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PEPTIDE DERIVATIVES OF THE CARCINOGEN 2-AMINOFLUORENE

A Dissertation Submitted to the

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Louis H. Rombach

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Sincere appreciation is extended

to Dr. I. R. MacGregor who served as research adviser during the course of this work;

to the Cancer Institute of the U. S. Public Health Service for awarding a fellowship (1951-1953).

DEDICATION

A mere acknowledgment would be
inadequate to express my gratitude
for the inestimable inspiration
and indefatigable interest manifested
by two persons who instilled in
me the undaunted courage to
persevere in this endeavor.

To these two, my Wife and my Mother,
I wish to dedicate this thesis.

CONTENTS

ABSTRACT

The versatility and importance of 2-aminofluorene and some of its derivatives has been well recognized in the field of oncology because of their ability to promote tumor formation in a variety of sites. In an effort to achieve a greater understanding of this phenomenon, a group of peptide derivatives of 2-aminofluorene was synthesized. * Because of the intimate relation which may exist between carcinogenesis and proteins, it seemed quite possible that some valuable information might be forthcoming from a study of the carcinogenic activity of these new derivatives.

The compounds which have been prepared during the course of this research for the first time are listed below.

2-glycylaminofluorene

2-phthalyldiglycylaminofluorene

2-diglycylaminofluorene

2-phthalylphenylalanylaminofluorene

2-phenylalanylaminofluorene

2-phthalyldiphenylalanylaminofluorene

2-diphenylalanylaminofluorene

* This project was supported by a Fellowship granted by the Cancer Institute of the U. S. Public Health Service. Biological testing of the compounds has been undertaken by Dr. F. E. Ray, Director, Cancer Institute, University of Florida, Gainesville, Florida.

ABSTRACT (continued)

α-amino-bis(2-acetamidofluorene)

2-phthalylglycylamino-7-acetamidofluorene

monohydrated dimer of 2-glycylamino-7-acetamidofluorene

In addition to the methods developed for the synthesis of these compounds, a number of other procedures for the preparation of intermediates have been modified to improve the yields. Of the twenty reactions described in detail twelve provided yields of 90-100% and another four, 80-90%, an important fact to consider in the synthesis of peptides.

I. INTRODUCTION

A. Carcinogenic Fluorene Derivatives

1. Discovery of Carcinogenic Activity

2-Acetamidofluorene, known also as 2-acetamino- or acetylaminofluorene or N-(2-fluorenyl)-acetamide, was first prepared as an insecticide and proved to be of value. In testing on rats it was found to be non-toxic if ingested for short periods in quantities 0.1% by weight of the diet, but 0.031% for 795 days led to the production of distant tumors in a variety of locations such as the external auditory canal, thyroid, breast, lung, pancreas, stomach, liver, intestine, kidney, bladder, renal pelvis and female genitalia (1). As a result numerous investigations of fluorene were commenced in order to determine the extent of its carcinogenic importance. Fluorene, 2-chlorofluorene, diethylaminoethyl-9-fluorene carboxylate and fluorenone were found to be non-carcinogenic for rats (2). Like the other aromatic amines only the 2-position proved to be active. 2-Nitro-, 2-amino-, 2-diacetamido- and 2-acetamido-7-hydroxy- fluorene, as well as N-(2-fluorenyl)-glycine and N-(2-fluorenyl)-hemisuccinamide were active, the latter two being water soluble (3). The acetamidofluorene was the most active and produced the largest variety of tumors. The materials were ingested, or with smaller quantities painted on, although only the 2-amine

produced skin tumors. Since the 2-acetamido but not the 2-amino compound was inactive when administered subcutaneously, it was thought that the latter was the carcinogen and the acetyl group merely acted as a carrier (4). This same hypothesis could apply to the other active fluorene compounds as well, i.e. hydrolysis to the free amine must occur before carcinogenesis. It is entirely possible that the nitro compound is reduced to the amine in vivo, at least in minute quantities since the nitro derivative was only slightly active and incapable of producing liver tumors whereas the amine could cause liver tumors after being painted on the stomach.

The amino and acetamido derivatives acted independently of the amount of protein in the diet and therefore were presumed to react by a different mechanism than p-dimethylaminoazobenzene (5). However, the ratio of liver protein to body weight was increased by acetamidofluorene. The plasma protein concentration remained constant unless larger quantities (0.15% by weight) of material were ingested (6). By incorporating both dried yeast and acetamidofluorene in the diet, the development of cancer could be delayed (7).

It was discovered that mice were more resistant than rats to the affects of acetamidofluorene although age, yeast (cf. above), cod liver oil and wheat germ apparently did not affect its action (8). If casein were added to the diet along with acetamidofluorene, a riboflavin deficiency was

noted which could be overcome by adding excess riboflavin (cf. page 83). Omission of the casein nullified the action of the excess riboflavin (9).

Thiouracil and allylthiourea were found to enhance the action of acetamidofluorene on the thyroid but to inhibit the formation of liver tumors (10).

The usual variations were observed with sex, age (cf. above) and strain, and examples were reported using co-carcinogens in conjunction with acetamidofluorene (11). The metabolism and/or detoxication of the amino and acetamido compounds, like some other carcinogens, also involved the formation of the hydroxy group since glucuronides of the 7-hydroxy derivatives were isolated (12).

2. Methods of Analysis

The quantitative procedure used to determine the fate of the fluorene derivatives involved the diazotization of the amine, as such or after hydrolysis or reduction of its precursor, followed by coupling to sodium-2-naphthol-3,6-disulfonate and then analyzing colorimetrically (13).

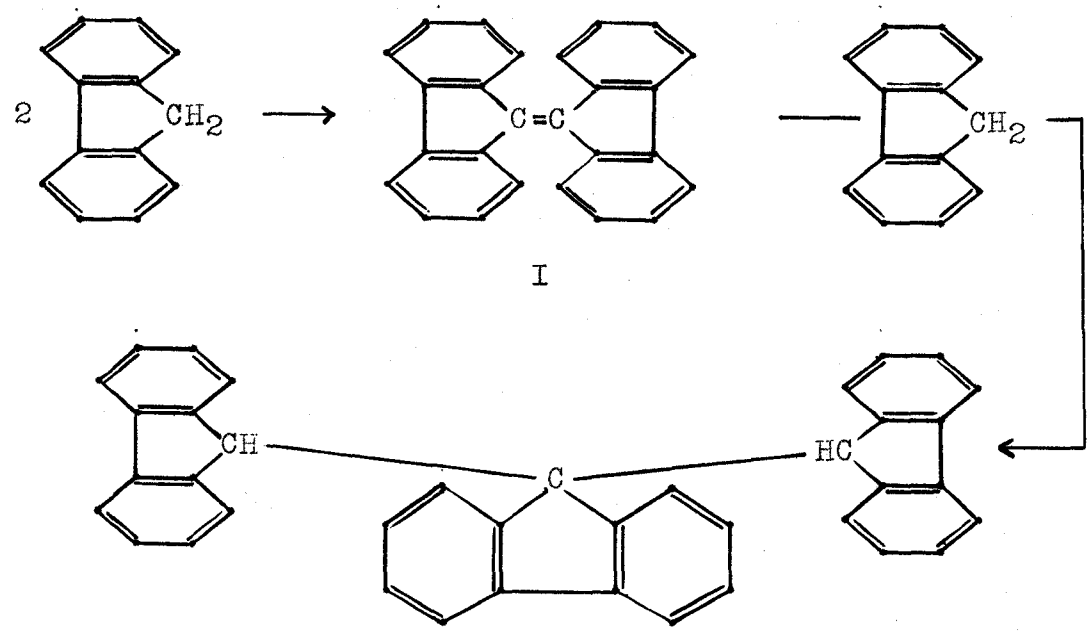
Initially it was noted that feeding of acetamidofluorene to rats resulted in an orange coloration of the underside fur. Diels had reported that a solution of the amine hydrochloride colored a pine stick an intense red which faded to orange on dilution (14). This served as additional proof that hydrolysis of the acetamido compound had been effected.

Since the usual methods of analysis could account for only about one-third of the carcinogen, a method was developed using fluorene derivatives containing a 9-C^{14} atom as well as acetamidofluorene containing an ω-C^{14} atom. This permitted a more efficient recovery of the total agent administered. It was found that the carbon dioxide exhaled contained appreciable quantities of radioactive carbon after experiments with the ω-C^{14} but not the 9-C^{14} compound. This was taken to indicate that hydrolysis of the acetamido compound occurred and the carcinogenic action might then be due to the free amine (15).

3. Mechanism of Action

Although relatively few mechanisms have been proposed to explain the action of carcinogenic aromatic amines, a hypothesis was developed following some preliminary work on fluorene, and this was subsequently extended to all four types of carcinogens. Since the reactions for each are similar, only the explanation for fluorene will be described (16).

