NEW COMPOUNDS FOR CANCER RESEARCH. THE SYNTHESIS AND METABOLIC STUDY OF ISOTOPIC DERIVATIVES OF FLUORENE

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A DISSERTATION PRESENTED TO THE GRADUATE COUNCIL OF
THE UNIVERSITY OF FLORIDA
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE
DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA
February, 1952

ACKNOWLEDGMENT

For his kind understanding and guidance, the author wishes to express her gratitude to Dr. Francis Earl Ray.

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INTRODUCTION

The student of cancer is interested in the origin, the characteristics, the development and the control of neoplastic growth. If study is limited to those tumors which arise spontaneously, the opportunity for study is likewise limited. It is of great value, therefore, that methods of inducing tumors experimentally in animals have been devised. This not only permits a study of the process of transformation from the normal to the cancerous state, but supplies a medium for the investigation of diagnostic and therapeutic agents.

A. Chemical Carcinogenesis

It is known that a number of physical, chemical and biological agents are capable of inducing cancerous growths in various tissues of animals and plants. These agents are of two general types: those which we consider foreign to the body and call extrinsic agents; and those produced by the organism itself, which we class as intrinsic agents. Certain specific classes of chemical compounds have been shown to be extrinsic agents capable of cancer production.

Several carcinogenic agents were known from clinical observation long before the extension of investigations to the laboratory. Perhaps the first was the description by Pott (63), in England, of scrotal carcinoma in men exposed to constant contact with soot. With the development of the coal tar industry during the nineteenth century an

increase in the number of skin cancers was noted in workers in this industry. The carcinogenic effect of coal tar was established when Yamagiwa and Ichikawa (90) reported, in 1918, that continuous painting of rabbits' ears with tar led to the appearance of carcinoma.

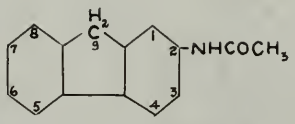
The successful search for the specific constituent active in coal tar was achieved by a group of London scientists under the leadership of Kennaway (35) and Cook (17). The active ingredient was found to be 3,4-benzpyrene. As a matter of fact, the first carcinogenic polycyclic hydrocarbon to be described was 1,2,5,6-dibenzanthracene reported by Clar (15). Further modifications of the benzanthracene nucleus led to the synthesis and biological testing of numerous related compounds. Particular interest was aroused when one of the most active of the carcinogenic hydrocarbons, 20-methylcholanthrene, was prepared from bile acids (16).

It has been shown that these compounds are detoxified and made noncarcinogenic during metabolism by the adding of hydroxyl groups in specific positions in the molecule (12.13).

The frequent occurence of cancer of the bladder in workers in the aniline dye industry led to the study of various chemicals in this group. Yoshida (91) in Japan found that both ingestion and subcutaneous injection of o-aminoazotoluene resulted in the development of hepatomas in rats and mice. In 1937 Kinosita (37) reported that p-dimethylaminoazobenzene when included in the diet of rats produced liver cancer in these animals. Since that time many compounds of this class have been found to be carcinogenic and a study of the

relation of the chemical structure of the azo dyes to their potency in inducing hepatomas in rats has been carefully investigated by Miller et al. (44,45,46).

While investigating the toxicity of the insecticide, 2-acetylaminofluorene, I, Wilson, DeEds and Cox (19,87,88) observed that this compound, when incorporated in the diet of rats, produced multiple and malignant tumors in a wide variety of sites.



I

Since subcutaneous implantation of the compound gave no evidence of tumors, the authors suggested that continued ingestion of the compound was necessary for carcinogenic activity. They also assumed that 2-aminofluorene was excreted; although they did not isolate the compound.

In 1914, Bielschowsky (5) fed 2-acetylaminofluorene at a rate of 4 mg. per day to a cancer-resistant strain of rats and found that ninety-four of one hundred and four rats tested developed both leukemia and cancers of the liver, mammary gland, ear duct, intestine, uterus and skin. Liver tumors were most numerous in male rats, while mammary cancer was quite uncommon. In female rats, however, mammary carcinomas were twice as frequent as liver carcinomas.

Bielschowsky reported that 2-acetylaminofluorene was ineffective when injected subcutaneously. He believed that such tissue was not capable of hydrolizing off the acetyl group to form 2-aminofluorene which he considered to be the active compound. Continued painting of epithelial tissue with 2-aminofluorene resulted in liver tumors in all of five rats tested. These two experiments cannot be compared directly, however, since the mode of application, namely subcutaneous and cutaneous, differed for each compound.

In a recent study Morris, Dubnik and Johnson (53) have shown that both 2-aminofluorene and 2-acetylaminofluorene are capable of producing tumors in a variety of sites after ingestion and after painting. Seven out of eleven rats developed tumors following painting with 2-aminofluorene, while five out of eleven developed tumors following feeding of the same compound. Painting with 2-acetylaminofluorene resulted in tumors in seven out of nine rats, and oral ingestion of this compound by twenty rats resulted in twelve developing tumors.

Armstrong and Bonser (3) studied the effect of feeding 2-acetylaminofluorene to five strains of mice and found that this resulted in the production of tumors in the bladder, liver, breast, forestomach, thyroid, renal pelvis and female genital tract.

Lopez (41), in England, has reported the induction of a brain tumor in a rat by including 2-acetylaminofluorene in the diet. This is unique since malignant tumors of nerve tissue are usually produced in mice and rats by direct implantation of a carcinogenic hydrocarbon.

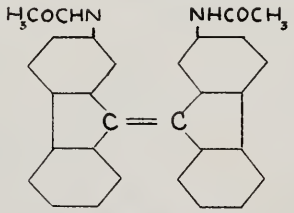
A recent biological investigation of 2-acetylaminofluorene has been made by Heiman and Meisel (29). Their results again show widespread incidence of tumor growth in various organs including the submaxillary, mammary, parathyroid and thyroid glands and the liver.

These numerous evidences of the high carcinogenic activity exhibited by 2-acetylaminofluorene have led to extensive study of this compound to determine what structure or structures of the molecule are responsible for its action.

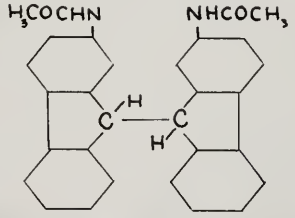
Miller, Miller, Sandin and Brown (43) have made studies to determine if the $-CH_2-$ bridge in 2-acetylaminofluorene is essential to its carcinogenic activity in the rat. Their data demonstrated that replacing the $-CH_2-$ by -S- as in 3-acetylaminodibenzothiophene did not alter the carcinogenicity of the molecule for either mammary gland or ear duct tissue. Substitution of $-\overset{O}{\overset{\|}{S}}-$ for the $-CH_2-$ as in 3-acetylaminodibenzothiophene-5-oxide greatly lowered the activity towards these two tissues, while insertion of an -O- bridge as in 3-acetylaminodibenzofuran only partially diminished the activity of the molecule in these respects. Unlike the control compound, 2-acetylaminofluorene, however, none of the three compounds had any carcinogenic activity toward the liver. The tumors produced by all these compounds appeared four to eight months after the beginning of the experiment.

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 eight to ten months from the beginning of the experiment.

In 1948 Pinck (61) postulated that 2,2'-diacetylamino-9,9'-bifluorylidene, II, is the essential intermediate in carcinogenesis. After considerable difficulty this compound and its reduction product, 2,2'-diacetylamino-9,9'-bifluoryl, III, were prepared (83). Morris and Dubnik (51) tested these derivatives on rats in feeding experiments exceeding periods of 30 weeks. They found 2,2'-diacetylamino-9,9'-bifluoryl to be ineffective while 2,2'-diacetylamino-9,9'-bifluorylidene had very little activity. These facts cast doubt on the possibility of the compounds being intermediates.



II



III

The only metabolite of 2-acetylaminofluorene that has been reported is 7-hydroxy-2-acetylaminofluorene (6). This compound is only weakly carcinogenic. The question arises whether the 7-position must be available for hydroxylation in order for the compound to exert powerful carcinogenic action. In an attempt to answer this question the 7-position of 2-acetylaminofluorene has been blocked with chlorine and iodine (67,82). A preliminary report by Morris (50) indicates that these compounds are not carcinogenic, although several brain abscesses were found when 7-iodo-2-acetylaminofluorene was fed. In

light of Bielschowsky's work this would indicate that an active hydrogen is necessary in the 7-position. The interposition of oxygen between the hydrogen and the nucleus, as in 7-hydroxy-2-acetylaminofluorene, reduces but does not destroy the carcinogenicity of the compound (32). In order to determine if nitrogen could be interposed with similar results, 2,7-diacetylaminofluorene has been prepared (67) and initial tests indicate it is a more active carcinogen than 2-acetylaminofluorene (51).

