2-Acetylaminofluorene, a carcinogen originally discovered by Wilson, DeEds, and Cox (28), has been shown by these and many other investigators to be a highly active carcinogen that attacks numerous sites in the rat and mouse. In addition, the parent hydrocarbon, fluorene, and several of its other derivatives have been tested for carcinogenic activity in the rat. Fluorene and its oxidation product, fluorenone, are inactive (30). While 2-nitrofluorene is only weakly carcinogenic (19) its reduction product, 2-aminofluorene, is highly active although somewhat less so than 2-acetylaminofluorene (2, 12, 19, 30). The activity of the latter compound is not greatly changed upon conversion to the diacetyl derivative (19). 7-Hydroxy-2-acetylaminofluorene, a metabolite of 2-acetylamino fluorene in the rat (3), has but little activity in this species (13). Similarly, the miscellaneous derivatives xanthone and 2-chlorofluorene are non-carcinogenic (30).

The objective of the study reported here was to determine if the -CH₂- bridge in 2-acetylamino fluorene is essential to its carcinogenic activity in the rat. Three analogues were tested in which the bridges consisted of either -S- as in 3-acetylamino dibenzothiophene, -O- as in 3-acetylaminobenzofuran (see Table I for structures). The activity of 4-dimethylaminobiphenyl is also described. This compound, a derivative of 2-amino fluorene in which the bridge is absent, was original-

METHODS

Preparation of compounds.—2-Amino fluorene, prepared from fluorene as described by Kuhn (14), was acetylated by dissolving it in excess hot acetic anhydride containing 1 to 2 per cent of redistilled pyridine. The 2-acetyaminofluorene was precipitated by the addition of 2 to 3 volumes of water, filtered, washed, and dried. It was then dissolved in hot ethanol, refluxed twice with 10 per cent of its weight of charcoal, and finally crystalized by the addition of water. The final yield, calculated from fluorene, was 50 per cent. The recrystallized product was nearly white and melted at 190°—191° C. 3-Acetylaminobenzenothiophene, m.p. 195°—198° C., and 3-acetylaminobenzenothiophene-5-oxide, m.p. 275°—276° C., were synthesized from dibenzothiophene as described previously (5). 3-Aminobenzofuran, prepared by the method of Gilman and Avakian (9), was acetylated by heating for 1 hour on a water bath a benzene solution of the amine with a 10 per cent excess of acetic anhydride. The acetylated compound which precipitated from the solution was dissolved in hot alcohol and decolored with charcoal. After recrystallization from 98 per cent ethanol the 3-acetylaminobenzofuran melted at 179°—180° C. since initial attempts to prepare 4-dimethylaminobiphenyl by methylation of 4-aminobiphenyl with aqueous alkali and methyl sulfate (26) were unsuccessful, the methylation was carried out by adding 0.6 mole of dimethyl sulfate during a half hour period to 0.2 mole of 4-aminobiphenyl while the temperature of the reaction mixture was maintained at 90° C. After allowing the mixture to cool to room temperature excess 10 per cent NaOH was added; the mixture was stirred for an hour and then filtered. The 4-dimethylaminobiphenyl melted at 120°—121° C. after being recrystallized twice from ethanol acetone by the addition of water. The final yield was 30 per cent of theoretical; the product was recovered unchanged after refluxing with acetic anhydride.

Method of assay.—In the first series 6 male and 6 female albino rats, each 175 to 200 gm. in weight, were fed a diet containing 0.005 per cent of each of the compounds being tested. The control rats in this study were killed at 500 days while the rats fed 7-hydroxy-2-acetylaminofluorene were killed at 700 days.
RESULTS

First series.—In this preliminary series 3 of the 6 female rats fed 3-acetylaminodibenzothiophene in the semi-synthetic diet developed tumors of the mammary glands after receiving the compound for 4 1/2 to 6 1/2 months. The other females and all of the males died tumor-free after receiving the compound less than 5 months. Tumors were found in the rats fed 2-acetylaminodibenzothiophene after the compound had been ingested for 8 to 11 months. Of the 6 females fed this diet one developed a tumor of the ear duct, another a liver tumor, and a third primary tumors of both the lungs and the ear duct. Of the 6 male rats fed this compound 3 developed tumors in the liver, and one of these rats also had a tumor of the ear duct.

Second series.—In this series the health and survival of the rats were better and the tumors developed earlier than in the first series. It is likely that this was due, at least in part, to the change from the semi-synthetic diet to the grain diet, since only 4 of the 10 female rats fed 3-acetylaminodibenzothiophene in the semi-synthetic diet survived for 4 months while 9 of the 10 females fed this compound in the grain diet were alive and in good health at this time.

