

The Carcinogenic Activity of 2-Acetaminofluorene

II. Effects of Concentration and of Duration of Exposure

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The production of carcinoma in rats fed diets containing 2-acetaminofluorene has been demonstrated by Wilson, DeEds and Cox (8). Albino rats were fed diets containing 0.031 per cent or more of acetaminofluorene (AAF) for periods ranging from 95 to 333 days. Growth was retarded, and hyperplastic and frequently carcinomatous lesions of a variety of tissues developed in all of the animals living for a long enough time. Bielschowsky and Green (4) fed AAF to rats and confirmed the effect on growth. Their data were expressed as mgm. of AAF per day, while we reported the AAF concentration in the diet. Considering average amounts of food ordinarily eaten by rats, it is probable that the growth effects were comparable in the two studies. Bielschowsky (2) later extended this work and in 93 rats found 105 malignant tumors. This work was very much in agreement with ours except that in the former no tumors of the urinary tract were found, and there were several cancers of the intestine, while we found that lesions of the urinary tract were frequent, but noted only one carcinoma of the colon. Armstrong and Bonser (1) gave AAF to mice of the CBA strain (mammary cancer-resistant); of the 12 animals, 10 survived for 32 or more weeks, and of these, 8 developed tumors. Five primary tumors, 4 of them malignant, were found in the urinary bladder. There were 5 tumors, 1 with malignant areas, in the livers, and 2 tumors, 1 malignant (sarcoma) in the uterus. There were no tumors of the ductus acusticus or breast, although a mild breast proliferation was noted. Neither Bielschowsky (3) nor Wilson, DeEds and Cox found any unusual growth in the thyroid, but if the animals were simultaneously treated with allylthiourea to obtain a goitrous gland, AAF led to benign and malignant tumors of the thyroid.

The experimental work on which this paper is based was completed in 1942, but because of the pressure of other work the authors were unable to prepare their results for publication.

As a consequence of the work reported in the first paper (8), further experiments were started to find out more details of the carcinogenic activity of AAF. Due to war-time interruption of the work, assembling of the data was delayed. This paper presents the work on the concentration of, and the duration of exposure to, AAF required to produce neoplastic changes. A comparison of the actions on rats and on mice is made. A generalized discussion of the microscopic appearance of tissue of animals in this and other papers is presented in a separate report (5).

EXPERIMENTAL

Rats.—The colony of rats was the same as that described previously (8). When some difficulty was experienced in obtaining effective transplants of tumor tissue, all breeding was changed to brother-sister matings. Some of the later animals were from this more highly inbred strain. The AAF was from the same batch used previously, and was furnished by the Insecticide Division of the Bureau of Entomology and Plant Quarantine, U. S. Dept. of Agriculture (8). The diet was the same as before. AAF was mixed with the diet in the desired concentrations and fed to the animals *ad libitum*. At approximately weekly intervals the rats were examined carefully. Ordinarily the animals were killed and autopsied when their condition became serious; a few died during the night and were autopsied the next day unless the viscera had been eaten by the other rats. Tissues were fixed in 4 per cent formaldehyde solution, and paraffin sections were stained with hematoxylin and eosin for microscopic examination.

Effect of concentration.—In the first paper (8) we presented data on rats receiving diets containing 0.031, 0.062 and 0.125 per cent AAF in the diet. All these animals, if they lived long enough, developed tumors, and the effectiveness was about the same on the lowest concentration as on the highest. It was considered desirable to find out how low a concentration of AAF would produce

tumors. The data have been extended to diets containing 0.016, 0.008, 0.004 and 0.001 per cent of AAF. There were 6 animals on each of these diets except the lowest, where there were 5. The animals were males, except for those on the 0.004 per cent diet. Tumors like those previously seen developed in all the rats on the 0.016 and 0.008 per cent concentrations. Five of the six rats on the 0.004 per cent diet had similar gross changes, although microscopically, incidence and degree of associated nodular hyperplasia were smaller than in animals fed large quantities of the compound. None of those on the 0.001 per cent AAF diet had lesions which resembled the usual cancerous tissues from animals on higher concentrations.