At this point it should be noted that Sieglitz and Schatzkes (71,72) have reported that 2,7-dichlorofluorene and 2,7-dibromofluorene exhibit a greater reactivity of the methylene group than fluorene itself. From this it would seem probably that the introduction of a halogen into the 7-position of 2-acetylaminofluorene would also activate the methylene group of this compound; and if the activity of the methylene group is the all important factor in its carcinogenicity as Pinck hypothesized, then 7-chloro- and 7-iodo-2-acetylaminofluorene should show greater carcinogenic action than 2-acetylaminofluorene. Since this is not the case it is indicated that Pinck has overlooked an important point in neglecting the evidently essential role of the 7-position.

In considering the possible function of the acetyl group in 2-acetylaminofluorene, the question arises whether conversion to the free amine is a necessary step in tumor incidence. It has been determined that the parent hydrocarbon, fluorene, and its oxidation product, fluorenone, are inactive (89); while 2-nitrofluorene is reported to be

weakly carcinogenic when fed (53). Here again it is evident that the mode of application is not unimportant since six out of ten rats developed cancer from painting with 2-nitrofluorene while two out of nine rats developed tumors after ingestion of the compound. 2-Aminofluorene is highly active, although maybe somewhat less than 2-acetylaminofluorene (5,28,53,89), but this may be due to its different solubility.

Hirs (31) has prepared N-(2-fluorenyl)-glycine and N-(2-fluorenyl)-hemisuccinamide in hope that their greater solubility in water over 2-aminofluorene would produce a more rapid carcinogenic response. Tests of these compounds reported by Twombly (80) show the compounds to be carcinogenic; but the relatively large amounts administered, i.e. 4 mg. per day for 288 days, do not lend themselves to any direct comparison with similar experiments involving 2-aminofluorene.

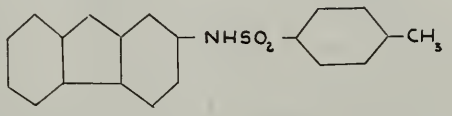
Bielschowsky (4) has reported that 2-methylaminofluorene and 2-dimethylaminofluorene are less carcinogenic than 2-aminofluorene.

A photometric method of estimation of 2-acetylaminofluorene in the tissues and excreta of animals administered this compound has been used by Westfall and Morris (86). Their procedure involves hydrolysis to the free amine, diazotization, and coupling with R salt to give a colored compound. By this method, however, only 32% of the ingested dose of 2-acetylaminofluorene is accounted for (55), indicating that in the animal body metabolites are formed which involve removal of the amino group or which tie up the nitrogen in such a manner that it cannot be hydrolized to a primary amine. Deacetylation

would be a process which could be assumed to precede the formation of such compounds.

Comparative studies which have been made on two radioactive derivatives of 2-acetylaminofluorene, namely 2-acetylaminofluorene-9-C^{14} and 2-acetylaminofluorene-ω-C^{14} (65), show that deacetylation of 2-acetylaminofluorene does take place in the rat (54,84). Six per cent of the C^{14} ingested as 2-acetylaminofluorene-ω-C^{14} is expelled as respiratory CO_2 within 6 hours, while over 40 per cent is excreted in this manner in 88 hours.

Another approach toward answering the question of possible conversion to the free amine has been made through the study of 2-benzoylaminofluorene and 2-p-toluenesulfonamidofluorene, IV. In general the acetyl group is readily removed from aromatic amines *in vivo*, while the benzoyl group is removed with difficulty. No enzyme is known to remove the tosyl ($CH_3C_6H_4SO_2$-) group. It was predicted that if conversion to the free amine is a necessary step, the carcinogenicity of these three derivatives of 2-aminofluorene would vary directly with the ease of removal of these various groups.



IV

In harmony with these predictions Morris (50) reports that preliminary experiments indicate that the 2-benzoyl compound is considerably less active than 2-acetylaminofluorene, while the tosyl derivative is noncarcinogenic.

The picture is still incomplete and further study of the carcinogenic mechanisms of fluorene derivatives is necessary if the important role of these compounds in chemical carcinogenesis is to be understood.

B. Chemical Detection and Therapy of Cancer

The need for a method of early diagnosis and treatment of internal cancer is most urgent today in the case of gastric carcinoma. Cancer of the stomach, a disease predominating in the male in a ratio of about 2 to 1, is the most common of all carcinomas in the human, causing almost 40,000 deaths every year in the United States and making up approximately one-fourth of all the deaths due to cancer (1).

Two methods that have been used most successfully in the treatment of malignant neoplasms are surgery and radiation. In gastric carcinoma, however, these methods find only limited effectiveness since their success depends primarily on diagnosis of the disease in the early stages prior to the development of metastases. Cancer of the stomach is a "silent" disease which usually does not display alarming symptoms until well advanced. In fact, no symptoms are recorded in 25-30 per cent of the autopsied persons dying of this disease (34), a fact which makes early diagnosis almost impossible. On the other

hand, gastric carcinoma has a tendency to metastasize early, involving vital organs of the body as the lymph nodes, liver, lungs, etc. It is evident that a simple method for the diagnosis of early gastric cancer would greatly reduce the mortality from this disease. X-ray and gastroscopy are used for the diagnosis of gastric disorders in general. Both are inconvenient, time consuming and relatively expensive. This militates against their early use.

For x-rays or radium to be effective in the treatment of cancer, conditions must be such that the neoplasm can be exposed to radiation of sufficient intensity to destroy the tumor without injury to the neighboring tissues. Gastric carcinomas do not readily lend themselves to such conditions.

If materials could be found, which on administration to tumor hosts, would localize with a certain degree of selectivity in the malignant neoplasm, and if radioactive atoms could then be incorporated into such materials, they might well be useful in the diagnosis and treatment of carcinoma in the stomach. There are short-range, beta-ray emitters available for this work at present which would adapt themselves well to the problem of radiation in a confined area of tissue. Bloch and Ray (8) point out, however, that this same short-range property of beta emitters would probably necessitate the devising of a method for measurement of the radioactivity in the stomach directly. They state that this could be accomplished by fastening a piece of photographic film or a small Geiger-Mueller counter in place of the bucket of a Rehfuss tube or on the end of a gastroscope. Such a device could then

be introduced into the stomach and the radioactivity measured. This inconvenience could be eliminated if the short-range, beta emitters were restricted to therapeutic use and the long-range, gamma-ray emitters employed for diagnostic work.

There are a considerable number of known instances where elements localize in certain organs and tissues with a high degree of selectivity. Outstanding examples are the deposition of calcium and phosphorus in bone and the uptake of iodine by the thyroid.

Studies on the use of radiophosphorus in the treatment of leukemia and lymphosarcoma have been carried out. Unfortunately bone takes up larger amounts of the element than do the malignant cells (36). Selverstone et al. (70) recently reported on the deposition of P^{32} in brain tumors. It was necessary in this case to devise a miniature Geiger counter that could be inserted directly into brain tissue, because the effective range of this beta emitter is only a few centimeters. Good differentials between normal and pathological areas were obtained. Localization of P^{32} in mammary and testicular tumors has also been reported (69).

Radioiodine has been used extensively in the investigation of the thyroid physiology. Hamilton and Soley (27) have measured the uptake of radioiodine by the thyroid in patients by placing a Geiger-Mueller counting tube against the neck, directly over the isthmus of the gland. Stevens et al. (75) have studied the distribution of I^{131} in mice with and without mammary carcinoma, 15091a, after injection of radioactive sodium iodide. Differences in the distribution of

the injected iodine among tissues of the tumor mice and the control mice were definite in the thyroid and spleen. Unlike the findings in previous experiments with rats (77), differences in the I^{131} content of the thyroids were not obviously related to any difference in the concentrations of I^{131} in the blood sera.

Gallium, a bone-seeking element, has been studied extensively by Dudley et al. (20,21,22). It is unquestionably taken up by bone tumors, but the diagnostic and therapeutic possibilities remain to be evaluated.

Among the organic compounds known to localize with a high degree of selectivity in certain organs are the contrast media used in x-ray diagnosis for the visualization of the gall bladder or urinary tract, such as sodium tetraiodophenolphthalein, sodium 2-keto-5-iododihydropyridine-N-acetate (Iopax), and sodium monoiodomethanesulfonate (Abrodil, Skiodan).

In attempting to develop a test for the early diagnosis of gastric cancer, Bloch and associates (9) studied three sulfonamides (sulfathiazole, sulfadiazine and sulfapyridine). While none of these was found applicable to the early detection of gastric malignancies, strikingly high sulfapyridine levels were found in the gastric contents of patients with normal gastric acidity.

Although no localization of the chemical material in malignant tissues is known to be involved, Boyland (10) reported the inhibition of tumors in mice following the administration of large and repeated doses of certain aromatic amines containing sulfur,

such as 4,4'-diaminodiphenylsulfoxide and sulfamyl sulfanilic acid. With further investigation, complete inhibition of growth of spontaneous tumors in four of four mice was obtained by Methylene Blue, 4,4'-diaminodiphenylsulfoxide and 4,4'-diaminodiphenyl ether (11).