The distribution of the tumors found in the rats fed the four compounds in the grain diet is given in Table I. Where more than one type of primary tumor was found on a single rat (for example, tumors from the ear duct and mammary glands) each tumor is listed in the table. Although multiple tumors of one kind, particularly of the mammary glands and ear ducts, occurred frequently they are not indicated. Where tumor incidences are presented in the text, they are based on the number of rats alive at 4 months. None of the tumors reported here has ever been observed when rats of this stock have been maintained for 12 months or longer on either the grain or semi-synthetic diets. After 15 to 20 months about 5 per cent of the female rats of this strain do develop spontaneous fibroadenomas of the mammary glands.

Each of these compounds induced tumors of the mammary glands in the female but not in the male rats. The first tumors were observed after 3 1/2 months and by 4 months the incidences were 67 and 57 per cent respectively for the rats fed 3-acetylamino fluorene and 3-acetylaminodibenzo-phen; the final incidences in these groups were 78 and 67 per cent respectively. In contrast, the sulfone of 3-acetylaminodibenzothiophene induced but one tumor at this site. While 3-acetylaminodibenzo-phen finally gave rise to a high incidence of mammary tumors (83 per cent), the latent period was 2 to 3 months longer than for the other compounds. Other workers (2, 6, 12, 29) have also observed few or no tumors of the mammary glands in male rats fed 2-acetylamino- fluorene, and 3-acetylaminodibenzo-phen; the final incidences in these groups were 78 and 67 per cent respectively. In contrast, the sulfone of 3-acetylaminodibenzothiophene induced but one tumor at this site. While 3-acetylaminodibenzo-phen finally gave rise to a high incidence of mammary tumors (83 per cent), the latent period was 2 to 3 months longer than for the other compounds. Other workers (2, 6, 12, 29) have also observed few or no tumors of the mammary glands in male rats fed 2-acetylamino fluorene, while these tumors usually develop in 30 (2, 6, 12, 29) to 100 (8) per cent of the female rats. Mammary tumors from 2 rats fed 2-acetylamino fluorene and from 5 rats fed 3-acetylaminodibenzo-phen were aseptically removed, minced in saline, and injected subcutaneously into 4 to 6 young female rats. With the exception of one tumor from a rat fed the thiophene derivative, successful transplantation...
was obtained in 25 to 100 per cent of the new hosts. Second generation transplants were attempted and successful in each of two cases.

2-Acetylaminofluorene and 3-acetylamino dibenzothiophene were also equally effective in inducing tumors of the ear ducts while 3-acetylamino dibenzothiophene-5-oxide and 3-acetylamino dibenzofuran were less active in this respect. Thus, by 6 months 22 to 37 per cent of the male and female rats fed the first two compounds had developed this type of tumor; these incidences rose to 38 to 75 per cent by 8 months. Only 14 and 23 per cent of the rats fed 3-acetylamino dibenzothiophene-5-oxide and 3-acetylamino dibenzofuran respectively developed these tumors. The incidence of this type of tumor did not seem to be influenced appreciably by the sex of the animals. While this type of tumor has been consistently observed in other experiments with 2-acetylaminofluorene (2, 12, 29), the incidence in this experiment was greater than has been reported previously. The mammary and ear duct tumors induced by each of the three new compounds were very similar, grossly and histologically, to those induced by 2-acetylaminofluorene.

In contrast to the ability of each of the four compounds to induce tumors of the ear ducts and mammary glands only 2-acetylaminofluorene gave rise to liver tumors and gross liver damage. In line with observations by other workers (2, 12, 25, 29) the livers of male rats appeared to be more susceptible to this carcinogen than the livers of female

<table>
<thead>
<tr>
<th>TABLE I</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>THE CARCINOGENIC ACTIVITIES OF ANALOGS OF 2-ACETYLAMINOFLUORENE WITH THE GENERAL FORMULA</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TUMOR SITE</th>
<th>MOS.</th>
<th>2-ACETYLAMINOFLUORENE</th>
<th>3-ACETYLAMINODIBENZOTHIOPHENE</th>
<th>3-ACETYLAMINODIBENZOTHIOPHENE-5-OXIDE</th>
<th>3-ACETYLAMINODIBENZOFLUORENE</th>
</tr>
</thead>
<tbody>
<tr>
<td>SURVIVAL</td>
<td>4</td>
<td>9</td>
<td>10</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>MAMMARY GLAND</td>
<td>4</td>
<td>6</td>
<td>0</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>6</td>
<td>0</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>7</td>
<td>0</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>EAR DUCT</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>4</td>
<td>6</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>LIVER</td>
<td>6</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>0</td>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NONE</td>
<td>4</td>
<td>3(3)</td>
<td>10(0)</td>
<td>3(3)</td>
<td>8(8)</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>2(2)</td>
<td>7(7)</td>
<td>1(1)</td>
<td>1(1)</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>1(0)</td>
<td>0</td>
</tr>
</tbody>
</table>