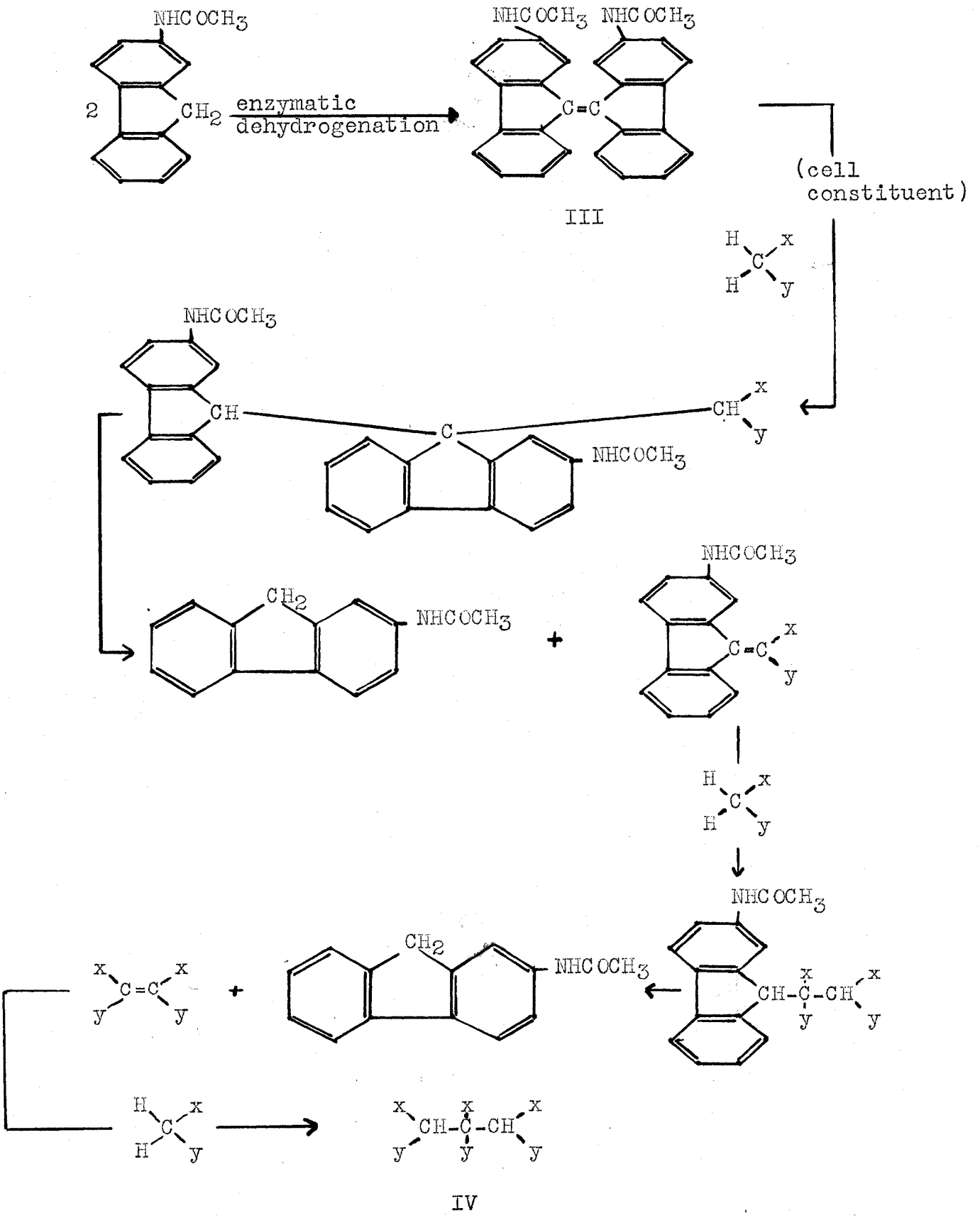
Fluorene was readily converted into $\Delta^{9,9'}$-bifluorene (I), also called dibiphenyleneethylene or bifluorylidene, and this in turn reacted with another molecule of fluorene in a Michael condensation to form 1,2,3-tribiphenylenepropane (II) (17). If the original fluorene molecules were substituted in the 2- or 2,7-positions by certain groups, the ethylenic compound disproportionated and reacted with unsub-



I

II

stituted fluorene in a series of steps such that, depending upon the conditions, the product contained none, one, two or three of the substituted fluorenes. The above reactions were presumed to be possible in vivo, so that after ingestion of acetamidofluorene the corresponding $\Delta^{9,9'}$-bi-(2-acetamidofluorene) would be formed (III). This substance may be capable of reacting with a cell constituent possessing active hydrogen atoms in a manner similar to that described for fluorene. However, instead of 1,2,3-tribiphenylenepropane as the final product, there would be formed analogously a modified cell constituent (IV). The important feature of this theory was the reaction of the substituted ethylene to produce an altered cell. Although the product here is represented as a propane derivative, it is entirely possible that this would



continue to react and eventually produce a modified cell constituent having a long carbon chain. This theory would appear to be quite similar to that of Haddow regarding environment (cf. pages 77, 87 and 88) except that some specific chemical reactions have been utilized. The sequence of reactions has been adapted to include all four types of carcinogens, so that not only an ethylenic group but an azo group might react similarly. If these two groups were not already present as in the stilbenes and azobenzenes, they could be formed by an enzymatic dehydrogenation similar to that postulated for acetamidofluorene. The polycyclic hydrocarbons containing an active methyl group would also react in this way, and even if the methyl group were absent, this, too, could be introduced in vivo since methylations already have been proven to be possible in the body (cf. page 84).

Although this mechanism appears plausible, it naturally is not in conformity with all of the other theories of carcinogenesis, but does serve to add to the oncological data being compiled.

B. Peptides

1. Synthesis

The relation of oncology to proteins has been emphasized frequently since cancerous cells as well as normal cells are composed of proteins. Although proteins have been degraded into their constituent amino acids by rupturing the peptide

linkages, it has thus far been impossible to synthesize them from the amino acids. Some results have been achieved since high molecular weight substances have been prepared from amino acids, but these usually were insoluble infusible resins which had little value. The other method of approach has been to prepare substances in a stepwise manner from amino acids, thereby resulting in just a few peptide linkages. In this way more information could be obtained about the polypeptides which may some day prove to be useful for the preparation of proteins.

For many years much work has been directed toward the formation of simple synthetic peptides. To give a complete description would be impractical. Hence, a brief resume will be presented which includes a reference to either the original work or to a review.

Some controversy ensued over the preparation of the first peptide when Fischer and Fourneau claimed this distinction after they had heated glycine anhydride with concentrated hydrochloric acid to give glycylglycine (18). The benzoyl derivative obtained from a reaction of the silver salt of glycine with benzoyl chloride had already been reported by Curtius (19).

Heating an α-amino acid ester led to the formation of a diketopiperazine, but from a β- or other amino acid was obtained a dipeptide ester (20).

α-Halogenated acyl halides have been utilized for the preparation of peptides. They have been coupled with other amino acids and the α-halogen was then removed by amination (21). By a combination of the above methods an octadecapeptide was prepared (22).

If the α-halogenated acyl halide were coupled to an amino acid ester, the halogen could be replaced by the azide group, reduced to the amine and the ester group could finally be removed by hydrolysis (23).

The azlactone synthesis of Erlenmeyer has been utilized by Bergmann to prepare peptides possessing more complex amino acids such as phenylalanine, tyrosine and histidine. In general it may be described as the condensation of an acylated amino acid with an aromatic aldehyde, followed by ring opening with an amino acid, hydrogenation, and then hydrolysis to the dipeptide (24).

Probably the most widely used synthesis has been that of Bergmann in which the amine was protected by the carbobenzyloxy group. This derivative could be converted to an azide, coupled with a second amino acid ester, hydrolyzed and hydrogenated to remove the protective group (25). The carbobenzyloxy protective group has been employed in many other types of reactions for peptide syntheses in addition to the azide method. In as much as some of the carbobenzyloxy derivatives were not obtained as crystalline solids, a method utilizing

the p-bromobenzyloxy group was devised and was found to alleviate the difficulty somewhat (26).

Some higher molecular weight peptides were obtained from a modified Leuch's reaction in which 2,5-oxazolidinediones, or N-carboxyanhydrides, were prepared by reacting amino acids or carbobenzyloxy amino acids with phosgene, thionyl chloride, methyl- or ethyl-chloroformate followed by thionyl chloride, or sodium methoxide and carbon dioxide followed by thionyl chloride. The ring could be split in a chain type reaction by water or a second amino acid to form a polypeptide (27). In a similar preparation the acyl halide of 2-benzylidene-4,5-diketo-3-oxazolidineacetic acid was split with an active hydrogen, this being a controlled stepwise process, however (28).

Some studies on the Lössen rearrangement using substituted hydroxamic acids led to the formation of polypeptides. This apparently proceeded via a rearrangement to the isocyanate, and then to the N-carboxyanhydride by ring closure (29). Although the N-carboxyanhydrides served as elegant precursors of peptides, their instability presented difficulties in preparation and storage. The 2-thio-5-thiazolidones came into use since they were not as reactive as the oxygen analogues (30).

A new method of increasing importance involved the use of the phthalyl group to protect the free amine. The acyl halide of the carboxyl function was reacted with another amino acid and the dipeptide was treated with alcoholic

hydrazine hydrate to remove the protective group as phthalhydrazide (31).

A number of mixed anhydrides were utilized since these could be split by an amino acid to yield a peptide linkage. The anhydrides consisted of a carbobenzyloxy amino acid and either a fatty acid, sulfuric acid or a carbethoxy group as the second component (32). Although a method was devised in which carbobenzyloxyamino acid ester was coupled to an isocyanate, this actually may be considered as another means of preparation for the mixed anhydride above (33).

The remainder of the methods discovered recently are of lesser importance and include the following: nitrosation of a diethylmalonate followed by decarboxylation, acylation, coupling to a second amino acid and reduction of the substituted oxime to an amine (34); condensation of the sodium salt of amides of hydroxy acids in a sealed tube (35); thiolesters of amino acids reacted slowly at room temperature in alkaline aqueous solution to yield dipeptide thiol esters (36).

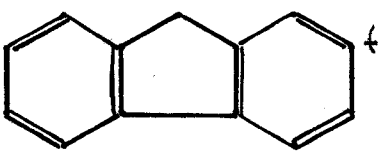
A final method may be cited as unique since the peptide linkage has been formed at either the amino or the carboxyl end of an amino acid. Diethylchlorophosphite was reacted with carbobenzyloxyamino acids to yield a mixed anhydride which in turn was reacted with the amino group of an amino acid ester. Alternatively, diethylchlorophosphite was reacted with an amino acid ester and this, with the carboxyl group of

a carbobenzyloxyamino acid (37).