Stevens et al. (76) have reported a high local concentration in cancer tissue of sulfapyrazine administered at a site distant from the tumor and followed by repeated glucose injections. Glucose increases the lactic acid formation by the cancer tissue and the sulfapyrazine was precipitated presumably because it is less soluble at acid pH than at pH 7.4.

Several characteristics of the sulfonamide linkage prompted Ray and Soffer (66) to continue investigation of these compounds for their diagnostic and therapeutic potentialities. Apparently the sulfonamide linkage is not attacked by enzymes. In addition to localizing in certain tissues sulfonamides have been shown to reduce the effective vitamin intake of animals by supressing the intestinal flora, and this reduction of essential vitamins in the diet of tumor-bearing mice is known to inhibit the growth of tumors (10). Ray and Soffer proposed the incorporation of S^{35} into these already promising compounds. This short-range beta emitter would not only exert possible additional therapeutic effects, but would also offer a means of detection. Procedures for the preparation of numerous compounds of this type are given, but as yet no animal experiments have been carried out.

Extensive investigations have been made concerning the possible selective localization of certain dyes in tumor tissue. Roosen (68)

and later Blatzer (7) reported a beneficial effect of Isamine Blue on certain tumors. The dose of dye required, however, proved to be rather toxic and the treatment severely stained the skin of the patients.

As early as 1909, Goldmann (26) noted that certain dyes seem to be concentrated by tumors. These findings have been confirmed by numerous other investigators (30,38,42).

In 1939, Duran-Reynolds (23) reported that T-1824 (Evans Blue) and other poorly diffusible dyes, injected intravenously, localized selectively in spontaneous and transplantable tumors growing in mice, rabbits and chickens. Brunschwig, Schmitz and Clark confirmed the findings of Duran-Reynolds in rats and rabbits bearing benzpyrene induced sarcomas and carcinomas and further extended the observations to humans, reporting that selective localization of the dye occurred in the majority of a series of human subjects presenting a variety of malignant neoplasms (14).

Wasserman et al., in 1911, attempted to use dyes which were selectively taken up by tumors as a vehicle for substances that are possible therapeutic agents if they enter the tumor (81). They prepared Selenium-Eosin compounds which were claimed to be highly successful in the treatment of animal tumors. Unfortunately, the methods of preparation and exact formulas of the compounds were not reported, and the work has not been substantiated.

Zahl and Cooper (92,93) injected lithium salts of various dyes such as Trypan Blue, Pontamin Sky Blue 6B, and carminic acid

into tumor-bearing mice. These compounds were to be used in the therapy of cancer in conjunction with slow neutrons, since the impact of such a neutron on a lithium atom leads to a nuclear reaction with a high energy release. The lithium salts of the dyes were supposed to localize selectively in the tumors, thereby producing a high concentration of lithium in the region where a maximum radiation effect was desired. As might have been expected, however, the lithium salts of these dyes dissociated rapidly in the body and the lithium ions did not remain with the dye moiety.

The first synthesis of a radioactive dye was reported by Tobin and Moore (79). They prepared radioactive dibromo-Trypan Blue and dibromo-Evans Blue by brominating o-tolidine with Br^{82} and coupling the resulting compound with H acid or Chicago acid. An investigation of the distribution of these dyes in tumor mice, however, showed that the liver took up about twice as much radioactivity as the tumor. While these findings cast some doubt on the selectivity of dye uptake by tumors, Moore and his associates point out that "the significant fact remains, that the colloid permeates into and therefore radiates tumor tissue wherever this tissue may be, and no matter how widespread the metastases are" (47).

The work of Moore and Tobin showed clearly that determination of radioactive compounds in tissues with Geiger counters is both simpler and more exact than the chemical or colorimetric analysis which had previously been employed in determining the localization of dyes. Bloch and Ray (8) point out that most observers merely

estimated the distribution of the dyes by macroscopic and microscopic inspection which resulted in many discrepancies. Grossly, the presence of dye can be seen more readily in light colored tissues or organs such as kidney cortex and tumor tissue, than in dark organs such as liver or spleen. Under the microscope, however, dye granules are most frequently observed in liver and spleen where they are stored in the reticulo-endothelial system, but rarely in the kidney. The use of a tracer element eliminates these difficulties and permits accurate distribution studies.

The radioactive element (Br^{82}) which Moore and Tobin used to make their dyes has the rather short half-life of 34 hours. To make radioactive organic compounds more generally available for tumor research, it is desirable to employ an isotope with a somewhat longer half-life, which has radiation suitable for biological work and which can be synthesized into organic compounds with a firm linkage. Bloch and Ray (8) recognized that I^{131} with a half-life of 8 days met these specifications and prepared a number of radioiodo derivatives of mono- and bis-azo dyes, covering a range of molecular weights, solubilities and degrees of acidity and basicity.

This idea was taken up by other investigators in the field. Lewis and co-workers (40), studying the effects of oxazine dyes, found that several structurally different compounds of this series stained and retarded implanted tumors in mice. Sloviter (73) prepared the radioactive iodine derivative of the oxazine dye, Nile Blue 2B. By determining radioactivity in various organs and tissues of

tumor-bearing mice receiving this compound, he was able to determine the distribution in the animal body. While significant concentration of the dye in the tumor was noted, the liver, kidney and spleen also showed a comparable affinity for the dye.

Moore (48) studied the localization of fluorescein in a wide variety of carcinomas and sarcomas, using as a criterion of uptake fluorescence under ultraviolet light. Lesions of the central nervous system took up the compound with an accuracy of the order of 90 per cent. The clinical application of this fact was greatly increased by incorporation of I^{131} into the fluorescein molecule thus permitting measurements to be made outside the skull, quite independent of operation procedures (49,78).

An extensive series of acidic and basic dyes tagged with radioactive I^{131} or S^{35} have been prepared by Myers and are being investigated for their selective affinity for various types of transplanted and spontaneous mouse tumors (57,58).

Stevens et al. (74) have recently studied the distribution of radioactive iodinated Trypan Blue, one of the compounds first prepared by Bloch and Ray (8). This compound was administered intravenously to mice bearing subcutaneous implants of tumor 15091a. The concentration of radioactivity in tumor tissue was several times greater than that in skeletal muscle or skin, but considerably less than in the liver, spleen or kidneys.

If a compound could be found which would display a greater affinity for the tumor than for such vital organs as the liver and

kidneys while at the same time maintaining the favorable ratio of concentrations in tumor and surrounding muscle displayed by Trypan Blue, such a compound would greatly increase the chances of accurate diagnosis and therapy of gastric cancer through the medium of chemical localization.

II

CARCINOGENIC DERIVATIVES OF 2-AMINOFLUORENE CONTAINING N^{15}

A. Statement of the Problem

As shown in Part IA, 2-acetylaminofluorene (AAF) has been definitely established as an active carcinogen, producing a variety of tumors in the animal body at numerous sites often far removed from the point of application (5,87,88). These characteristics of the compound make it useful for studying some of the fundamental factors of carcinogenesis.

A satisfactory method for its quantitative estimation in the animal body is necessary for such a study. A photometric method of estimation has been previously used, which involves hydrolysis of AAF to 2-aminofluorene (AF), diazotization, and coupling with a known naphthol derivative to give a colored compound (86). By this method, however, only a 32 per cent recovery of AAF is obtained, indicating that in a very short time two-thirds of the material no longer has a diazotizable primary amino group (55).

Recently, preparation and investigation of the radioactive carcinogens, 2-acetylaminofluorene-9-C^{14} and 2-acetylaminofluorene-ω-C^{14}, have greatly facilitated and metabolic studies of AAF. Since well over 90 per cent of the radioactivity may be accounted for in the animal body, these compounds have permitted a study of the pathways taken by the fluorene radical and the acetyl group of the

compound (54,65,84).

The fate of the nitrogen atom, however, is yet to be determined. As has been indicated before, a diazotizable amino group is necessary for AAF estimation by the photometric method. The low percentage recovery by this method indicates, therefore, either that the nitrogen atom, to a large extent, has been removed from the fluorene molecule or that the primary amino nitrogen has been rendered non-diazotizable, possibly by being converted to a secondary or tertiary amino group. This second possibility may involve the formation of a nitrogen ring compound similar to that prepared in vitro by the reaction of AF with pyruvic acid (59).

The importance of finding a method which would enable a decision to be reached between these two alternatives is evident. Synthesis of AAF with a labeled nitrogen atom which could be traced in the animal body was therefore undertaken. Since no suitable radioactive form of nitrogen is available, the heavy isotope, N^{15}, was employed. The concentration of the isotopic nitrogen can be determined in various animal tissues by means of the mass spectrograph. By comparing the pathway of 2-acetylaminofluorene-N^{15} with that of 2-acetylaminofluorene-9-C^{14}, it will be possible to determine if and where in the animal body nitrogen is removed from the fluorene radical or where the nitrogen undergoes the metabolic change which renders it incapable of diazotization.