*NUMBER LIVING AT 4 MONTHS OVER NUMBER AT START

†TOTAL NUMBER OF TUMOR-FREE RATS (NUMBER OF TUMOR-FREE RATS STILL ALIVE)

††ONE RAT, NOT INCLUDED HERE, DEVELOPED A TUMOR AT ANOTHER SITE (SEE TEXT)
rats. Although 6 female rats survived more than 6 months and 2 of these more than 8 months, none developed detectable liver tumors, while the incidence for the male rats was 70 per cent at 8 months. Further, the gross damage was usually less in female than in male rats fed 2-acetylamino-
fluorene for the same length of time.

In addition to tumors of the ear duct, mammary gland, and liver, single tumors were also found at three other sites. One female rat fed 3-acetylamino-
dibenzoazothiophene for 4 months developed an "oat-cell" carcinoma of the lung. This tumor was first discovered as a metastasis in the spleen, and one month after removal of the spleen the animal died with numerous metastases in the liver and mediastinum. A tumor of endothelial origin was found in the subcutaneous tissue over the forehead of a male rat fed 3-acetylamino-
dibenzoazothiophene-5-oxide for 8 months. An epidermoid carcinoma of low grade malignancy arising from or near the vagina was found on autopsy of a female rat fed 3-acetylamino-
dibenzoazofuran for 8 months. Although this rat also had mammary gland and ear duct tumors, all of the three tumors were primary cancers.

4-Dimethylaminobiphenyl.—Twelve of the 14 male rats fed 4-dimethylaminobiphenyl survived for at least 8 months, the time at which the first tumor was found (Table II). By 10 months, 3 of the 12 rats had died without developing tumors. Between 8 and 10 months, 13 primary tumors were found in the remaining 9 rats. These included 4 adenocarcinomas of mammary gland origin, 1 sarcoma arising in the mediastinum and invading through the sternum, 3 tumors of the ear duct, 3 benign liver tumors, and 2 fibromas arising in or near the vertebral canal. The fibromas were found in 2 rats which developed a paralysis of one of the hind legs. Severe urine retention was present in these rats during the last week of life. These effects were presumably due to pressure by the tumor on the cord. The occurrence of mammary tumors in these male rats is of interest since none of the male rats fed the other compounds developed this type of tumor.

TABLE II

<table>
<thead>
<tr>
<th>Tumor Site</th>
<th>Diagnosis</th>
<th>Months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mammary gland</td>
<td>Adenocarcinoma</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>Mediastinum</td>
<td>Sarcoma</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Ear duct</td>
<td>Epidermoid</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Carcinoma</td>
<td>3</td>
</tr>
<tr>
<td>Liver</td>
<td>Benign</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Hepatoma</td>
<td>3</td>
</tr>
<tr>
<td>Vertebral canal</td>
<td>Fibroma</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>None</td>
<td></td>
<td>11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
</tr>
</tbody>
</table>

DISCUSSION

The data presented in Table I demonstrate that the substitution of a thio ether linkage, -S-, for the methylene linkage, -CH2-, in the 2-acetylamino-
fluorene molecule did not alter its carcinogenicity toward the mammary gland and ear duct tissue of the rat. On the other hand, the introduction of the sulfoxide linkage, -SO-, produced a compound which was only feebly carcinogenic. Replacement with the ether linkage, -O-, led to a compound which eventually exhibited a high activity towards the mammary gland and a somewhat reduced activity towards the ear duct tissue. The inability of each of the three analogues of 2-acetylaminofluorene to induce tumors in the liver is noteworthy since the latter compound is quite active in this respect.

Somewhat similar observations have been made on the substitution of oxygen for sulfur, or vice versa, in several naturally occurring molecules. Thus the replacement of -S- by -O- in the ring of the biotin molecule does not seriously alter the ability of the resulting compound to act as a vitamin for several microorganisms as well as for certain higher species (1, 15, 31). However, oxidation of biotin to the sulfone destroys the ability of the molecule to act as a vitamin and, in fact, the sulfone can serve as a biotin antagonist (7). Conversely, the insertion of -S- for -O- in the ether linkage in thyroxine yields a molecule which can still accelerate metamorphosis in tadpoles (11). On the other hand, in methionine where the lability of the S-CH3 bond is important to its activity in vivo, the replacement of -S- by -O- leads to an anti-
metabolite (24).