Histological sections were made of the heart, lung, liver, spleen, kidney, adrenal, testis, bladder, pancreas and thyroid of each of the 5 rats fed 0.001 per cent of the compound for 741 days. There was atrophy of some of the organs, probably explained by the advanced age of the animals when they were killed. Several had chronic inflammatory changes in the lungs, and one showed bronchial epithelial metaplasia to stratified squamous cells, but this did not suggest a specific effect. Similarly chronic inflammation of the pancreas in 2 animals could not be related to the treatment. No distinct hyperplastic nodules of liver cells or cysts were seen in any of the animals, and the bladders were normal in all. One or both of these organs have been altered in practically all animals receiving as little as 0.004 per cent of AAF in the diet. Therefore, the significance of a single nodule composed of fibrous tissue and bone in the liver of one animal and of a small nodule of fibrous tissue and blood vessels resembling an angioma in the spleen of another animal is questionable. These were the only tumor-like lesions in this group of animals. Even assuming that AAF influenced the development of these 2 nodules, which were different from any tumors developing in the other experimental animals, it must be concluded that the effect of this quantity of the material is at most minimal.

The effect of the concentration of the compound in the diet is shown not only by the incidence of tumors, but also by the length of time the rats could be kept on the diet before the tumors necessitated killing the animals. The average time on the diet for the rats receiving the 0.125, 0.031 and 0.016 per cent diets were, respectively, 211, 313 and 305 days. A concentration of 0.008 per cent allowed the animals to live for an average of 407 days; the time was 645 days for the 0.004 per cent diet, and the animals on the 0.001 per cent diet

were sacrificed without signs of tumor after 741 days.

Duration of administration.—In the previous report (8) it was mentioned that 5 rats that had received AAF in their diet for 95 days and then were placed on control diet subsequently developed cancer. It was stated also that a single, large gastric administration of the compound did not seem to be carcinogenic. The 4 animals still alive when that report was written were autopsied from 15 to 22 months after the AAF was administered. One had a mammary adenoma in the groin and some cortical nodules in one adrenal. Some of the others had cystic ovaries or pneumonia, neither of which seemed to be attributable to the AAF.

Since 95 days' exposure will produce tumor and 1 day's will not, the question arises as to how long the animals must eat the substance to initiate those changes which will ultimately develop into malignancy. The periods of exposure selected were 25, 50, 75, 100, and 150 days. The concentration of AAF at the start was 0.125 per cent. Because of the poor physical condition of the animals still on the experimental diet, the concentration was decreased after 54 days to 0.031 per cent. All animals were returned to the control diet at the conclusion of the respective feeding periods. Each group consisted of 9 to 12 animals with approximately equal numbers of each sex. With the exception of 1 rat, which died of pneumonia 112 days after the beginning of the experiment, all of the animals receiving AAF for 75 or more days developed macroscopic tumors. Microscopic examination regularly showed characteristic changes in the tissues of 15 of the 16 rats examined, the 16th being the rat that died of pneumonia.

Of the 11 rats in the 50 day group, 6 had gross tumors, 3 had lesions of questionable character, and 2 were grossly normal. Tissue sections from 10 of these animals were studied. Less frequent alteration was found than in the rats receiving the diet for a longer time, but every animal showed some change. All the livers contained nodules of hyperplastic hepatic cells or cysts, although only 2 bladders showed definite hyperplastic changes. The bladder of a third animal had a grossly visible nodule but this was not included in the section. There were 3 mammary adenomas, 2 subcutaneous fibromas, 1 gross nodule suggesting mammary tumor which was not sectioned, 1 adenocarcinoma of the colon with metastases, 1 papillary carcinoma of the renal pelvis, 1 adenoma of kidney tubules, and 1 unclassified malignant tumor of the mediastinum suggesting origin from the thymus gland. Three rats from this group had lung tumors; 1 was

a small-cell carcinoma surrounding a bronchus, and the others were nodules in which the aveoli were almost filled by proliferated lining cuboidal cells. In the 2 uteri from the animals examined there was local proliferation of endometrial glands.