B. Procedure

The usual method of preparation of AAF is the nitration of fluorene to 2-nitrofluorene (NF), reduction with Zn and 78 per cent ethanol to AF (39), and acetylation with acetic anhydride. Because of the high cost of N^{15} compounds, the ordinary methods of nitration which use 200-300 per cent excess concentrated nitric acid could not be employed. In addition, the N^{15} nitric acid is a dilute (approximately 2M) solution, so it was necessary to work out a satisfactory method of eliminating water and of using equal molar quantities of fluorene and $HN^{15}O_3$. This was accomplished by employing acetic anhydride as a solvent and dehydrating agent and concentrated H_2SO_4 as a dehydrating agent and catalyst. The yield based on nitric acid was 78.9 per cent, as compared to a calculated 21.4 per cent by previous methods (39).

Since the two intermediates, NF and AF, have also been shown to be carcinogenic (5,53), samples of these were isolated for study.

A process was also worked out for a one-step reduction and acetylation of NF to AAF. This procedure conserves both time and reagents, although it does not substantially improve the yield as had been hoped.

C. Experimental Details

1. 2-Nitrofluorene-N^{15}. Fluorene, 10.47 gm. (0.063 moles), was dissolved in acetic anhydride, 131.4 ml. (1.39 moles), which was

sufficient to take care of the 1.35 moles of water in the nitric acid. The solution was heated to 55°, and 26.47 ml. (0.063 moles) of 2.39M HNO_3 containing 62 atom per cent N^{15} was added dropwise over a period of 1 hour during which the temperature was kept between 50-60°. Then, concentrated sulfuric acid, 5 ml. (0.08 moles), was added dropwise at 55° to react with the water formed and to catalyze the reaction. This precipitated the 2-nitrofluorene-N^{15}. The filtrate was removed by means of a filter stick, and the yellow product washed by stirring with water and 1 per cent sodium acetate until neutral; the melting point was 154°; Kuhn (39) found 155-156°; the yield was 78.9 per cent.

2. 2-Aminofluorene-N^{15}. A paste of 2-nitrofluorene-N^{15}, 3.3 gm. (0.015 moles), and 78 per cent ethanol, 110 ml., to which was added Zn dust, 33 gm., $CaCl_2$, 1.1 gm. (in 1.5 ml. of water), and charcoal, 1 gm., was heated to reflux with a microburner. Until the mixture began to reflux, the flask was shaken with a rotary motion to keep the contents well mixed. The mixture was boiled vigorously for 2 hours. Zn dust, 10 gm., was again added and refluxing continued for an additional 2 hours. The hot filtrate, which was removed by means of a filter stick and illuminating gas pressure to avoid contact with air, was added to 600 ml. of water to precipitate the white product; the melting point was 126°; Kuhn (39) found 127.5°; the yield was 78.5 per cent.

3. 2-Acetylaminofluorene-N^{15}. 2-Aminofluorene-N^{15}, 5 gm. (0.027 moles), was dissolved in 20 ml. benzene, and heated to reflux. Acetic anhydride, 7 ml. (0.074 moles), was added dropwise and the mixture refluxed 30 minutes. The product precipitated on cooling and was recrystallized by dissolving in hot 95 per cent ethanol, adding water until turbid, filtering hot, and allowing the product to crystallize; the melting point was 191°; Porai-Koshits and Nikiforova (62) reported 186°; Morris and Westfall (55) gave 194°; the yield was 90 per cent. As no extraneous nitric acid was used, the compound should contain 62 atom per cent of N^{15}.

4. 2-Acetylaminofluorene directly from 2-nitrofluorene. Glacial acetic acid, 250 ml. (0.023 moles), 2-nitrofluorene, 5 gm. (0.023 moles), Zn dust, 20 gm., and charcoal, 2 gm., were heated by means of a microburner to reflux with vigorous shaking. After hard refluxing for 2 hours, more Zn dust, 10 gm., was added and the mixture refluxed an additional 2 hours. Acetic anhydride, 10 ml. (0.077 moles), was added dropwise and the refluxing continued 1 more hour. The mixture was filtered hot and the product precipitated by addition of the filtrate to 700 ml. of water and recrystallized as described above; m.p. 190.5°; yield, 66 per cent.

D. Results

A successful synthesis of the carcinogens, 2-nitrofluorene, 2-aminofluorene, and 2-acetylaminofluorene, labeled with isotopic

nitrogen (N^{15}) was carried out (2). In a preliminary study of the distribution of 2-acetylaminofluorene-N^{15} in the rat (24) it has been reported that large portions of the carcinogen, measured both as N^{15} and as diazotizable nitrogen, pass through the bile. The results also support the theory that a large portion of the nitrogen of the carcinogen is present in the tissues of the rat in a form that is not detectable by the diazotization method.

III

THE METABOLISM, DISTRIBUTION AND EXCRETION OF 2-p-TOLUENESULFONAMIDOFLUORENE-S^{35} IN THE RAT

A. Statement of the Problem

Recently the preparation (65) and investigation (54,84) of the radioactive forms, 2-acetylaminofluorene-9-C^{14} and 2-acetylaminofluorene-ω-C^{14} have greatly extended our knowledge of the metabolism of 2-acetylaminofluorene (AAF). Among the conclusions established is that 6 per cent of the C^{14} ingested in the acetyl-labeled derivative is expelled as respiratory CO_2 within 6 hours, while more than 40 per cent was found in the breath after 88 hours. This shows that deacetylation of AAF in the animal body can take place.

The observation by Morris et al., (52) that 2-aminofluorene (AF) is also carcinogenic raised the question whether conversion to the free amine is a necessary prelude to tumor incidence.

In general, the acetyl group is readily removed from aromatic amines. If the acetyl group of AAF could be replaced with groups which are less readily hydrolized or not removed at all, the study of such derivatives would throw light on the subject. It was predicted that, if conversion to the free amine is a necessary step, the carcinogenicity of such derivatives would vary directly with the ease of removal of these groups. The benzoyl group is hydrolized with

difficulty, while no enzyme is known to remove the tosyl group ($CH_3C_6H_4SO_2$-). Accordingly, 2-benzoylaminofluorene and 2-p-toluenesulfonamidofluorene were prepared.

In harmony with the predictions, Morris (50) reports that the 2-benzoyl compound is considerably less active than AAF, while the tosyl derivative is noncarcinogenic.

Does the difficulty of hydrolysis of the tosyl group retard the absorption and metabolism of this derivative to such an extent that it is eliminated unchanged from the animal body? To endeavor to answer this question, 2-p-toluenesulfonamidofluorene-S^{35} ($TS^{35}AF$) was administered to rats and the compound traced by the radioactivity in organs and excreta. The urine and feces were also examined to determine if the compound had undergone metabolic change.

B. Experimental Details

Four-month-old Sprague-Dawley (Holtzman) strain rats with an average body weight of 350 gm. were employed. The $TS^{35}AF$ (m.p. 157-159° for the first and second runs; m.p. 160-161° for the third experiment) was used in the form of a coconut oil solution, 10 mg. $TS^{35}AF$ per ml. oil. Administration was by stomach tube. Each rat was fasted 24 hours prior to receiving a 2 ml. dose of the $TS^{35}AF$-coconut oil solution and then permitted unlimited food (Purina Dog Chow) through the remainder of the experiment. The animals were allowed water at all times.

Following treatment, each rat was placed in an especially constructed cage which facilitated the separation and collection of urine and feces samples. The floor of the cage was hardware cloth (6mm. mesh) below which was a fine-mesh screen tray for the collection of feces. Under this was a funnel through which the urine was collected into a graduated container. The fine-mesh tray was replaced after each defecation to prevent the urine from coming in contact with the feces.

The animals were kept in an air-conditioned room at 24° except in the case of the third run where unavoidable conditions necessitated keeping the animal at a room temperature of about 27°.

Feces samples were weighed immediately following collection and then air dried.

In the first run urine and feces samples collected for a 54 hour period were studied. The animal was deprived of food at 8 a.m. and the $TS^{35}AF$ solution administered at 8 a.m. the following day. The animal was under observation between the first and twelfth hours, the twenty-fourth and thirty-sixth hours, and the forty-eighth and fifty-fourth hours, during which intervals urine and feces were measured immediately on excretion. Collective samples for the 12 hour periods between the twelfth and twenty-fourth hours and the thirty-sixth and forty-eighth hours were made.

