or more of fluorenone significantly decreased the rate of growth, especially in the latter half of the 100 day feeding period. The livers, kidneys, and perhaps testes of these rats were significantly heavier than in the appropriate controls. The fur was stained yellow or yellow-brown. There were no indications of tumor or of nodular hyperplasia. Six female rats were placed on a 0.25 per cent fluorenone diet for periods ranging up to 600 days. Among these, 2 of the animals had small thyroid follicles and 1 showed a cystic endometrium.

Twenty milligrams of fluorenone dissolved in propylene glycol was injected subcutaneously into each of 4 female rats 3 times during a 6-month period. The animals were observed for 373 to 600 days. Two of the animals had breast adenomas—in 1 there was nodular hyperplasia of additional mammary tissue. One animal had leukemia. There were no other hyperplastic lesions like those in the AAF animals.

3-Chlorofluorene.—The compound differs from fluorenone in that an oxygen in an ether linkage is inserted between the 2 benzene rings. Xanthone affected the rats in a way similar to fluorenone. There was occasional yellow or orange

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2-The diethylaminoethyl-fluorene-9-carboxylate hydrochloride was furnished to us by Dr. P. J. Hanzlík, Dept. of Pharmacology, Stanford University School of Medicine, who received it through the courtesy of G. D. Searle & Co. We have no knowledge of the purity of this compound.
coloration of the fur. Animals on the diet containing 0.5 per cent of the compound had a significantly slower rate of growth than did control rats, and growth on the 1.0 per cent diet was practically stopped. After 100 days on the diet, rats eating 0.25 per cent or more of the compound in the diet had livers heavier than normal for their body weights, and their spleens were slightly lighter. The organs appeared normal. Five female rats were kept on the 0.25 per cent diet for over a year, and 3 of them for 640 days. The organs of all appeared normal at autopsy.

**DISCUSSION**

As had been anticipated, AF proved to be carcinogenic. There are, however, certain apparent discrepancies. If AAF is active through the AF formed from it by hydrolysis in the intestinal tract, then AF should be at least as active as AAF. This was not the case with the rats. Lesions were slower in developing, and at the 0.016 per cent level, were less numerous. This may be due to differences in solubility. AF is considerably more soluble than AAF. Perhaps in the rat it was absorbed soon after ingestion and excreted quickly thereafter, so that the tissues were not continually exposed to the compound. The AAF, on the other hand, may have given a more continuous exposure, because AF could be absorbed only as fast as the acetylated compound was hydrolyzed in the intestine. In mice, on the other hand, the AF seemed to be as effective as AAF, and perhaps a little more so, as judged by the time of development of tumors. Possibly a difference in eating habits could explain the divergent findings of rats and of mice.

The comparatively slight effect noted in the 1 rat that survived 2 subcutaneous implantations of crystalline AF may also be explained by an influence of solubility and rate of excretion upon carcinogenic effect. That the AF was absorbed to a considerable extent is clear from the fact that after the second implantation, all 5 of the rats were sick and 4 of them died. Similar implantation of AAF crystals in other rats had no observable effect.

Bielschowsky (1) has described an experiment in which he obtained distant malignant tumors in rats that had their skins painted with AF. A 4 per cent solution in acetone was applied thrice weekly for 210 days. This experiment differed in certain important respects from the 1 described in this paper where crystals of AF were implanted subcutaneously. The frequent painting and the presumably slow absorption from the skin might keep a continuous stream of the compound passing through the animal. That AF is carcinogenic is clear from the work of Bielschowsky and the data presented in this paper.

If 2-chlorofluorene is changed at all to AF by the animal, the extent of the change is probably very slight. The hydrocarbon fluorene, the 2 fluorene compounds in which substitution was at the 9-carbon, and xanthone, which differs from fluorenone by having an oxygen atom introduced into the central ring, all proved to have no carcinogenic action when given to the animals together with the food. As mentioned earlier, others have found that fluorene is not carcinogenic when applied to the skin or implanted underneath it.

In an earlier paper (12) we suggested that an amino group in the 2-position might be of importance in determining carcinogenicity of certain compounds—2-aminofluorene, 2-acetaminofluorene, beta-naphthylamine (5), 2-aminoanthracene (10), 2, 2'-diamino-1, 1'-dinaphthyl, etc. (2). The present paper does not give any final answer to this suggestion, but it does show that aminofluorene is carcinogenic, that replacement of the amino group by chlorine destroys that activity, and that certain substitutions at positions other than the 2-carbon give non-active compounds.

A number of the compounds were given subcutaneously in propylene glycol solution. In several instances, and apparently without regard to the amount or character of the dissolved compound, death of the animal from leukemia occurred. Indeed leukemia developed in 1 of 3 rats given subcutaneous injection of propylene glycol alone. Altogether there were 21 control and experimental rats that received propylene glycol subcutaneously; 6 of these developed leukemia in 202 to 600 days. While occasional instances of leukemia have been noted in AAF animals, the incidence was much lower than for the rats receiving propylene glycol solutions of the various substances; in non-experimental rats and in rats used for other studies, leukemia has not been observed. Propylene glycol has been studied somewhat extensively and has not been considered a very toxic substance. Dogs have been kept for 5 to 9 months on a regimen in which their fluid requirements were met by a 5 per cent solution of propylene glycol (11). Hanzlik, Lehman, Van Winkle and Kennedy (4) fed rats for 5½ months on a diet that was 25 per cent propylene glycol. Kesten, Mulinos and Pomerautz (6) administered the glycol in drinking water to rats for times up to 234 days. It is possible that in these instances the animals were not observed for a long enough period to permit the development of leukemia. However, this cannot be said
of the study by Morris, Nelson and Calvery (9), who kept rats on diets containing 2.45 and 4.9 per cent of the compound for 24 months. The high incidence of leukemia in the propylene glycol-injected animals of this colony deserves further study.

SUMMARY

1. 2-Aminofluorene when incorporated in the diet and fed for a considerable period to rats and mice, produced carcinomas resembling those formed by 2-acetaminofluorene. Implanted crystals of aminofluorene were quite toxic; in the rat that lived, lesions were slight. This may be due to the elimination of the compound from the body before malignant processes were well initiated.

2. 2-Chlorofluorene, fluorene, fluorenone, diethylaminoethylfluorene-9-carboxylate and xanthone were nor carcinogenic when administered in the diet to rats.

3. It is suggested that an amino group in the 2-position is important in determining carcinogenic activity of certain types of compounds.

4. Leukemia developed in a number of rats given propylene glycol solutions subcutaneously. The literature does not record any leukemias in animals given the compound by mouth. It should be determined more definitely whether the glycol was the causative factor.

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REFERENCES


The Carcinogenic Activity of 2-Acetaminofluorene. IV. Action of Related Compounds

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