Since most of the above procedures have some limitations, their usage is usually determined after a consideration of the compounds desired.

II. DISCUSSION

Much work has been done in the past regarding the role of fluorene in the field of oncology. The most emphasis has been placed on 2-substituted derivatives containing a nitrogen atom linked to the ring since it has already been shown that this is required for carcinogenic activity in the fluorene series. The relation of carcinogenesis to proteins has long been recognized because of the intimate connection between cancer, cellular growth and the protein constituents of the cell. After a consideration of these facts it seemed only logical that some valuable information might be forthcoming from a study of various 2-substituted aminofluorenes which contain one or more peptide linkages (I), the fundamental building blocks of protein molecules.



I

Sufficient background for this research has already been given in the descriptions of the carcinogenic activity of fluorene derivatives and the synthesis of simple peptides as a means of investigating the chemistry of polypeptides and proteins. Since carcinogenesis itself deserves an explanation in order to permit a greater understanding of the fluorene

discussion, an additional section on this topic has been included as a supplement. Two additional subjects, namely the chemistry of fluorene and the chemistry of amino acids and proteins, have not been included because they have no direct relation to the problem and are of such a length that any attempt to incorporate them would be unjustified.

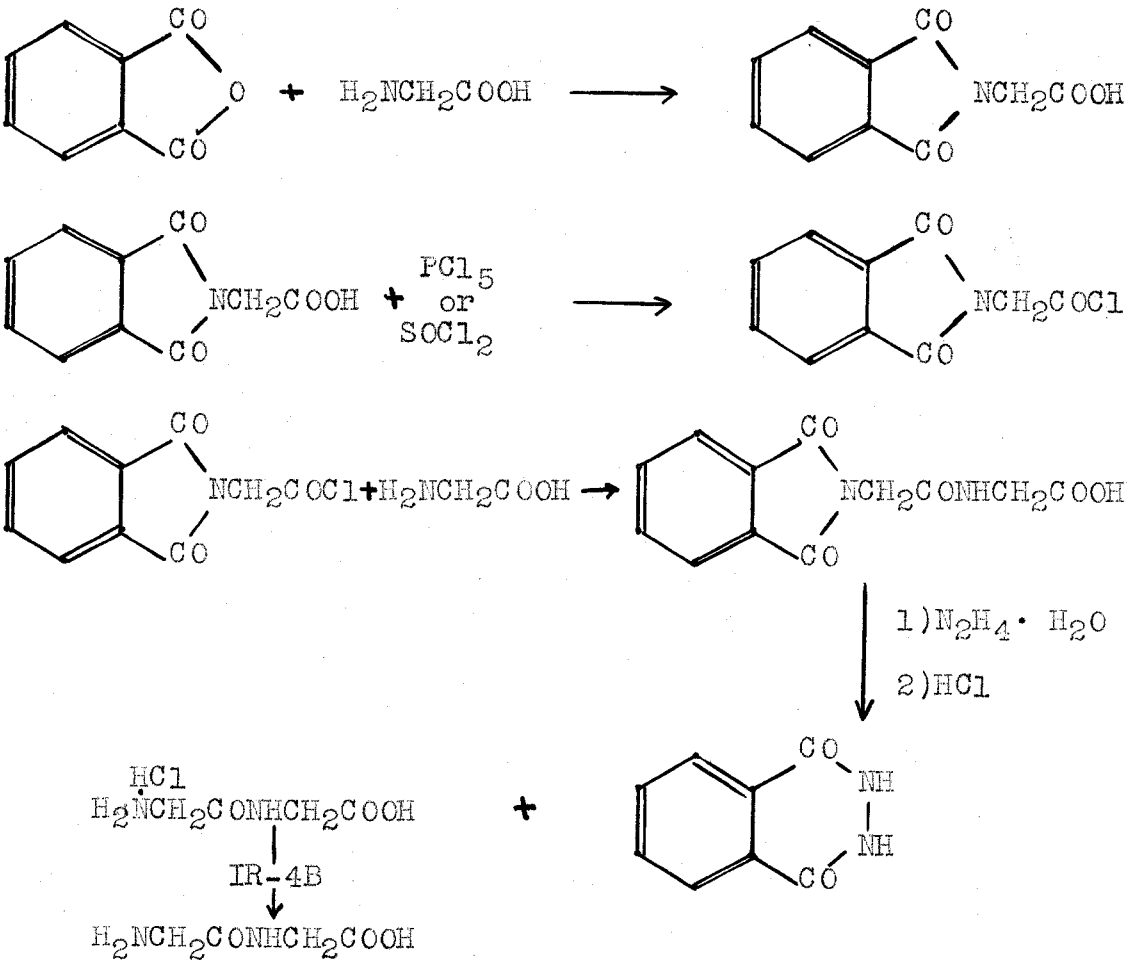
A. 2-Glycylaminofluorene*

1. From Phthalylglycine

The initial phase of this undertaking involved a survey of the literature for a method which might be utilized for the formation of the desired peptide linkages. Selected from a number of possibilities was the relatively new method of Sheehan and Frank (38) outlined below. Treatment of an amino acid with phthalic anhydride led to the production of a phthalylamino acid. The free carboxyl group was coupled via the acyl halide to a second amino acid or peptide containing a reactive amino group. This provided a ready means for the stepwise formation of a peptide link between two substances which possessed the necessary reactive groups. The removal of the phthalyl group was accomplished by means of alcoholic

* The peptide link has often been described diagrammatically as an amide link (NHCO), without stipulating that it exists between two amino acids. In order to utilize a uniform terminology throughout the thesis, the amides formed in this research between an amino acid and 2-aminofluorene or 2-amino-7-acetamidofluorene have been called peptides. However, no attempt was made to utilize the term dipeptide until a compound was prepared which contained two amino acid moieties in addition to the aminofluorene.

hydrazine hydrate. After vacuum distilling the solvent the solid residue was treated with dilute hydrochloric acid to convert the amine to its soluble hydrochloride, and the insoluble phthalhydrazide, a by-product of the hydrazinolysis, was easily separated by filtration. The filtrate was passed through an IR-4B ion exchange resin to recover the free amine.

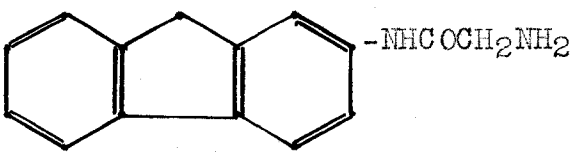


The advantages of such a procedure readily became apparent. Whereas some of the other methods were not applicable to all amino acids, no such restriction was imposed here. The

phthalyl protected amino acids gave crystalline solids, quite unlike the substances obtained when some of the other protective groups were employed. Stability of the intermediates was a prime consideration, and although the acyl halides described above were moderately reactive, they were much more stable than many of the intermediates encountered in other peptide syntheses. In addition, the danger and inconvenience in handling such reactants as phosgene, isocyanates, sulfur trioxide and chlorophosphites provided difficulties which were unencountered in the procedure selected. Ease of splitting the protective group from the amine was an important factor, and the use of hydrazine hydrate to effect the removal appeared straightforward. Removal of the protective group is important especially when the racemization of optically active compounds must be considered. Examples of retention of activity were reported by Sheehan (39) and further served to increase the value of the method chosen. Finally, the overall yields usually were superior to any of the other procedures reviewed. Hence, in view of the previous considerations the selection of the phthalyl method was unquestionably the best.

The fact that 2-aminofluorene proved to be carcinogenic provided the basis for an interesting series of reactions. The free amine offered the group necessary for coupling with a phthalylamino acid acyl halide. By removing the phthalyl group with hydrazine hydrate a new amine was formed by a

process which could be repeated as often as desired to produce a substituted 2-aminofluorene containing one or more peptide links. It should be noted that by utilizing glycine as the amino acid, the first free amine to be formed was 2-glycylaminofluorene (II), a compound very



II

similar to 2-acetamidofluorene, the most potent carcinogen in the fluorene series.

The presence of fluorene in coal tar provided a ready source of starting material. Mononitration of fluorene was carried out using concentrated nitric acid in an acetic acid solvent (40). Large quantities of fluorene were nitrated in 76% yields to provide an ample supply of material for reduction with powdered zinc to the 2-aminofluorene. The reduction was limited since the optimum yield of 93% obtained with small quantities of 2-nitrofluorene decreased to 76.5% when the amounts were increased to 0.15-0.20 mole. The method employed was similar to that of Sampey and Reid (41) using powdered zinc and acetic acid in alcohol. However, it was observed that a more efficient reaction occurred if the zinc dust and nitro compound were mixed intimately prior to addition. Furthermore, mechanical stirring was utilized

throughout the reaction to maintain a suspension of the reactants, and although the reaction commenced with greater vigor, suitable temperature control was afforded by the water bath.

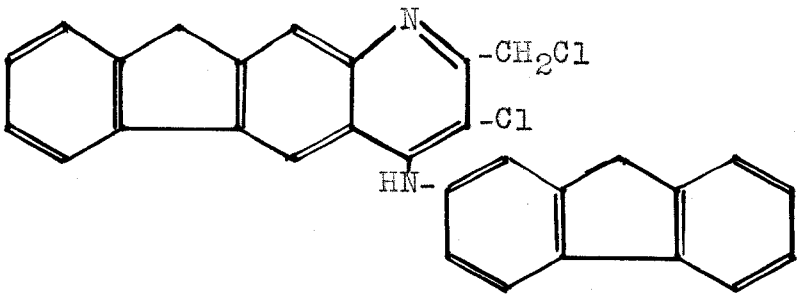
The catalytic hydrogenation of 2-nitrofluorene using Adams' Catalyst (platinum oxide) provided a means of reducing 0.5 molar quantities in excellent yields. The amounts of material which could be employed were limited only by the physical size of the hydrogenator. Adams' Catalyst was prepared using the description given in the literature (42). Some difficulty was experienced in determining the high temperatures required for the process. However, by using a Pyrex glass tube (with one end sealed) containing an iron-constantan thermocouple, a temperature indicator as well as a stirring rod was provided. The thermocouple was attached to a Leeds-Northrup Potentiometer and the temperature was calculated from a temperature-e.m.f. conversion table.