Twenty-five days' feeding of the compound led to 6 animals with gross tumors, 1 with possible tumor, and 4 with grossly normal tissues. Microscopic examination disclosed occasional changes like those in the other treated animals. They were less frequent and in most animals were minimal. The 9 livers studied histologically exhibited occasional small cysts and 3 showed distinct nodules of hepatic cells. Two female rats showed local proliferation of glands beneath the endometrium similar to those previously described. Only 1 ovary was sectioned from this group; there was a serosal cyst, and in addition, there was marked swelling of the ovarian stromal cells. No follicles were seen in the section. There were 2 mammary fibroadenomas in 1 animal. Two bladders from this group contained papillomas; one spleen showed a fibrous nodule, apparently a fibroma. Two lungs showed focal proliferation of alveolar epithelium like those described in the previous group. A period of 25 days seems to be an approach to the lower limit of exposure necessary for carcinogenic action. Although nodular hyperplasia and neoplasia were not as frequent in this group of animals as in those receiving AAF for a longer time, there was nevertheless a definite carcinogenic effect.

Time of exposure as well as concentration of the agent, is a factor in determining the time required for development of the lesions. The groups receiving AAF for 75 or more days developed tumors in about equal times. The average for these groups from beginning of experimental feeding to the time of autopsy varied from 227 to 295 days. For a 50 day feeding period, the interval increased to 406 days, and for the 25 day period, to 554 days.

In the AAF feeding experiments to determine the minimum effective concentration and the shortest time of exposure, 108 rats were used—53 males and 55 females. Microscopic examination was not made on all animals, so the following figures cannot be interpreted to mean number of malignancies, since some malignant lesions were observed which were not apparent grossly and some large tumors were found to be benign. The principal tumors noted on gross examination were: 44 per cent of the animals had tumors of the bladder, and 7 per cent had renal tumors. The incidence and time of development of these 2 types of lesions were about the same for both sexes. Strikingly abnormal livers were found in 20 per cent of the

females and in 55 per cent of the males, a little earlier in the males than in the females. The average time for males was 317 days from the start of feeding of AAF; for females it was 411 days. Tumors of the mammary type were noted in 34 per cent of the females and 8 per cent of the males, the time of development being about the same for both sexes. The subcutaneous tumors of the head were found in 29 per cent of the females, 17 per cent of the males, and with equal time intervals. Apparently the liver of the male rat is more susceptible than that of the female, whereas the opposite is true for the head and the mammary type of tumor. Bielschowsky (2) gave the incidence of malignant tumors in his rats; he also found a sex difference for liver, mammary gland and head. The greatest difference between Bielschowsky's results and ours is the complete absence of tumors of the urinary tract in his animals.

An impression is obtained, when conducting experiments like those just considered, that the mammary, and particularly the head, tumors develop earlier than do tumors of the internal organs. This impression is not clearly borne out by the assembled data. Obviously, the external tumors are more easily noted than are those of the internal organs, and they ordinarily appear to grow more rapidly, and so, being more spectacular, are more quickly recognized.

Mice.—Since this colony of rats had not been used for cancer studies previous to the work reported earlier (8), it was considered advisable to check the results by using some of the strains of mice frequently employed for carcinogenic experiments.¹ Five levels of AAF-containing diets were fed to C57 mice. The AAF concentration varied from 0.031 to 0.5 per cent of the diet. Four females and 2 to 4 males were started on each level. Liver, bladder or kidney lesions, grossly similar to those developing in the rats, were found in most (11 of the 16 mice alive at the time the first tumor was discovered) of those receiving 0.125 per cent or more of AAF and in some of those receiving the 0.062 per cent diet. Pathological changes not clearly tumorous were found in the mice given the lowest concentration of AAF. The first tumors did not appear quite as early in mice as in rats, and the experiment had to be terminated sooner because of the poor condition of the remaining mice. The average length of time from the start of the AAF diet to autopsy was 349 days.

¹We are indebted to Dr. John F. Menke of Stanford University School of Medicine for furnishing us with these mice.

The histological findings were more specific. Of the mice that received 0.5 per cent of the substance in the diet only the 1 animal surviving as long as 248 days showed any specific tissue change, and this was a moderate nodular epithelial proliferation in the liver. The 2 animals that lived less than 250 days on the 0.25 per cent diet showed no liver changes; however, 1 had a carcinoma of the bladder. The 4 mice that lived longer showed proliferated nodules of hepatic epithelial cells, although in one animal the proliferated structures were only small ducts. Three of the livers showed distinct adenomas. Three other bladders from this group showed irregular epithelial proliferation. One of these was carcinomatous. One animal had a mass of chronic inflammatory tissue in the perinephric region but no tumor tissue could be identified here.