The second run animal was treated at 8 p.m. and urine and feces samples were collected as described above, but for the 12 hour periods alternate to those of the first run. At the sixty-

sixth hour this rat was anesthetized with ether and the pericardial cavity opened. The heart was exposed, the left ventricle was slit with a scissors, and the heart was allowed to pump the blood into a graduated centrifuge tube containing 1 ml. of 1.1% sodium oxalate. The 5.7 ml. of blood collected was centrifuged to separate formed elements from plasma. The contents of the stomach, small intestine and large intestine including the cecum were obtained quantitatively. Ligatures were placed as follows: one just superior to the esophageal valve, two (5 mm. apart) just inferior to the pyloric sphincter, two at the junction of the ileum and the ascending colon, and one on the rectum as close to the anus as possible. The complete gastrointestinal tract was removed keeping all ligatures intact. By cutting between the ligatures at the pyloric sphincter and the ileocecal junction the three regions were separated. Each was carefully milked and then opened and scraped free of contents. These contents samples were each weighed and allowed to dry in air.

During the third run individual urine and feces samples were collected for a 24 hour period. At the twenty-fourth hour the blood and contents of the stomach, small intestine and large intestine were removed as before. The liver and kidneys of this animal were also removed and weighed.

The various samples and organs were then prepared for radioactivity determination in the following manner. After drying, the feces, and stomach and intestine contents samples were ground to a fine powder in a mortar. A suspension of each in 1% sodium hydroxide

(containing 5 drops of the wetting agent, Tergitol, to each 25 ml. of solution) was then prepared by mixing in a Waring blendor for thirty minutes. Fifty ml. of sodium hydroxide solution was used for each 10 g. of sample. A few drops of capryl alcohol was added to prevent foaming. The suspension was allowed to stand twenty-four hours at 5°. The mixture was then brought to room temperature and again agitated in a Waring blendor. A 1 ml. portion was placed on a shallow aluminum planchet and allowed to dry at room temperature. The liver and kidneys were prepared one-half as concentrated by macerating the tissue in a Waring blendor in the presence of 1% sodium hydroxide solution (25 ml. of solution to 10 g. of tissue). Planchets were prepared from this suspension as described above. One ml. portions of the urine, blood plasma and blood cell samples were plated directly on planchets.

Radioactivity measurements were made in an internal-type counter (Q-gas chamber and Nuclear Instrument and Chemical Corporation Scaler, Unit Model 162) with an efficiency of 45 per cent. The $TS^{35}AF$ had an activity of 49,670 counts per min. per mg. Each sample was counted for three ten minute intervals and the net counts per minute above background recorded for each sample.

The concentration of $TS^{35}AF$ in these samples was determined by direct comparison with standard planchets prepared in the same manner and containing known concentrations of $TS^{35}AF$. Feces standards were used for determining the feces, and stomach and intestine contents; liver standards for the liver, kidneys and blood

cells; and urine standards for the urine and blood plasma samples. In order to eliminate correction for the decay of S^{35}, the standards were counted on the same day as the samples. The total amount of $TS^{35}AF$ present in the whole sample was calculated by multiplying the concentration by the total weight of the sample. The total blood volume was calculated on the basis of 6.7 ml. per 100 gm. body weight (25).

The procedure used for the estimation of AF in the urine was the photometric method of Westfall and Morris (86). This method employes extraction by acetone; hydrolysis with hydrochloric acid; diazotization and coupling with R salt. Preliminary experiments carried out on TSAF solutions in urine showed that the hydrolysis procedure used is not capable of breaking this sulfonamido linkage. Subsequent photometric analysis of the urine samples were carried out eliminating hydrolysis and thus permitting estimation of free AF. Urine, obtained from an animal of the same strain and age to which 2 ml. of coconut oil had been administered was treated in the same manner as the experimental samples and used as a blank in the colorimetric analysis. Standards containing varying known concentrations of freshly prepared AF (m.p. 127^{o}) per ml. of urine were made up. These were subjected to the same procedure as the experimental samples and a standard curve plotting mg. AF per ml. urine against optical density was prepared. From this chart the concentration of the experimental samples was obtained after their optical density was determined. Urine for the standards was obtained under the same conditions from untreated animals. The instrument employed for the

photometric analysis was a Beckman Quartz Spectrophotometer, Model DU.

Material for the isotope dilution experiments was obtained in the following manner. The suspension of third run feces in 1% sodium hydroxide was evaporated in air to dryness. A sample of TSAF which had previously been allowed to stand a comparable period of time in 1% sodium hydroxide was found to be unaffected. The dried feces was then extracted with 10 ml. portions of acetone until no activity was detectable in the residue. Ten mg. of the active residue obtained by evaporation of the acetone filtrates was successively recrystallized with 40 mg. of base TSAF from 70% ethanol. The recrystallizations were carried out in a tared, 15 ml., graduated centrifuge tube by solution in a minimum of boiling 70% ethanol; filtering hot; and then allowing to cool slowly to room temperature for 2 hours. After 8 hours at 5° the material was centrifuged and the supernatant decanted. The precipitate was oven dried at 100° and weighed. One ml. of acetone was added for each 10 mg. of precipitate and one-fourth ml. of this solution plated on a planchet. In this manner 2.5 mg. of the material was counted each time. Eight recrystallizations were carried out. The active material from the urine was obtained by evaporating the pooled urine samples to dryness in air and extracting with three 10 ml. portions of acetone and two 10 ml. portions of 70% ethanol. This was recrystallized with base TSAF as described for the material obtained from the feces. Isotope dilution experiments of the active material from the urine

with base Na-p-toluenesulfonate were also carried out. The base compound and active material were first refluxed together for 2 hours to bring about ion exchange. The recrystallization procedure was the same as described before except that 95% ethanol was used as the recrystallizing medium and water as the solvent for the preparation of planchets.

To test for radioactivity in the inorganic sulfate, 16 ml. from pooled samples of urine from the first and second run animals was used. To this was added 9 ml. of aqueous carrier solution containing 3.5 mg. sodium sulfate per ml. Following the addition of 2 ml. M sodium acetate, the pH was adjusted to 2 with N HCl or N NaOH. The volume was then brought to 80 ml. with water. Fifteen ml. of benzidine reagent (0.088 M benzidine dihydrochloride in 0.16 M HCl) was added and the pH 2 value re-established with M sodium acetate. After 10 minutes, 10 ml. of acetone was added. After standing over night, the benzidine sulfate was collected, air dried and counted.

C. Results

The distribution of radioactivity in the organs and excreta of rats following administration of $TS^{35}AF$ is given in Table I. The first animal was observed for 54 hours, and it may be seen that, while 67 per cent of the material was excreted in the feces, only 0.5 per cent appeared in the urine.

The time for the second animal was extended to 66 hours. At the end of this time no radioactivity was detectable in the blood.

TABLE I

DISTRIBUTION OF RADIOACTIVITY IN THE RAT FOLLOWING ORAL ADMINISTRATION OF 2-p-TOLUENESULFONAMIDOFLUORENE-S^{35}
(20 mg. $TS^{36}AF$ in 2 ml. coconut oil)

	Rat No. 1; 54 hours		Rat No. 2; 66 hours		Rat No. 3; 24 hours	
	Mgs. Recovered	% Recovered	Mgs. Recovered	% Recovered	Mgs. Recovered	% Recovered
Urine	0.12	0.50	0.08	0.40	0.056	0.275
Feces	13.42	67.10	18.11	90.57	12.742	63.710
Stomach	—	—	0.00	0.00	0.432	2.160
S. Int.	—	—	0.09	0.46	1.672	8.360
L. Int.	—	—	0.19	0.93	6.051	30.250
Bl. Plasma	—	—	0.00	0.00	0.011	0.057
Bl. Cells	—	—	0.00	0.00	0.000	0.000
Liver	—	—	—	—	0.196	0.980
Kidney	—	—	—	—	0.008	0.038
Total	13.54	67.60	18.47	92.36	21.168	105.830

There was a small amount of the material in the intestines, but none remained in the stomach. Over 90 per cent had been eliminated in the feces and 0.4 per cent in the urine. A total of 92.4 per cent of the sulfur was accounted for.

Because the material had been completely eliminated from the blood at 66 hours, the distribution of $TS^{35}AF$ was examined at an intermediate time - 24 hours. In this third animal, a little less than 0.3 per cent was eliminated in the urine and 63.7 per cent in the feces. Two per cent remained in the stomach; the small intestine contained 8 per cent, and the large intestine 30 per cent. The liver had about 1 per cent and the kidney less than 0.1 per cent. The blood plasma now showed definite evidence of a low concentration of the compound, with none in the blood cells. This agrees with the findings of Morris and Westfall (55), since all the AAF detected in the blood by diazotization was found in the plasma. The total S^{35} activity accounted for in the third experiment was 105.8 per cent. This error on the positive side is just about the same as that of the preceding sample on the negative side.

The peak in the elimination in the feces comes at 16 hours after administration. The amounts of radioactive material found in the urine at intervals following feeding of $TS^{35}AF$ reach a maximum at about 6 hours and then gradually drop off. Morris and Westfall (56) found that the peak in the concentration of diazotizable AAF in the rat urine came at the 4-6 hour period.