The 2-aminofluorene from either of the above methods was coupled with phthalylglycine by means of a Schotten-Baumann reaction to form the initial peptide linkage. Phthalylglycine was obtained from phthalic anhydride and glycine according to the procedure described by Sheehan and Frank (loc. cit.). Since phthalic anhydride sublimed readily upon heating, a slight excess was used. To minimize sublimation loss even further the anhydride was introduced into a flask which was

immersed in an oil bath that had been heated previously to 190-200°. Fusion occurred rapidly whereupon the glycine was introduced into the melt in small portions. The completion of the reaction was determined by the discontinuance of the evolution of water vapor. Mechanical stirring proved useful while the reaction was in progress; shortly after the water evolution ceased, stirring was terminated as the melt began to solidify. Before becoming completely solid the product was agitated by hand to prevent a large cake from forming. This facilitated removal from the flask and the slight amount adhering to the walls was dissolved easily with boiling water. The solid residue was extracted repeatedly with boiling water. By cooling the filtrate after each extraction, the product precipitated and was removed. This modified procedure provided phthalylglycine in 98% yields.

Two methods were employed for the preparation of phthalylglycyl chloride. Phosphorus pentachloride was added to a stirred suspension of phthalylglycine in dry benzene heated to 60° (38). A scrubber was utilized to remove the hydrogen chloride boiled off. After completion of the reaction the benzene and phosphorus oxychloride were vacuum distilled, and the product was recrystallized from benzene-ligroin (40-60°). Although the yield was satisfactory, the use of phosphorus pentachloride resulted in the formation of the oxychloride as a by-product and required additional care to insure complete

removal. The use of an excess of phosphorus pentachloride also caused difficulty since it had to be removed by filtration. Feldman (43) reported cyclization products such as III

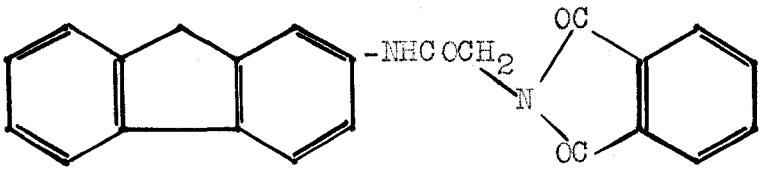


III

derived from 2-chloroacetamidofluorene in the presence of phosphorus pentachloride and phosphorus oxychloride. Thionyl chloride offered an elegant method for the preparation of acyl halides because the by-products were gases, sulfur dioxide and hydrogen chloride, and any excess low boiling thionyl chloride was removed by vacuum distillation. In addition, the possibility for the reactions reported by Feldman was minimized, an important factor here in as much as the compounds prepared in this work would be expected to react similarly to the chloroacetamidofluorene. The formation of the acyl halide from thionyl chloride was carried out with or without (44) dry benzene as a solvent. As long as pure thionyl chloride was used, the reaction went to completion in less time than with phosphorus pentachloride with the same or greater yield.

The ordinary procedure for the Schotten-Baumann reaction involves the coupling of an acyl halide to the amine in an

alkaline aqueous solution to neutralize the hydrochloric acid formed. Phthalyl protected amino acids are unstable in strong alkali, decomposing to substituted phthalamidic acids. Magnesium oxide was used as the base according to the method of Sheehan and Frank (loc. cit.), but 2-phthalylglycylaminofluorene (IV) was obtained in only 25% yield. Emerson (44)



IV

utilized sodium bicarbonate but this likewise proved unsatisfactory. Because of the insolubility of 2-aminofluorene in water, most of the acyl halide was hydrolyzed before it could react with the amine. By employing an anhydrous solvent this difficulty was eliminated. When acetone or dioxane was used as the solvent, an immediate increase in the yield was noted. By running the reaction in benzene in the absence of a base to remove the hydrogen chloride, a yield of 79% was obtained; this corresponded to 95% on the basis of 2-aminofluorene recovered as the hydrochloride. Various proportions of pyridine were then included among the reactants; it was discovered that a two molar excess of pyridine provided the best yield (95%).

Considerable difficulty was experienced in the purification of 2-phthalylglycylaminofluorene because of its insolubility

in most organic solvents. Ethanol, methanol, chlorobenzene, dibutyl ether, diethyl ether and glacial acetic acid-diethyl ether all proved unsatisfactory. Glacial acetic acid alone was somewhat useful, but the discovery of dimethylformamide offered an answer to the solubility problem. It should be emphasized that in several instances during the course of this research the lack of a suitable solvent seemed to preclude the continuance of further work. In each of the unsolved cases, however, dimethylformamide proved to be an effective solvent.

Since the phthalyl derivative was insoluble in ethanol, it was recovered from hot dimethylformamide by the dropwise addition of hot ethanol until the solution just became cloudy. Slow cooling permitted the formation of white crystalline plates which were filtered and washed free of excess dimethylformamide with ethanol.

Treatment of 2-phthalylglycylaminofluorene in ethanol with hydrazine hydrate led to the formation of the free amine and phthalhydrazide. Various criteria were used to determine the completion of the reaction. For best results an excess of hydrazine hydrate was employed. The starting material, being only slightly soluble in ethanol, gradually dissolved as the reaction proceeded, provided a sufficient quantity of solvent was used. The completion could be determined in smaller quantities of ethanol by using a time limit. This

was determined from several trials by varying the time of reaction and analyzing the products for unconverted starting material. The former method proved the most satisfactory.

The products from this reaction, being an acid and a base, reacted with each other to form a salt. The solid residue obtained after removal of the solvent by vacuum distillation was a mixture of this salt, the free amine and phthalhydrazide. The relative amounts depended upon the conditions under which the mixture was isolated.

It was also noted that if too great an excess of hydrazine hydrate were introduced, a precipitate formed after completion of the reaction even in larger quantities of solvent. This proved to be the salt formed from phthalhydrazide and hydrazine. It was identified by comparing it to the known sample which was prepared by reacting phthalhydrazide in ethanol with hydrazine.

The mixture which remained after vacuum distillation of the solvent following hydrazinolysis was treated in a variety of ways in an attempt to recover 2-glycylaminofluorene. The procedure of Sheehan and Frank (loc. cit.) required the formation of 2-glycylaminofluorene hydrochloride by digesting the mixture with dilute hydrochloric acid. The amine hydrochloride was not sufficiently soluble in aqueous solution, however, and merely resulted in the formation of a new mixture of amine hydrochloride and phthalhydrazide. In an effort to

separate these a study was made with the following solvents (both hot and cold): methyl, ethyl, n-butyl and t-amyl alcohols, acetone, methyl ethyl ketone, dioxane, methyl and ethyl cellosolve, benzene, benzene-hexane, chlorobenzene, mixed xylenes and carbon tetrachloride. Since negative results were obtained, further exploration of this procedure was discontinued.

Barber and Wragg (45) suggested that the hydrochloric acid treatment was unnecessarily harsh in some cases. They proposed that the amine could be separated from its salt by thermal dissociation, solvent extraction or by the addition of a stronger base.

Using the original mixture obtained from the hydrazinolysis reaction, a survey was conducted in order to utilize the solvent extraction procedure. Solubility tests (hot and cold) were made with the solvents listed below: methyl, ethyl and t-amyl alcohols, methyl and ethyl cellosolve, ethyl acetate, dioxane, acetone, methyl ethyl ketone, diethyl ether, benzene-hexane, ligroin (90-120°), chlorobenzene, mixed xylenes, carbon tetrachloride and glacial acetic acid. The latter, although it dissolved phthalhydrazide in the cold, merely made possible the formation of a second amine salt.

A method which proved of value involved extraction of the mixture with hot chlorobenzene. Phthalhydrazide was only slightly soluble in hot chlorobenzene while the amine was

moderately soluble; both were practically insoluble in cold chlorobenzene. After filtering the hot solution to remove the phthalhydrazide, the filtrate was cooled to give a mixture richer in the amine. After repeating several times a pure sample of 2-glycylaminofluorene was obtained although this procedure was tedious and inefficient.

A third general method was employed for the recovery of 2-glycylaminofluorene. Hot and cold ethanolic and aqueous solutions of the salt mixture were treated with a number of organic bases such as piperidine and pyridine, but to no avail. Repetition with warm (50°) aqueous and alcoholic sodium hydroxide brought about hydrolysis of the peptide link.

The hydrazinolysis itself was performed in the presence of the above organic bases, but this, also, was unsatisfactory.

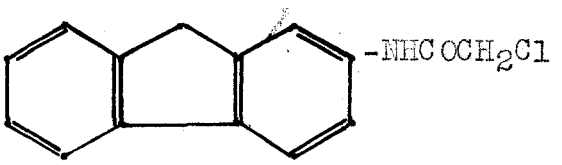
A new method appeared recently (46) in which phenylhydrazine was used to remove the phthalyl protective group from the amine. It had the advantage that a soluble by-product was formed which facilitated its separation from the peptide. Treatment of 2-phthalylglycylaminofluorene (IV) with phenylhydrazine for 7.75 hours led only to the recovery of the reactants.

Finally, it was noted that the salt mixture could be suspended in a cold (10-15°) 30% aqueous solution of sodium hydroxide without getting hydrolysis of the peptide link. Vigorous agitation with an efficient mechanical stirrer for ten hours produced the soluble sodium salt of phthalhydrazide.