The carcinogenic activity of 4-dimethylaminobiphenyl is of considerable interest; for even though the activity of this compound, on both a time and molarity basis, was relatively low, it induced a variety of tumors in a high percentage of the surviving animals. Of particular interest is the fact that this compound induced mammary tumors in male rats; such tumors are rare in male rats fed 2-acetylamino-
fluorene (2, 6, 12, 29). The sites which this molecule attacks show that it is more closely related to the aminofluorenes than to the aminoazo dyes. In comparison with the former...
class the -CH$_2$- bridge is missing and in comparison with the latter class the azo, -N=N-, linkage is absent. However, the omission of the bridge in the fluorene molecule is probably the less serious of the two since here the inter-atomic distances are probably not greatly changed. This follows from the fact that biphenyl and some of its derivatives generally tend to assume a coplanar configuration (27) which is, very probably, rigidly maintained in the fluorene molecule. Arguments have been presented in favor of this uniplanar structure (22) as well as a labile folded ring structure for fluorene (28). The present authors consider it possible that the greater biological activity of the fluorene derivatives versus the biphenyl derivative (Tables I and II) is associated with the -CH$_2$-, -S-, and -O- groups which help maintain a coplanar arrangement of the benzenoid nuclei. One consequence of this arrangement is that the structure of the molecule would then be represented to a greater extent by resonating quinonoid structures such as:

\[
\begin{array}{c}
\text{N} \\
\text{X} \\
\text{Y} \\
\text{C} \\
\text{C} \\
\text{C} \\
\text{C} \\
\text{C} \\
\text{C} \\
\text{C} \\
\text{C} \\
\end{array}
\]

It is also interesting that Haddow et al. (10) found 4-dimethylaminobiphenyl to be inactive as a tumor inhibitor. Apparently it is an exception to the approximate correlation which Haddow and his group have observed to hold between the growth-inhibitory power and the carcinogenicity of certain polycyclic aromatic hydrocarbons. Later these studies led to the discovery of the carcinogenic activity of 4-dimethylaminostilbene and some of its derivatives. In this connection it is notable that while the 4-aminoazobiphenyls are isomeric with the 4-aminostilbenes, they have carcinogenic activities (10) which ally them more closely with the azo, -N=N-, linkage isomeric with the 4-aminoazobiphenyl which has no bridge connecting the 2 and 2' positions even more difficult to explain on this basis. Aside from these objections we are in accord with the general idea that chemical carcinogens may act through combination with certain cell constituents, especially protein, and have presented evidence for this in the case of azo dye induced liver tumors (16, 18).

**SUMMARY**

1. The carcinogenic activities of 3-acetylanilinobenzothiophene, 3-acetylanilinobenzothiophene-5-oxide, and 3-acetylanilinobenzofuran were directly compared with that of 2-acetylaminofluorene in male and female rats. A fourth compound, 4-dimethylaminobiphenyl, was tested for carcinogenic activity in male rats. All of the compounds were fed for 8 months.

2. The data demonstrate that replacing the -CH$_2$- bridge in 2-acetylaminofluorene by -S- as in 3-acetylanilinobenzothiophene did not alter the carcinogenicity of the molecule for either mammary gland or ear duct tissue. Substitution of -S- for the -CH$_2$- as in 3-acetylanilinobenzothiophene-5-oxide greatly lowered the activity towards these two tissues, while insertion of an -O- bridge as in 3-acetylanilinobenzofuran only partially diminished the activity of the molecule in these respects. Unlike the control compound, 2-acetylaminofluorene, however, none of these 3 compounds had any carcinogenic activity towards the liver. The tumors produced by all of these compounds appeared 4 to 8 months after the beginning of the experiment.

3. 4-Dimethylaminobiphenyl, a derivative in which the -CH$_2$- bridge is absent, produced tumors in the mammary glands, ear duct, liver, and vertebral canal of male rats. These tumors appeared...
REFERENCES


The Carcinogenic Activities of Certain Analogues of 2-Acetylamino fluorene in the Rat

E. C. Miller, J. A. Miller, R. B. Sandin, et al.

Cancer Res 1949;9:504-509.

Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/9/8/504

Sign up to receive free email-alerts related to this article or journal.

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.