In Figure 1 a comparison of the distribution of $TS^{35}AF$ and AAF in the rat is made. The data for AAF was determined on the basis of diazotizable nitrogen (55,56), on the activity of the C^{14}-labeled compound (54), and on mass spectrographic analysis of $AAF\text{-}N^{15}$ (24).

After 16 hours Morris and Westfall (55) found 7-25 per cent of the ingested dose of AAF still remaining in the stomach. At 24 hours, about 2 per cent TSAF was found in the stomach, but none remained in this organ at 66 hours.

Using a dose of 16 mg. AAF per 100 gm. of rat, which was comparable to the dose of TSAF, Morris and Westfall (56) found that 28-45 per cent was eliminated in the urine, as compared with 0.5 per cent of our material, at 24 hours. Using the radioactive AAF, 6-7 per cent of the material was recovered in the urine after a 6 hour period. The greatest part of this compound, 75 per cent, was still in the stomach. Over 1 per cent of the AAF was found in the kidney, whereas less than 0.1 per cent of the tosyl compound was in this organ. This is to be expected, in view of the much higher proportion of AAF eliminated in the urine.

The carrier experiments carried out on the material eliminated by the intestine showed it to be unchanged TSAF. Examination of the material excreted in the urine, however, showed that it had been metabolized, the original compound not being present.

When the urine was subjected to the modified diazotization and coupling procedure (86) it was found that free AF was present as

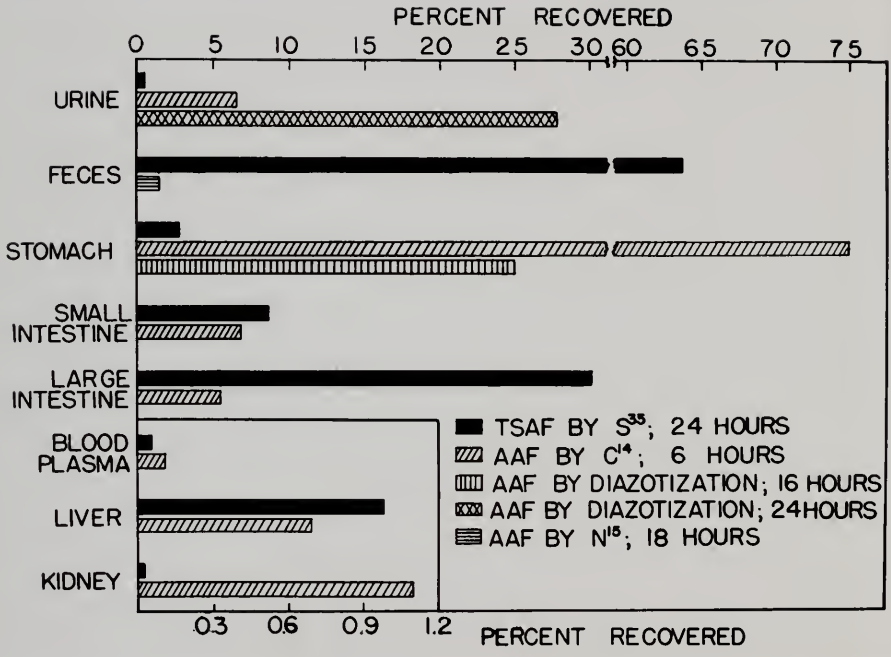


Figure 1. - A comparison of the distribution of TSAF and AAF in the organs and excreta of the rat. The scale for the blood plasma, liver, and kidney recovery has been proportionally magnified.

0.48 per cent of the ingested TSAF. It is thus evident that all the compound accounted for in the urine was in the completely hydrolized form.

In an attempt to determine what other metabolite of TSAF was present in the urine, the urinary inorganic sulfate was precipitated as benzidine sulfate. No radioactive inorganic sulfate was present.

The hydrolysis of TSAF would be expected to yield, in addition to AF, p-toluenesulfonate. Carrier experiments carried out with this compound failed to establish its presence. Either before or after hydrolysis, the p-toluenesulfonic acid moiety was metabolized. Oxidation of an aromatic methyl group to the carboxy acid is a known metabolic process. It is possible that the other metabolite of TSAF is p-sulfobenzoic acid.

Since these studies show that over 90 per cent of the ingested TSAF is excreted unchanged in the feces, it may be suggested that the compound is noncarcinogenic because it is not absorbed in sufficient concentrations by organs most likely to be attacked. The following facts, however, contradict this idea. The amount of radioactive AAF in the liver at 6 hours was 0.7 per cent, which is comparable with the TSAF value (1.0 per cent) at the end of 24 hours. After 6 hours, however, Morris and Westfall (55) found only 0.2 per cent of the AAF in the liver by diazotization. This difference in the two values for AAF is significant. In the first case, the AAF was determined in terms of the C^{14}. The second value is based on diazotizable AF available by extraction and hydrolysis. The fact that less is accounted for by

the diazotization method indicates that a substantial portion of the AAF in the liver is in a modified form. Thus, the carcinogenicity of AAF in the liver is not the result of its concentration per se but is a consequence of the state of the compound in the liver. We find that the tosyl derivative is present in the liver in larger concentrations and for a longer period of time than AAF. If TSAF were capable of cancer production, it has sufficient contact with this organ to display this activity. A probable explanation for this lack of carcinogenesis is that the TSAF, unlike AAF, is a more stable compound and is not metabolized in the liver.

Another noteworthy result is that the concentrations of both TSAF and AAF in the blood plasma are equal. Morris and Westfall (55) report 100 μg. AAF per 100 ml. blood plasma, 16 hours after ingestion. We found this same level (100 μg. TSAF per 100 ml. blood plasma) even after 24 hours.

The question still remains whether AF is the primary carcinogen, and whether conversion of substantial amounts of a derivative to the free amine is a necessary prelude to carcinogenesis. An objection to this theory is the evidence that AAF is the primary carcinogen. It is known that a variety of aromatic amines are acetylated in vivo. In addition, Wilson, DeEds and Cox (89) report that in the rat AF is a slightly slower-acting carcinogen than AAF, a fact which would not at first be expected if AAF is active only through conversion to AF. In view of these facts, then, AAF could be considered the primary carcinogen, no matter in what form the AF was supplied,

provided that AF could be formed *in vivo* from the other derivatives. Yet even if this were the case, the necessity exists for the hydrolysis of a fluorene derivative to AF prior to acetylation.

On the other hand, Morris *et al.* (54,84) have shown that AAF undergoes deacetylation in the animal body. The lower activity of AF compared to AAF in the rat is not too indicative of primary AAF activity. Animals ingesting 2-diacetylaminofluorene (di-AAF) develop tumors in a shorter time than is necessary for either AF or AAF (53). It cannot be inferred, nevertheless, that di-AAF is the primary carcinogen! The lower activity of AF in the rat may be due to solubility differences. Since AF is considerably more soluble than AAF (85), it may be excreted more quickly; hence, the tissues are not exposed as long as with AAF, which is excreted at a rate depending on its hydrolysis to AF. Furthermore, Wilson, *et al.* (89) point out that in the mouse AF is possibly more active than AAF, since it shows a slightly shorter period of incubation.

From the evidence available at present, it does not seem possible to reach a final conclusion in this matter; however, the results of these experiments with TSAF support the theory that unless a fluorene derivative can be converted in substantial amounts in the organism to the free amine, the substance will not be carcinogenic (64).

IV

THE DISTRIBUTION OF DISODIUM FLUORENE-2,7-DISULFONATE-S^{35} IN MICE BEARING A TRANSPLANTABLE STOMACH CARCINOMA

A. Statement of the Problem

In Part IA the need for a simple method of early detection and therapy of gastric cancer has been pointed out. Materials, which on administration to tumor hosts, would localize with a certain degree of selectivity in the malignant tissues have been sought for many years. The incorporation of radioactive elements into such compounds might well be useful in the diagnosis and treatment of gastric carcinoma.

Extensive investigations have been made concerning the possible selective localization of dyes in tumor tissue (7,14,23,26,68, 81,92,93). Tobin and Moore (79) were the first to synthesize a radioactive dye. They prepared Br^{82} derivatives of Evans Blue and Trypan Blue. An investigation of these dyes in tumor mice, however, showed that the liver took up about twice as much of the radioactivity as the tumor (47). Their work, nevertheless, indicated clearly that determination of radioactive compounds in tissues with Geiger counters is both simpler and more exact than the chemical or colorimetric analysis which had previously been used in determining the localization of dyes.

Bloch and Ray (8) selected I^{131} to label a number of mono- and bis-azo dyes. This element with a half-life of 8 days has a radiation suitable to biological work and can be synthesized into organic compounds with a firm linkage. Recently Stevens et al. (74) studied the distribution of radioactive iodinated Trypan Blue, one of the compounds first prepared by Bloch and Ray. This compound was administered intravenously to mice bearing subcutaneous implants of tumor 15091a. The concentration of radioactivity in tumor tissue was several times greater than that in skeletal muscle or skin, but considerably less than in the liver, spleen or kidneys.