After diluting the solution to four times the original volume with water the insoluble amine was removed by filtration and washed with a large quantity of water until the washings were no longer alkaline as they came through the filter. Pure 2-glycylaminofluorene (II) was obtained after recrystallization from chlorobenzene.

2. From Chloracetyl Chloride

An alternate procedure was utilized in an attempt to prepare 2-glycylaminofluorene, 2-Aminofluorene was reacted with chloroacetyl chloride; it was thought that the 2-chloroacetamidofluorene (V) could be aminated to form 2-glycylaminofluorene. The preparation of chloroacetamidofluorene has been

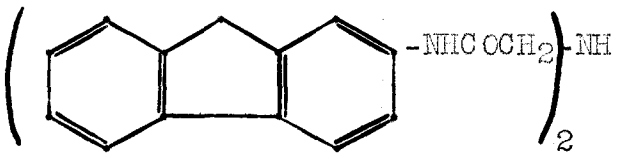


V

reported by Buu-Hoï (47) and Feldman (loc. cit.). Several attempts to repeat the first method failed; the latter procedure was followed to better advantage. Chloroacetyl chloride dissolved in ether was added to a cold ethereal solution of 2-aminofluorene. Not only could the by-product hydrogen chloride be neutralized by the presence of an equimolar excess of 2-aminofluorene, but by pyridine as well. Both the yield (84%) and the melting point (190.5°) for 2-chloroacetamidofluorene were higher than those given in the literature;

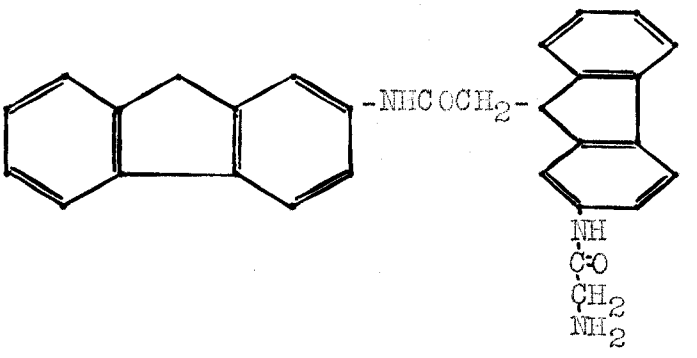
Feldman reported 73.5%; m.p. 183-185°.

Numerous attempts were made to aminate 2-chloroacetamidofluorene. Trials with sodamide or liquid ammonia failed to produce any product, and only starting material was recovered. Refluxing the chloro compound in concentrated ammonium hydroxide led to the formation of a small quantity of secondary amine, namely α-amino-bis(2-acetamidofluorene) (VI). Although



VI

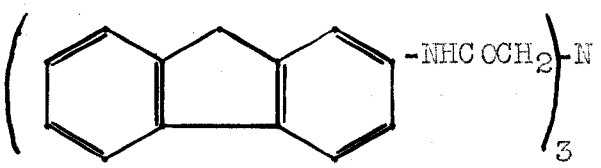
the analyses for carbon, hydrogen and nitrogen agreed with the calculated results, another compound (VII) having the same molecular formula was theoretically possible from the amination reaction which was carried out in a large excess of ammonia. Qualitative tests were made in order to eliminate



VII

VII. The carbylamine test for a primary aliphatic amine proved to be negative, although a positive test was obtained for a secondary amine when nitrosation yielded a yellow-brown

oil. Larger quantities of VI were formed from pressure reactions at 100° using chloroacetamidofluorene and ammoniated ethanol. This was performed in small runs in glass pressure bottles or in larger amounts employing an Aminco High Pressure Hydrogenator sealed with plugs after removal of the hydrogen lines. Although this reaction proved interesting from the standpoint of providing a substituted 2-aminofluorene containing two peptide links (but not adjacent), it was unsatisfactory for the formation of a peptide chain containing successive amino acid moieties. For this reason no attempt was made to identify the product obtained from 2-chloroacetamidofluorene and ammonia in a pressure apparatus with cuprous oxide as a catalyst. Smaller quantities of this same material were separated from the α-amino-bis(2-acetamidofluorene) previously described. Preliminary investigation of the solubility and melting point (about 300°) indicated that it probably was the tertiary amine VIII.



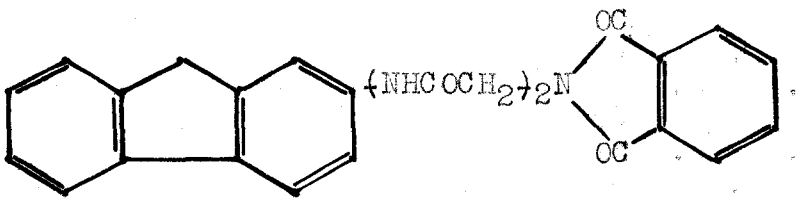
VIII

B. 2-Diglycylaminofluorene

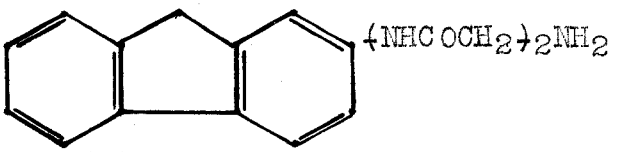
1. From Phthalylglycine

The 2-glycylaminofluorene was utilized in a second modified Schotten-Baumann reaction with phthalylglycyl chloride. The procedure for the synthesis of 2-phthalyldiglycylaminofluorene

(IX) with its subsequent conversion to 2-diglycylaminofluorene (X) was analogous to that for the glycyl compounds. Two



IX



X

significant differences were noted with this pair of compounds. The increased length of the polar sidechain served to overcome to a slight extent the inherent insolubility of the fluorene nucleus. For the same reason, namely increased chain length, the primary amine group of 2-diglycylaminofluorene became less stable toward air oxidation. To obtain a sample for analysis the 2-diglycylaminofluorene was recrystallized from chlorobenzene in an atmosphere of nitrogen.

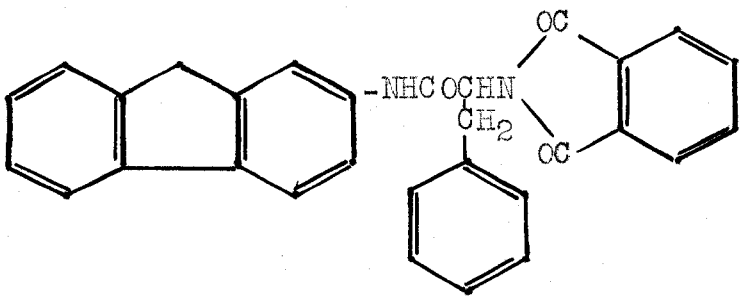
C. 2-Phenylalanyl- and 2-Diphenylalanylaminofluorene

1. From Phthalylphenylalanine

In an effort to procure some data regarding the effect of an essential amino acid in place of glycine, another series of substituted aminofluorenes was prepared in which phenylalanine was incorporated into the peptide linkage.

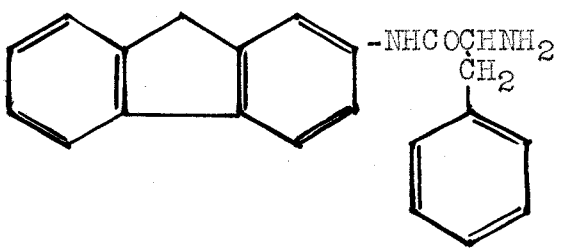
Phthalylphenylalanine was formed in a manner essentially

the same as that appearing in the literature (38). Although this reference included a method for the conversion to the acyl halide using phosphorus pentachloride, in view of the previous considerations for phthalylglycyl chloride, it was decided to carry out the reaction using thionyl chloride. The coupling of phthalylphenylalanyl chloride with 2-aminofluorene proceeded in a satisfactory manner, as did the hydrazinolysis of 2-phthalylphenylalanylaminofluorene (XI).



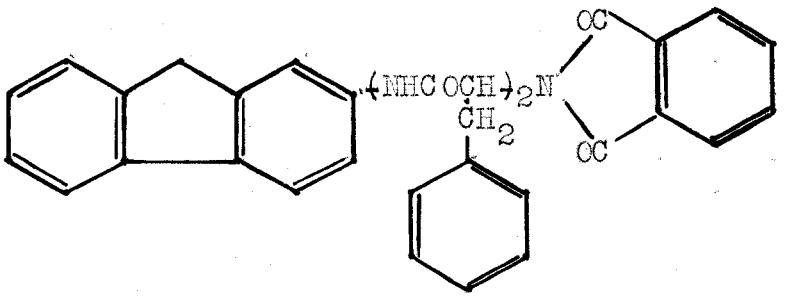
XI

The free amine isolated, 2-phenylalanylaminofluorene (XII),

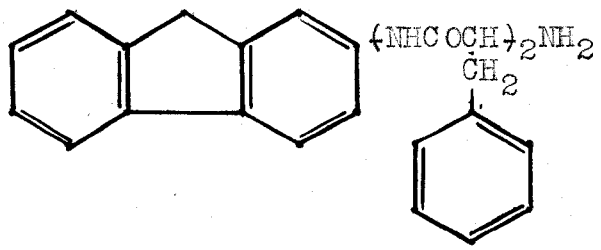


XII

was coupled again with phthalylphenylalanyl chloride to yield 2-phthalyldiphenylalanylaminofluorene (XIII). Hydrazinolysis of this compound led to the formation of 2-diphenylalanylaminofluorene (XIV). As in the case of the glycyl derivatives an increase in the length of the side chain increased the solubility



XIII



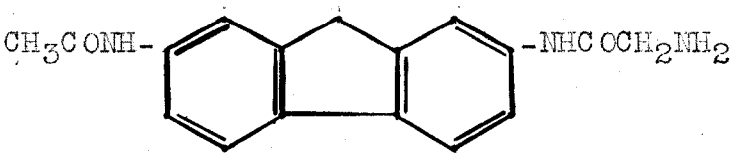
XIV

in polar solvents. However, no abrupt change in the stability toward air oxidation was noticeable between the two peptides of phenylalanine. Both phthalyl derivatives and both free amines had lower melting points than the corresponding glycine analogues.