Of the group receiving 0.125 per cent of AAF in the diet, 1 animal died of leukemia in less than 100 days. It had no other lesions attributable to the treatment, although prominent epithelial cells were seen lining pulmonary alveoli throughout most of the lung section. Another animal died in 228 days with no characteristic lesions. The remaining two animals in this group as well as the 3 receiving 0.062 per cent AAF, all lived 248 days or more, and all showed proliferative epithelial lesions. All of the livers showed epithelial nodules of hepatic cells, the extent of which bore no apparent relation to the length of life. Two were classed as adenomas. Two animals showed focal, non-malignant proliferation of the bladder epithelium, whereas in 2 others there was a questionable epithelial thickening of the bladder. One animal had an unexplained hydronephrosis.

Of the 8 mice which received 0.031 per cent AAF in the diet, 6 showed no recognizable epithelial proliferation of the liver, and in the other 2 there were only questionable changes. Two had irregular, non-malignant epithelial proliferation in the bladder, and one showed early bladder carcinoma. One animal had cellular infiltrations in the kidney and lymph nodes suggesting leukemia. Another showed an aggregate of small glands with a small cyst at one side of the endometrial lumen, similar to the lesions described for the rat uterus. This was 1 of only 2 mice from which the uterus was examined histologically. One mouse had a chronic skin ulcer without any evidence of tumor.

Three male C3H mice given 0.125 per cent AAF for 294 to 372 days were autopsied. In these 3 mice there were 2 instances of nodular hyperplasia of liver cells with adenoma formation, 2 bladder carcinomas, 2 instances of slight irregularity in the size of the pancreatic acini, 2 of unexplained hydro-

nephrosis and 1 pulmonary nodule in which the alveoli were partly collapsed and lined by cuboidal epithelial cells. This was similar to the benign pulmonary nodules in several of the rats.

Twenty mice of the Bagg albino strain, half males and half females, were kept on diets containing 0.062 or 0.125 per cent AAF until death or to the end of the experiment, which lasted 322 days. There was no striking difference between the animals on the 2 concentrations. Seven of the animals developed grossly visible tumors, 7 more had other grossly visible lesions. Two of the females had mammary tumors. Six of these animals were examined histologically. Only 2 animals showed recognizable nodular proliferation in the liver. Chronic cystitis was present in all, but only 2 showed distinct bladder epithelial proliferation, and 1 of these had early carcinoma of the bladder. Four of the mice showed slight irregularity of pancreatic acini, the significance of which is uncertain. Two had subcutaneous carcinomas, probably of mammary origin, and one of these had nodular hyperplasia with cyst formation in another sectioned mammary gland. In 1 animal from which sections of the uterus were studied there was irregularity in the endometrial glands, some of which were cystic.

Transplantation of tumors.—Several attempts to transplant the AAF-produced tumors into rats were made. When the parentage of the recipient differed from that of the donor, only 2 of 22 into which transplants were made gave a positive response. However, when the parentage of the animals was the same, 6 of 12 recipients developed positive tumors. Of 12 transplants which were examined histologically, 7 showed abnormal tissue growth suggesting tumor, and one, after secondary transplantation, showed a cyst lined by folded columnar epithelium. In most cases the growth at the site of the transplantation was similar to that of the primary lesion, although 1 animal into which an adenocarcinoma was transplanted developed a sarcoma composed of bundles of closely packed, rather small spindle cells. Of particular interest is the observation that two transplants in this animal grew and sections of both transplanted tumors had the same structure. It may be of significance that the peripheral portion of the primary tumor, from which the transplants were obtained, showed unusual amounts of very cellular fibrous stroma containing mitotic figures. This phenomenon of sarcomatous growth following transplantation of epithelial tumors has been described in transplantable spontaneous mammary tumors of mice (*see* reference 6, page 29). The specific tumors from which secondary growths

were obtained on transplantation were: squamous cell carcinoma of the head, subcutaneous tumors probably of mammary origin, and liver tumors. One of each of these types was retransplanted, satisfactorily in each case, at least in so far as gross appearance indicated, and there was microscopic verification in the case of the head tumor transplant.