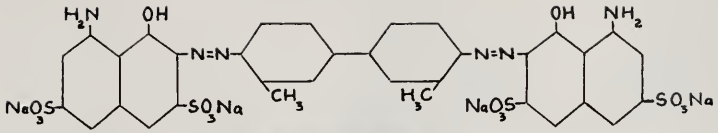
If a compound could be found which would display a greater affinity for the tumor than for such vital organs as the liver and kidneys, while at the same time maintaining a favorable ratio of concentrations in tumor and surrounding tissue, it would greatly increase the chances of accurate diagnosis and therapy of gastric cancer. Since radioactivity offers a ready means of detection, the need for a colored derivative no longer exists and other types of labeled compounds may be studied.

Several characteristics of the sulfonamido linkage endow these substances with diagnostic and therapeutic potentialities. So far as could be found in the literature the work reported in Part III (64) describes the only case of in vivo hydrolysis of a sulfonamido linkage and this hydrolysis occurred to the extent of only 0.5 per cent. The azo linkage which occurred in the compounds of Tobin and Moore, and Bloch and Ray is quite susceptible to enzy-

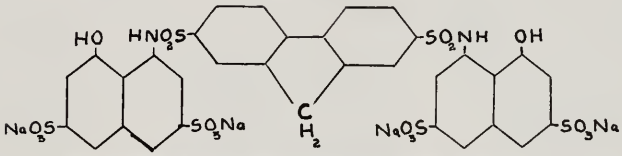
matic fission. In addition to localizing in certain tissues (9,76) sulfonamides have been shown to reduce the effective vitamin intake of animals by suppressing the intestinal flora, and this reduction of essential vitamins in the diet of tumor-bearing mice is known to inhibit the growth of tumors (10). Ray and Soffer (66) proposed the incorporation of S^{35} into these already promising compounds. This short-range beta emitter would not only exert possible additional therapeutic effects, but would also offer a means of detection.

Since certain nitrogen derivatives of fluorene are known to produce tumors in various sites in the animal body, it is possible that other derivatives of this molecule might be directed to tumors already present. If such fluorene derivatives contained one or more sulfonamide linkages labeled with S^{35} the possible diagnostic and therapeutic effects pointed out above could be utilized.

For example, by condensing the acid chloride of fluorene-2,7-disulfonic acid with H acid or Chicago acid, compounds analogous to Trypan Blue or Evans Blue could be obtained. In these compounds the dimethylbiphenyl group would be replaced by the biphenylenemethane (fluorene) group and the readily-broken azo linkages by sulfonamido linkages.



Trypan Blue



Fluorene-2,7-disulfonamido Analogue

The possible compounds which could be successfully condensed with fluorene-2,7-disulfonyl chloride are by no means limited to the naphthylamine sulfonic acids, and disulfonamido derivatives of fluorene with a wide range and variety of physical properties may be conceived.

The selection of a derivative with the best possible physical qualities for specific affinity to cancer tissue presents a problem. Such a large molecule with numerous functional groups makes it difficult to determine what specific alteration in structure should be made to obtain the desired tumor localization. If the molecule is built up step by step it should be possible to determine which structures contribute to the desired properties and which are responsible for the objectional qualities.

The fundamental structure upon which a molecule of this type could be built up is fluorene-2,7-disulfonic acid. To this end disodium fluorene-2,7-disulfonate-S^{35} (2,7-FDS^{35}) has been prepared and its distribution in tumor-bearing mice investigated.

B. Experimental Details

1. Disodium fluorene-2,7-disulfonate-S^{35}. Fluorene, 50 gm. (0.03 moles), together with concentrated H_2SO_4, 69 ml. (1.20 moles), containing S^{35} in weak HCl, 0.3 ml. (8 mc.), was warmed on a steam bath. After one-half hour the fluorene dissolved. The solution was warmed an additional one and one-half hours during which time a white precipitate formed. Sufficient ice was added to dissolve the product and the solution was freed of any undissolved residue by filtering through a filter stick. The product was precipitated by the slow addition of solid Na_2CO_3. Two recrystallizations were carried out by dissolving the product in a minimum of boiling water, filtering hot, adding absolute ethanol until turbid and allowing the white product to crystallize; the yield was 95.42 per cent based on fluorene and 47.71 per cent based on $H_2S^{35}O_4$; sulfur analysis gave 17.23 per cent S; the calculated value is 17.31 per cent S. The p-toluidine derivative melted at 326°; the melting point of the disulfonyl chloride was 225-226°; Courtot and Geoffroy (18) found 225-226° for fluorene-2,7-disulfonyl chloride.

2. Animal studies. Five to six week old Strain A (Bar Harbor) mice were employed as twenty-third generation hosts for the subaxillary transplantation of a keratinizing squamous cell carcinoma (Line A, stomach carcinomata originally obtained from The Animal Supply and Research Units of the British Empire Cancer Campaign). When the tumors were 10 days old, each mouse was administered, by tail vein injection, 0.25 ml. of saline solution containing 5.0 mg. 2,7-FDS^{35} having a specific activity of 39,660 counts per min. per mg. Base experiments in which the animals received twice this concentration of the compound failed to reveal any toxicity symptoms.

The animals were sacrificed at 2-, 8-, and 32-hour intervals following treatment and pooled samples from 2 animals were used for each determination. The mice were anesthetized with Nembutal 10 minutes before the time of death and blood removed by heart puncture prior to death. A 1.1 per cent solution of sodium oxalate (0.5 ml. to 1 ml. blood) was used as an anticoagulant.

The tumor, liver, spleen, kidneys, stomach with contents and leg muscles were removed, immediately weighed and suspended in a 1 per cent sodium hydroxide solution containing 1 ml. of Tergitol per 250 ml. One ml. of this solution was used for each 100 mg. tissue. The suspension was allowed to stand 48 hours at 5°. The mixture was then brought to room temperature and thoroughly homogenized for 20 minutes. A 1 ml. portion was placed on a shallow alumimum planchet and allowed to dry at room temperature. One ml. samples of blood were plated directly on planchets.

Radioactivity measurements were made in an internal-type counter (Q-gas chamber and Nuclear Instrument and Chemical Corporation Scaler, Unit Model 162) with an efficiency of 45 per cent. Each sample was counted for three ten minute intervals and the net counts per minute above background recorded for each sample.

The concentration of 2,7-FDS^{35} in these samples was determined by direct comparison with standard planchets prepared in the same manner and containing known concentrations of the radioactive compound. Liver standards were used for the liver, spleen and kidneys; muscle standards for the muscle and tumor; and rabbit blood standards for the blood. In order to eliminate correction for the decay of S^{35}, the standards were counted on the same day as the samples. The total amount of 2,7-FDS^{35} present in the whole sample was determined by multiplying the concentration by the total weight of the sample. The total blood volume was calculated on the basis of 63.2 ml. per kg. body weight (60).

C. Results

The distribution of radioactivity in the organs and tissues of tumor-bearing mice following injection of 2,7-FDS^{35} is given in Table II. Two hours after administration, the concentration of 2,7-FDS^{35} in the tumor (0.152 mg. per gm. tissue) was higher than in any organ. This concentration was four and one-third times as great as that found in skeletal muscle and more than twice that in the spleen. While comparatively substantial amounts of S^{35} were

TABLE II

DISTRIBUTION OF RADIOACTIVITY IN TUMOR-BEARING MICE FOLLOWING INTRAVENOUS INJECTION OF DISODIUM FLUORENE-2,7-DISULFONATE-S^{35} (5.0 mg. 2,7-FDS^{35} in 0.25 ml. saline)

	Conc. in Mgs. Per Gm. Tissue or Ml. Blood	Total Mgs. Recovered	% Recovered
Two hours:			
Blood	0.164	0.331	3.31
Liver	0.130	0.295	2.95
Kidneys	0.136	0.066	0.66
Spleen	0.068	0.024	0.24
Stomach + c	0.143	0.067	0.67
Tumor	0.152	0.058	0.58
Leg muscles	0.035	———	———
Eight hours:			
Blood	0.079	0.174	1.74
Liver	0.050	0.108	1.08
Kidneys	0.062	0.035	0.35
Spleen	0.030	0.011	0.11
Stomach + c	0.077	0.028	0.28
Tumor	0.058	0.016	0.16
Leg muscles	0.052	———	———
Thirty-two hours:			
Blood	0.002	0.004	0.04
Liver	0.007	0.011	0.11
Kidneys	0.026	0.011	0.11
Spleen	0.005	0.001	0.01
Stomach + c	0.028	0.006	0.06
Tumor	0.018	0.006	0.06
Leg muscles	0.000	———	———

TABLE III

RATIOS OF THE CONCENTRATION OF RADIOACTIVITY IN TUMOR TISSUE TO THE CONCENTRATION IN OTHER TISSUES FOLLOWING A SINGLE INJECTION OF LABELED COMPOUND TO TUMOR-BEARING MICE

	^{35} 2,7-FDS		^{131} I -	^{131} I -
	2 hrs.	32 hrs.	Trypan Blue 24 hrs.	Nile Blue 2B++ 2.4 hrs.
Blood	0.93	9.00	----	0.71
Liver	1.17	2.57	0.15	1.37
Kidneys	1.12	0.69	0.34	0.84
Spleen	2.24	3.60	0.38	1.46
Stomach	1.06	0.64	0.87	----
Muscle	4.34	+	3.33	3.61

+ No radioactivity remained in the muscle tissue.