D. Monohydrated Dimer of 2-Glycylamino-7-acetamidofluorene

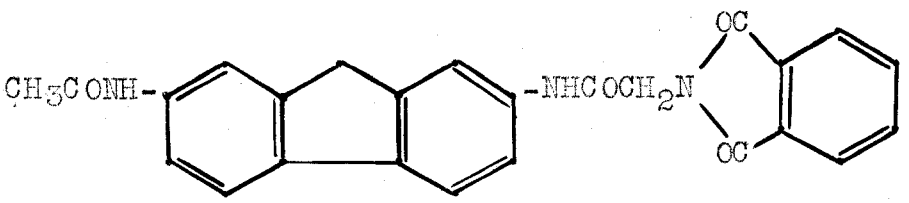
1. From Phthalylglycine

As a means of further correlating 2-glycylaminofluorene and 2-acetamidofluorene, an attempt was made to synthesize 2-glycylamino-7-acetamidofluorene (XV). Instead of using 2-aminofluorene as one of the reactants in the modified Schotten-Baumann reaction, 2-amino-7-acetamidofluorene was employed. This was prepared according to the procedure of



XV

Schulman (40), starting with fluorene. Phthalylglycyl chloride reacted with the 2,7-disubstituted fluorene to yield 2-phthalylglycylamino-7-acetamidofluorene (XVI).

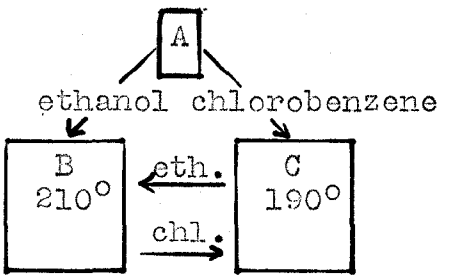


XVI

Hydrazinolysis of the latter followed by the customary treatment with cold 30% aqueous sodium hydroxide solution was expected to give the desired compound (XV). The fact that the initial carbon, hydrogen and nitrogen analyses failed to agree with the theoretical values promoted an intensive study of the material. A series of postulates were proposed which applied not only to this compound (XV) but to the other peptides as well.

The first analyses showed that the sample could be the monohydrated dimer of 2-glycylamino-7-acetamidofluorene. It was then discovered that repeated recrystallizations gave melting points which were not constant but which varied over a range of several degrees. The material did retain a definite high or low range, however, depending upon the particular

solvent used. For example, the sample which was thought to be 2-glycylamino-7-acetamidofluorene had a melting point of approximately 190° when recrystallized from chlorobenzene, but 210° when recrystallized from ethanol. Additional analyses showed that for the lower melting material the results, although still in agreement with the theoretical values for a monohydrated dimer, were closer to the theoretical calculations for an unhydrated monomer. The analytical evidence for dimerization was supported in part by a study of the melting points since it was expected that the dimer would have a melting point somewhat higher than that of the monomer. A sample of the original material before recrystallization (A) was divided into two parts; one part was recrystallized from chlorobenzene, the other, from ethanol. It was found that recrystallization from ethanol gave a product (B) melting higher than the product (C) obtained from chlorobenzene recrystallization. Furthermore, when the lower melting product (C) was subsequently recrystallized once or twice from ethanol, the melting point of the new material was in sharp agreement with that of the sample (B) obtained from ethanol recrystallization. Conversely, when sample B was subsequently recrystallized once or twice from chlorobenzene, the melting point now dropped to that found for the sample (C) obtained from the chlorobenzene recrystallization. It might also be mentioned that the



original material (A) separated from phthalhydrazide by the sodium hydroxide treatment had a melting point less than B or C. This lower value could have been caused by impurities, but it also suggested that the sample consisted of even less dimer than the products obtained after recrystallization from ethanol or chlorobenzene.

To obtain further evidence to corroborate the unexpected phenomenon a molecular weight study was made on the sample having the higher melting point and suspected of being a monohydrated dimer. Using chlorobenzene as the ebullioscopic solvent an experimental value of 578 was obtained; theoretically the molecular weight should have been 608. By employing the same sample in acetone the experimental molecular weight was 387 as compared to the theoretical 295 for the unhydrated monomer. This was entirely in agreement with expectations since the more polar solvent, acetone, would tend to dissociate the dimer more than chlorobenzene.

The sharp distinction provided by the molecular weight evidence arose from the fact that the two solvents used had a wide variation in dipole moments. The differences in the recrystallization data were slight, but the evidence was the

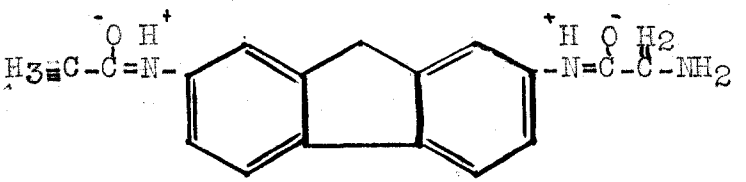
same. Chlorobenzene was just slightly more polar than ethanol, so the melting point and analyses on the compound from this solvent were only slightly in favor of the monomer. A consideration of the theoretical carbon values for the monohydrated dimer and the unhydrated monomer will help to emphasize this fact. The former is 67.09% as compared to 69.13% for the monomer. Even though the difference is approximately 2%, the experimental difference between the carbon values for the high and low melting materials was only about 0.2%. This would still be sufficient to account for the change in melting points.

If the conversion to the dimer in ethanol were incomplete, it might be expected that some separation could be made due to the variations in solubility, the dimer being less soluble than the monomer. Evidence was obtained to substantiate this when the filtrates remaining from the ethanol recrystallizations were evaporated. The recovered samples had melting points in agreement with that of the product which was obtained from chlorobenzene recrystallization and which was thought to contain a larger amount of monomer.

In summary, what is being asserted is not that 2-glycylamino-7-acetamidofluorene has been isolated as a pure monomer and a pure monohydrated dimer. What is being asserted is that 2-glycylamino-7-acetamidofluorene can exist either as a monomer or a monohydrated dimer, but depending upon the polar nature of the solvents used, for example for molecular weight

determinations or for recrystallizations, the relative amounts of the two in a mixture can be varied. However, it would appear from analytical data that the material obtained through the alcohol recrystallization could very well be the pure monohydrated dimer. On the other hand it seems quite probable that the pure monomer would be more difficult to obtain, since even the molecular weight data from the strongly polar acetone failed to show the complete dissociation of the dimer.

Hydrogen bonding is well recognized in such fields as proteins, peptides and polymers and often affects the properties of such materials. In protein chemistry hydrogen bonding is considered to exist between peptide linkages on adjacent chains. More specifically it is described as an interaction between the carbonyl group of one peptide link and the amide portion of another. This interaction may occur directly between peptide chains or indirectly through a third component such as water. In addition, it is known that the carbon to nitrogen bond of a peptide linkage possesses a certain amount of double bond character and that it is planar. If 2-glycylamino-7-acetamidofluorene is analyzed carefully, it will be seen that it contains two peptide linkages which may be written with double bonds between each carbon and nitrogen to represent the olefinic character. Furthermore, the hyperconjugative forms may be included in the diagram (XVII). It can be seen that a conjugated system extends through almost



XVII

the entire structure. Such a system might be so stabilized that hydrogen bonding sufficient to cause dimerization could easily occur. In fact, if true, this mechanism would serve to offer additional proof for the planarity of the fluorene nucleus.

E. Additional Conclusions

Some consideration must now be given to the other peptides prepared during this project. Two of these, namely 2-glycylaminofluorene (II) and 2-diphenylalanylaminofluorene (XIV) were recrystallized several times from ethanol and were found to exhibit the same phenomenon, i.e. an increase in melting point, but to a lesser degree. This would be expected since the nucleus carries only one peptide side chain thereby providing a shorter conjugated system, making dimerization less probable.

III. EXPERIMENTAL

All melting points were taken with a calibrated 76 mm. immersion thermometer at a depth of 76 mm. in a 2.5" x 3.5" cylindrical iron block.

A. 2-Nitrofluorene (40)

A four liter beaker was immersed in a deep water bath on a hotplate, and drainage tubes were arranged so that hot water could be withdrawn from the bath as cold water was introduced, thus permitting a more efficient control of temperature. The entire assembly was located in a hood to insure removal of the oxides of nitrogen. A mixture of technical fluorene (240 grams, 1.44 moles) and two liters of 99% acetic acid was agitated with a heavy efficient stirrer at a temperature of 60-65° until the solid dissolved. From a dropping funnel nitric acid (320 ml., 5.1 moles, sp. gr. 1.42) was added in a slow stream, and as the reaction suddenly commenced with evolution of heat, the temperature was regulated by means of the water bath so as not to exceed 80°. When the product precipitated, stirring became extremely difficult. After thirty minutes the bath was removed and the temperature was allowed to drop to 60°, at which time the solid was filtered, pressed dry and washed with three 100 ml. portions of 99% acetic acid, the first two each containing two grams of potassium acetate. The 2-nitrofluorene was suspended in three

liters of water, stirred mechanically, filtered and the process was repeated. The dried product from three runs averaged 232 grams (76%); a fourth trial with 1.5 times the above amounts yielded 333 grams (72%) after recrystallization from acetone; m.p. 156°.