Twenty-one C57 mice received transplants and growths developed in 18 of them. Gross observation indicated successful transplantation from bladder, kidney and liver, and one bladder tumor transplant was satisfactorily retransplanted. Histological study showed successful transplantation of bladder and liver carcinomas; no kidney tumor transplants were examined microscopically. There was one case of leukemia from which lymph node lesions were transplanted and retransplanted successfully.

DISCUSSION

The carcinogenic action of AAF on rats has been found to be present when the concentration in the diet was as low as 0.004 per cent, and, with higher concentrations, the material did not have to be administered for longer than 25 days. It is interesting, although perhaps accidental, that the amount of AAF ingested by the animals eating for the minimum time and by those eating a minimum concentration, was about the same. Assuming that each rat ate 10 gm. of food a day, a rat on the diet for 25 days would ingest about 0.3 gm. of AAF, and one on the 0.004 per cent diet about 0.25 gm. However it must be remembered that 1 gm. in a single dose was not effective in producing carcinoma.

Mice were apparently somewhat more resistant to the action of AAF than were rats. They tolerated a higher percentage of the substance in the diet, fewer lesions appeared than in rats given the same dose, and the time of appearance was longer than in rats fed moderate doses. However, the types of tissue change were similar to those seen in rats, and many distinct tumors were present. Some were malignant as judged by the invasive property of the abnormal cells and by transplantation experiments. No metastatic tumors were recognized in mice. Most of the organs frequently affected in the rats were also the seat of nodular epithelial hyperplasia and tumor formation in the mice, but the relative frequency and severity of the changes was different in the two species. The most striking differences were the complete absence of squamous cell tumors of the side of the head in mice, absence of distinct metaplasia of the epithelium of the renal pelvis while bladder tu-

mors were common, and the much smaller incidence of mammary tumors in this species.

The presence or absence of mammary tumors warrants further consideration. In our rats, especially females, breast tumors were quite common. In the mice, only 2 of the female Bagg albinos developed tumors and none of the C57 animals. Armstrong and Bonser (1) found no mammary tumors in their mice, but there was a mild breast proliferation. Bielschowsky (2) discussed this question in some detail. He found no estrogenic activity induced by AAF, and he stated that if a highly estrogenic substance were responsible for the mammary tumors, they should be found with equal frequency in male and female rats. However, he discovered, as we did, that there were many more tumors in females than in males. Novelli and Giunti (7) have reported that certain derivatives of fluorene have a weak estrogenic activity in rats. They did not study any aminofluorene compounds. In a preliminary experiment we were unable to find any estrogenic effect with AAF; however, the rats were in poor nutritional condition and this could have influenced the results. One of us (A. J. C.) later observed normal estrual cycles in several rats on AAF. This will be reported in greater detail later.

In the first paper on AAF (8) it was stated that occasionally in older breeding females of this colony, there has appeared a large, localized mammary tumor. These have been found to be benign adenomas or fibroadenomas. The number of such tumors has now reached a total of 14, from 500 breeding females. One unclassifiable malignant tumor with numerous metastases was found in a female rat well over 2 years of age. This animal was a laboratory pet and her diet was unconventional, but in no case was she subjected to any experimental diets or procedures. No spontaneous tumors have been seen in males.

Our observations suggest that there may be a relationship between the frequency of development of mammary tumors after AAF feeding and the incidence of spontaneous tumors of the mammary gland. In the few strains of animals studied, epidermoid tumors of the head occurred only in those animals (rats) that showed frequent mammary tumors.

SUMMARY

1. 2-Acetaminofluorene (AAF) had carcinogenic action on rats when its concentration in the diet was as low as 0.004 per cent. A diet containing 0.001 per cent had no recognizable carcinogenic effect.

2. When the concentration in the diet was

0.125 per cent, 25 days feeding was sufficient to initiate changes which showed up some time later as cancers. A single large dose into the stomach was not carcinogenic.

3. As the concentration of AAF decreased, or the time of administration became shorter, there was an increased interval before tumors were observed.

4. Tumors like those produced in rats were observed in 3 strains of mice. The mice were more resistant than the rats as judged by frequency of tumors, time for tumor development, and concentration of AAF which the animals could tolerate. Mammary tumors in C57 mice were absent, and infrequent in the Bagg albino strain; epidermoid tumors of the head were not seen in any of the mice, although they were frequent in the rats.

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