++ After 24 hours no radioactivity from Nile Blue 2B was detectable in the animal body.

accounted for in the liver and kidneys, these values were less than for the tumor tissue. The blood showed a slightly higher concentration (0.164 mg. per ml.) than did the tumor.

After 8 hours the localization of 2,7-FDS^{35} in the tumor was still greater than in the liver, spleen or muscle, but slightly less than in the kidneys and blood.

The distribution studies after 32 hours reveal that none of this radioactive compound remained in the muscle tissue. The concentration of 2,7-FDS^{35} in the tumor was over three and one-half times that in the spleen and two and one-half times that in the liver. The kidneys still showed a higher localization than the tumor, but the concentration in the blood was now only one-ninth that in the tumor. These ratios are given in Table III. Comparable ratios are also given for I^{131}-labeled Trypan Blue and Nile Blue 2B. The data for radioactive iodinated Trypan Blue was obtained from the work of Stevens et al. (74); the distribution studies for I^{131}-Nile Blue 2B were made by Sloviter (73).

Contrasting the distribution of 2,7-FDS^{35} with that of I^{131}-labeled Trypan Blue and Nile Blue 2B, it is evident that the fluorene derivative has not only maintained but has bettered the ratio of concentrations in tumor and muscle tissue. This ratio is 4.34:1 for the 2,7-FDS^{35} two hours after injection. The ratio for Nile Blue 2B is 3.61:1 after 2.5 hours while Stevens and associates report a 3.33:1 ratio for Trypan Blue at 24 hours. Thirty-two hours after injection of 2,7-FDS^{35} considerable radioactivity remained in the tumor while

it was undetectable in the muscle. Sloviter found no radioactivity in the animal body 24 hours after administration of radioactive iodinated Nile Blue 2B.

One of the chief disadvantages in the use of the dyes tested for tumor localization studies is the fact that such vital organs as the liver, kidneys and spleen take up large quantities of the dye. For example, with Trypan Blue six times as much radioactivity was accounted for in the liver as in the tumor 24 hours after injection. This ratio still persisted after 5 days when the final distribution study was made. With this same compound the kidneys and spleen concentrated over three times as much of the dye as did the tumor.

A much more favorable situation is found in the distribution of 2,7-FDS^{35}. At 8 and 32 hours only the kidneys had a slightly higher concentration of this compound than did the tumor. At no time was the localization of the fluorene derivative greater in the liver than in the tumor, and after 32 hours the concentration was two and one-half times greater in the tumor tissue than in the liver. The spleen showed a substantially lower localization of the S^{35}-labeled compound than did the tumor at all of the time intervals studied.

This data for 2,7-FDS^{35} also compares favorably with Sloviter's findings for Nile Blue 2B. After 2.5 hours the kidneys showed a greater localization of this dye than did the tumor tissue, but at 2 hours the concentration of the fluorene derivative was 1.12 times greater in the tumor than in the kidneys. The ratio of concentration

of radioactivity in tumor tissue over the concentration in the spleen at 2.5 hours was 1.46 for Nile Blue 2B, while this same ratio was 2.24 and 3.60 at 2 and 32 hours for 2,7-FDS^{35}. The proportion of labeled compound in the tumor as compared to the liver was about the same for the two compounds at 2 and 2.5 hours (1.17 for 2,7-FDS^{35} and 1.37 for Nile Blue 2B); however, this ratio more than doubled for the fluorene derivative at 32 hours.

The small percentages of radioactivity accounted for in the animal body indicate that 2,7-FDS^{35} is readily excreted. This would be expected since the disulfonic acid groups impart a high degree of solubility to the fluorene molecule. Such a fairly rapid excretion is a definite advantage over the high molecular weight Trypan Blue which remains in the animal body after 5 days in concentrations only slightly less than at 24 hours. On the other hand Nile Blue 2B is excreted so rapidly that none remains in the animal body after 24 hours while the fluorene compound is still detectable in the tumor at 32 hours.

By comparison with the dyes studied, 2,7-FDS^{35} has greatly increased the ratio of localization in tumor tissue compared to liver, kidneys, spleen and blood while at the same time increasing the favorable balance between tumor and muscle tissue. It is retained by the animal body for a length of time sufficient to enable detection and treatment of malignant tissue without persisting in high concentrations for a prolonged period. The ratio between tumor and kidney tissue of

1.12 at 2 hours falls to 0.69 at 32 hours. In Part III (64) it was found that a sulfonamido derivative of fluorene was eliminated by the kidneys in small amounts. It is possible that the unfavorable ratio found with the 2,7-FDS^{35} could be greatly improved by converting it into a sulfonamide.

This fluorene compound, therefore, offers a promising basis upon which sulfonamide derivatives may be built and tested in tumor diagnosis and therapy.

Gastric secretion. A noteworthy result was the high level of localization of 2,7-FDS^{35} in the stomach and its contents. In every case the concentration of radioactivity in the stomach was comparable to or higher than that in the tumor. Since the labeled compound was administered by intravenous injection, it is necessary that it be secreted by the stomach wall to reach the gastric contents. Such secretion of a highly acid compound is not in agreement with the results obtained in studies on the elimination of dye stuffs by the gastric glands. Ingraham and Visscher (33) investigated a large number of dyes and found that the only ones secreted by the stomach were those capable of acting as basic dyes. Forty acid or amphoteric dyes studied were not eliminated by the gastric glands.

Stevens and co-workers (74) found that radioactive iodinated Trypan Blue also localized in the stomach in concentrations comparable to that in tumor tissue, but much less than in the liver, kidneys and spleen. While Trypan Blue is an acid dye by virtue of its sulfonic acid groups, it also contains basic functional groups. Moreover,

splitting of the azo linkage with the formation of amino groups is known to take place in the animal body. The radioactive iodine could have been directed to the stomach by a fragment of the dye made more basic by this cleavage of the azo linkage. However, in view of the results reported above for 2,7-FDS[36] it may be necessary to revise present ideas of the secretory mechanism of the stomach.

V

SUMMARY

1. A synthesis is described which incorporates isotopic nitrogen (N^{15}) into the molecule of the carcinogens, 2-nitrofluorene, 2-aminofluorene, and 2-acetylaminofluorene. By mass spectrographic analysis the hitherto unknown fate of the nitrogen during metabolism may be determined.

2. The distribution of radioactivity was studied in the organs and excreta of the rat at 24-, 54-, and 66-hour intervals following oral administration of a single dose of noncarcinogenic 2-p-toluenesulfonamidofluorene-S^{35}.

The amount found in the liver (1.0 per cent) and in the blood (100 μg. per 100 ml. plasma) was comparable to the amounts of 2-acetylaminofluorene found in similar experiments. The noncarcinogenicity of 2-p-toluenesulfonamidofluorene, therefore, is not caused by insufficient concentration of the compound in the liver (a predominant site for 2-acetylaminofluorene-induced tumors) or in the blood. Over 90 per cent of the ingested dose was found to be eliminated unchanged through the gastrointestinal tract. Only 0.5 per cent of the radioactivity was found in the urine. All the compound accounted for in the urine was in the form of 2-aminofluorene. This minute quantity of 2-p-toluenesulfonamidofluorene which undergoes metabolism contrasts with the relatively large amount, approximately 30 per cent, of the carcinogenic 2-acetylaminofluorene appearing in the urine.

3. A synthesis is described which incorporates radioactive sulfur (S^{35}) into the molecule of disodium fluorene-2,7-disulfonate. The distribution of radioactivity in the tissues of tumor-bearing mice following a single injection of this compound was studied at 2-, 8-, and 32-hour intervals. The ratios of concentration of radioactivity in tumor tissue to the concentration in other tissues were determined. By comparison with similar ratios for I^{131}-labeled Trypan Blue and Nile Blue 2B, the fluorene compound has greatly increased the ratio of localization in tumor tissue compared to liver, kidneys, spleen, blood and muscle. It offers a promising basis upon which sulfonamido derivatives may be built and tested in tumor diagnosis and therapy.

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BIOGRAPHICAL SKETCH

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This dissertation was prepared under the direction of the Chairman of the candidate's Supervisory Committee and has been approved by all members of the Committee. It was submitted to the Graduate Council and was approved as partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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