B. 2-Aminofluorene

1. Zinc Reduction (41)

A series of two liter, three-necked round bottom flasks each equipped with a condenser and a mechanical stirrer were arranged in water baths as described above. The reduction was carried out using recrystallized 2-nitrofluorene (36 grams, 0.17 mole), 60 grams of zinc dust, 15 grams of calcium chloride in 20 ml. of water, 15 ml. of 99% acetic acid, 920 ml. of methanol and 180 ml. of water. The nitrofluorene and zinc were mixed intimately prior to addition. The stirred mixture was heated until the reaction commenced, at which time extreme care was used to control the temperature with the water bath. Three additional 10 gram portions of zinc were added periodically during the next four hours while refluxing was continued. The gray solution was filtered while hot through Super-Cel; the residue was refluxed in 100 ml. of methanol and refiltered. The combined filtrates were poured into two liters of water; the amine was filtered, washed with water containing a trace of sodium bisulfite, pressed and dried. The average yield of 2-aminofluorene from twelve runs of the

above was 23.6 grams (76.5%); m.p. 126.5-127°. The average from three small runs using 2/3, 2/3 and 4/9 the given amounts was 93%. In addition, four trials resulted in incomplete reduction (solutions became yellow but not gray) and only small amounts of product were obtained.

2. Hydrogenation

The one liter glass liner of an Aminco High Pressure Hydrogenator equipped with gas inlet tubes was charged with crude 2-nitrofluorene (42.4 grams, 0.2 mole), 0.5 grams of Adams' Catalyst (42) and 900 ml. of 95% ethanol. After flushing the apparatus twice with hydrogen, the reduction was performed at 80° and 100 p.s.i. When the pressure remained constant (within twenty minutes), heating was discontinued and the material was removed after cooling. The solution was heated, filtered to remove the catalyst, and the filtrate was poured into two liters of water. After a second filtration the product was washed with water containing a trace of sodium bisulfite and dried to yield 33.9 grams (93.5%); m.p. 126.5-127°. A similar run using 2.6 times the above amounts of reactants (with the same volume of solvent) gave a 92.2% yield of 2-aminofluorene.

C. 2-Chloroacetamidofluorene

This experiment was conducted according to the procedure of Feldman (43). A one liter, three-necked round bottom flask was fitted with a rubber-sealed stirrer, dropping funnel

and calcium chloride filled drying tube. Into the flask was introduced 2-aminofluorene (23.5 grams, 0.13 mole) and 500 ml. of dry diethyl ether. After cooling the ether solution chloroacetyl chloride (7.5 grams, 0.066 mole) dissolved in 250 ml. of dry diethyl ether was added dropwise in about thirty minutes. The mixture was filtered and the solid residue was washed with ether, dried, washed with water and redried. The material was extracted with boiling toluene several times (total about one liter) and the latter was then vacuum distilled to recover the slightly tan crystalline 2-chloroacetamidofluorene which weighed 14.4 grams (84%). The material was recrystallized from glacial acetic acid; m.p. 190.5°.

D. α-Amino-bis(2-acetamidofluorene)

Approximately 20% ammoniacal ethanol solutions were prepared by bubbling gaseous ammonia through ethanol in a series of bubblers. Such a solution (330 grams) containing ammonia (17 grams, 1 mole) and 2-chloroacetamidofluorene (5 grams, 0.019 mole) was placed in the one liter glass liner of an Aminco High Pressure Hydrogenator equipped with plugs instead of gas tubes. The solution could also be divided equally among five, 250 ml. glass pressure bottles (caution). After being heated for four hours at 100°, the solution was cooled and vacuum distilled to remove the solvent. The white α-amino-bis(2-acetamidofluorene) was stirred in water, filtered and dried (5.3 grams, 60.5%); m.p. 278.5-279.5°

after recrystallization from dimethylformamide.

Anal. calc'd. for $C_{30}H_{25}O_2N_3$: C, 78.41; H, 5.48; N, 9.15.

Found: C, 78.69; H, 5.60; N, 9.29.

E. Phthalylglycine (38)

A 500 ml., two-necked round bottom flask provided with a stirrer and powder funnel was immersed in an oil bath heated to 190-200°. Phthalic anhydride (88.8 grams, 0.6 mole) was introduced and a mobile melt was formed. To the melt glycine (38 grams, 0.5 mole) was added in small portions with stirring in about twenty minutes. After maintaining this temperature for an additional twenty minutes, the evolution of water ceased and solidification occurred. The material was removed from the flask and extracted repeatedly with two liters of boiling water. The combined portions of phthalylglycine obtained by cooling the filtrate after each extraction totaled 93.6 grams (91.5%); m.p. 194-198°. From a similar preparation using 0.5 times the above amounts the yield was 98%.

F. Phthalylglycyl Chloride

1. Using Phosphorus Pentachloride (38)

A 500 ml., three-necked round bottom flask equipped with a thermometer, rubber-sealed stirrer and dropping funnel was immersed in a water bath. Dry benzene (100 ml.), phthalylglycine (10.5 grams, 0.05 mole) and phosphorus pentachloride (10.5 grams, 0.05 mole) were introduced and the mixture was heated to 60° with stirring. If complete solution did not occur within thirty minutes, additional phosphorus pentachloride

was added until solution was complete. Heating was continued for another 1.5 hours before the solvent was removed by vacuum distillation. The phthalylglycyl chloride was recrystallized from benzene-ligroin (40-60°) to yield 10.7 grams (95.5%); m.p. 84-85°.

2. Using Thionyl Chloride (44) (cf. F-1 for apparatus)

Phthalylglycine (20 grams, 0.1 mole) and thionyl chloride (35 ml., 0.47 mole) were refluxed about two hours. Ligroin (40-60°) was added to precipitate the phthalylglycyl chloride which was then recrystallized from benzene-ligroin to give 21.3 grams (95.1%; an average from five runs).

G. 2-Phthalylglycylaminofluorene

A one liter, three-necked round bottom flask provided with a dropping funnel, rubber-sealed stirrer and calcium chloride filled drying tube was placed into an ice bath (5°). Dry benzene (400 ml.), 2-aminofluorene (3.7 grams, 0.02 mole) and pyridine (1.6 grams, 0.06 mole) were introduced. Phthalylglycyl chloride (4.5 grams, 0.02 mole) dissolved in 80 ml. of dry benzene was added dropwise with stirring in about twenty minutes. After one hour the ice bath was removed and stirring was continued for an additional hour. The mixture was filtered and the solid product was washed successively with benzene, alcohol, water and alcohol. The dried 2-phthalylglycylaminofluorene weighed 7 grams (95%); m.p. 302-302.5° after recrystallization from dimethylformamide-ethanol.

H. 2-Glycylaminofluorene

A solution containing 2-phthalylglycylaminofluorene (3.5 grams, 0.0095 mole) and hydrazine hydrate (1 gram, 0.02 mole) in 500 ml. of 95% ethanol was refluxed until clear, usually 1.5-2 hours. The solvent was removed by vacuum distillation, and the solid, after being washed with a small quantity of water to remove the excess hydrazine, was filtered and dried. The phthalhydrazide-amine salt mixture (33 grams) was suspended in a cold solution of 225 grams of sodium hydroxide in 525 ml. of water and stirred vigorously for ten hours. The temperature was maintained at 10-15° with an ice bath. After diluting with water to four times the original volume, the suspension was filtered; the product was washed with water until all the sodium hydroxide was removed, and dried. The yield of 2-glycylaminofluorene, calculated from 2-phthalylglycylaminofluorene, was quantitative; m.p. 183-184° after recrystallization from chlorobenzene.

Anal. calc'd. for $C_{15}H_{14}ON_2$: C, 75.61; H, 5.92; N, 11.76.

Found: C, 75.61; H, 5.82; N, 11.70.

I. 2-Phthalyldiglycylaminofluorene (cf. G for apparatus)

The reaction was carried out using crude 2-glycylaminofluorene (17 grams, 0.072 mole) and pyridine (16.95 grams, 0.21 mole) in 500 ml. of dry benzene. To the cold (5°) stirred solution was added slowly phthalylglycyl chloride (15.93 grams, 0.072 mole) in 100 ml. of dry benzene. The

product was filtered and washed successively with benzene, alcohol, water and alcohol. The dried 2-phthalyldiglycyl-aminofluorene weighed 24.5 grams (80.5%); m.p. 301-302° after recrystallization from dimethylformamide-ethanol.

Anal. calc'd. for $C_{25}H_{19}O_4N_3$: C, 70.58; H, 4.50; N, 9.88.

Found: C, 70.46; H, 4.66; N, 9.72.

J. 2-Diglycylaminofluorene

A solution containing 2-phthalyldiglycylaminofluorene (0.77 grams, 0.002 mole) and hydrazine hydrate (0.1 grams, 0.002 mole) in 300 ml. of 95% ethanol was refluxed for 2.5 hours. After two successive 1.5 hour periods additional hydrazine hydrate (0.1 grams, 0.002 mole) in 50 ml. of ethanol was introduced. Within another hour a clear solution resulted and the solvent was removed by vacuum distillation. The salt mixture was washed with a small quantity of water, filtered and dried. It was then efficiently stirred in a cold solution of 35 grams of sodium hydroxide in 95 ml. of water for six hours at 10-15°. The suspension was diluted to four times the original volume and filtered; the filter cake was washed with water to remove the alkali and dried. The 2-diglycyl-aminofluorene weighed 0.46 grams (85% from 2-phthalyldiglycyl-aminofluorene); m.p. 187-188° after recrystallization from chlorobenzene under a nitrogen atmosphere.

Anal. calc'd. for $C_{17}H_{17}O_2N_3$: C, 69.13; H, 5.80; N, 14.23.

Found: C, 69.39; H, 5.84; N, 13.93.

K. Phthalylphenylalanine (38) (cf. E for apparatus)

The temperature of the oil bath was maintained between 170-210°; phthalic anhydride (44.4 grams, 0.3 mole) was introduced into the flask and melted. In small portions phenylalanine (41.3 grams, 0.25 mole) was added with stirring over a period of twenty minutes. After an additional thirty minutes with stirring the mixture was cooled, removed from the flask and extracted repeatedly with two liters of boiling water. The combined portions of phthalylphenylalanine obtained by cooling the filtrate after each extraction totaled 70.4 grams (95.5%); m.p. 175-180°.

L. Phthalylphenylalanyl Chloride (cf. F-1 for apparatus)

1. Using Thionyl Chloride

Phthalylphenylalanine (8.72 grams, 0.03 mole) and thionyl chloride (10 ml., 0.134 mole) were heated to reflux temperature. The material dissolved within thirty minutes and after another thirty minutes of heating ligroin (40-60°) (90 ml.) was added to precipitate the product. The phthalylphenylalanyl chloride was filtered, washed with ligroin and dried (9.07 grams, 98%); m.p. 123-125°.

M. 2-Phthalylphenylalanylaminofluorene (cf. G for apparatus)

To the cold (5°) solution containing 2-aminofluorene (5.06 grams, 0.028 mole) and pyridine (6.64 grams, 0.084 mole) in 420 ml. of dry benzene was added dropwise with stirring phthalylphenylalanyl chloride (9.07 grams, 0.029 mole) dissolved in 150 ml. of dry benzene. This required fifty

minutes and after two more hours, one hour without the ice bath, the solution was filtered. The solid was washed with small quantities of benzene and alcohol, then a large volume of water, followed again by a minimum amount of alcohol. The dried 2-phthalylphenylalanylaminofluorene weighed 10.6 grams (84%); m.p. 229.5-230° after recrystallization from dimethylformamide-ethanol.

Anal. calc'd. for $C_{30}H_{22}O_3N_2$: C, 78.58; H, 4.84; N, 6.11.

Found: C, 78.78; H, 4.81; N, 5.99.

N. 2-Phenylalanylaminofluorene

A solution containing 2-phthalylphenylalanylaminofluorene (1.29 grams, 0.003 mole) and hydrazine hydrate (0.14 grams, 0.003 mole) in 300 ml. of 95% ethanol was refluxed one hour, whereupon more hydrazine hydrate (0.14 grams, 0.003 mole) in 25 ml. of 95% ethanol was added. After refluxing another thirty minutes the solvent was removed by vacuum distillation; the solid was washed with a small quantity of water, filtered and dried. This was suspended in a cold solution of 32 grams of sodium hydroxide in 75 ml. of water and stirred for 8.5 hours at 10-15°. After diluting to four times the original volume with water the product was filtered, washed free of sodium hydroxide and dried. The yield of 2-phenylalanylaminofluorene was quantitative from the phthalyl derivative; m.p. 160-160.5° after recrystallization from alcohol.

Anal. calc'd. for $C_{22}H_{20}ON_2$: C, 80.46; H, 6.14; N, 8.53.
Found: C, 80.50; H, 6.26; N, 8.49.

O. 2-Phthalyldiphenylalanylaminofluorene (cf. G for apparatus)

Into a cold solution of 75 ml. of dry benzene containing 2-phenylalanylaminofluorene (1.34 grams, 0.004 mole) and pyridine (0.97 grams, 0.012 mole) was added dropwise a solution of phthalylphenylalanyl chloride (1.28 grams, 0.004 mole) in 60 ml. of dry benzene. Stirring was continued for 2.5 hours, the last hour without an ice bath. After filtering, the precipitate was washed successively with small amounts of benzene and alcohol, then a large quantity of water followed by a small portion of alcohol. The dried 2-phthalyldiphenylalanylaminofluorene weighed 1.14 grams (46%); m.p. 234.5-235.5° after recrystallization from alcohol.

Anal. calc'd. for $C_{39}H_{31}O_4N_3$: C, 77.34; H, 5.16; N, 6.94.
Found: C, 77.59; H, 5.06; N, 6.69.

P. 2-Diphenylalanylaminofluorene

A solution containing 2-phthalyldiphenylalanylaminofluorene (0.85 grams, 0.0014 mole) and hydrazine hydrate (0.14 grams, 0.0028 mole) in 200 ml. of 95% ethanol was refluxed for three hours; more hydrazine hydrate (0.14 grams, 0.0028 mole) in 25 ml. of 95% ethanol was added. This addition was repeated three hours later; following two more hours of refluxing the solvent was removed by vacuum distillation. The solid was stirred in 100 ml. of cold 28% sodium hydroxide for 7.5 hours, the ice

bath being removed for the final three hours. After diluting to four times the original volume with water, the 2-diphenylalanylaminofluorene was filtered, washed free of alkali and dried to give a quantitative yield from the phthalyl compound; m.p. 185.5-186° after recrystallization from ethanol.

Anal. calc'd. for $C_{31}H_{29}O_2N_3$: C, 78.29; H, 6.15; N, 8.84.

Found: C, 78.55; H, 5.96; N, 8.61.

Q. 2-Amino-7-acetamidofluorene

This was prepared according to the procedure of Schulman (40) by the following series of reactions. Dinitration of fluorene produced the 2,7-compound which was reduced to the diamine and converted to the dihydrochloride. After monoacetylation and neutralization of the hydrochloride the desired 2-amino-7-acetamidofluorene was obtained.

R. 2-Phthalylglycylamino-7-acetamidofluorene (cf. C for apparatus)

A suspension of 2-amino-7-acetamidofluorene (6.6 grams, 0.03 mole) in 300 ml. of dry benzene containing pyridine (6.6 grams, 0.08 mole) was cooled to 5°. A solution of phthalylglycyl chloride (6.2 grams, 0.03 mole) in 200 ml. of dry benzene was added dropwise with stirring in thirty minutes. After stirring an additional hour the mixture was filtered; the cake was washed successively with benzene, alcohol, water and alcohol. The dried 2-phthalylglycylamino-7-acetamidofluorene weighed 8.3 grams (70.5%); m.p. 344.5-345.5° after recrystallization from dimethylformamide-ethanol.

Anal. calc'd. for $C_{25}H_{19}O_4N_3$: C, 70.58; H, 4.50; N, 9.88.

Found: C, 70.38; H, 4.38; N, 9.60.

S. Monohydrated Dimer of 2-Glycylamino-7-acetamidofluorene

A solution containing 2-phthalylglycyl-7-acetamidofluorene (2.92 grams, 0.007 mole) and hydrazine hydrate (0.34 grams, 0.007 mole) in 600 ml. of 95% ethanol was refluxed. After 2.25 hours a similar quantity of hydrazine hydrate in 50 ml. of 95% ethanol was added; this was repeated twice more within the next 7.75 hours. Refluxing was continued another two hours and the clear solution was vacuum distilled to remove the solvent. The solid weighing 3.15 grams was stirred efficiently for 7.75 hours at a temperature which rose from 5° to room temperature during that time. After dilution to four times the original volume with water, the suspension was filtered and the solid residue was washed to remove the alkali. The dried monohydrated dimer of 2-glycylamino-7-acetamidofluorene was obtained in quantitative yield; m.p. 189-192°, from chlorobenzene; 210-211°, from ethanol.

Anal. calc'd. for

$(C_{17}H_{17}O_2N_3)_2 \cdot H_2O$: C, 67.09; H, 5.96; N, 13.81.

Found: m.p. 189-192°: C, 67.48; H, 5,83; N, 13.74.

C, 67.46; H, 5.86; N, 13.45.

C, 67.46; H, 5.71; N, 13.68.

m.p. 210-211°: C, 67.29; H, 6.25; N, 13.59.

C, 67.15; H, 6.06; N, - .

IV. SUMMARY AND CONCLUSIONS

The purpose of this research, as the name implies, has been to synthesize some peptide derivatives of the carcinogen 2-aminofluorene. That this has been accomplished is evident from the following list of compounds which are being reported for the first time.

2-glycylaminofluorene

2-phthalyldiglycylaminofluorene

2-diglycylaminofluorene

2-phthalylphenylalanylaminofluorene

2-phenylalanylaminofluorene

2-phthalyldiphenylalanylaminofluorene

2-diphenylalanylaminofluorene

α-amino-bis(2-acetamidofluorene)

2-phthalylglycylamino-7-acetamidofluorene

monohydrated dimer of 2-glycylamino-7-acetamidofluorene

In the preparation of the above, new methods were devised in order to circumvent some inherent difficulties, the principal one being the insolubility of either the products or the reactants.

Coupling of phthalyl protected amino acid acyl halides to aminofluorene derivatives was carried out in an anhydrous benzene solution in the presence of an organic base. Although Yingst (unpublished U.C. thesis) reported 2-phthalylglycyl-aminofluorene in 81% yield, no results greater than 25% could

be obtained. His yield was calculated from the total weight of material recovered from the reaction mixture. However, he did mention that the yield of purified 2-phthalylglycyl-aminofluorene was 70% based upon the amount of 2-aminofluorene used. This conformed with the 25% yield reported in this work since most of the 2-aminofluorene was recovered from the mixture after completion of the reaction. The new process described here provided a 95% yield of 2-phthalylglycylamino-fluorene melting seven degrees higher than Yingst's product.

A special study was made on the hydrazinolysis of the phthalyl group to determine the optimum ratio of reactants to each other and to the solvent. A means of separating the desired amine from the phthalhydrazide formed in the reaction was also perfected. This involved the use of a cold, vigorously stirred 30% aqueous solution of sodium hydroxide in which the peptide linkage was unreactive.

The 2-glycylaminofluorene reported by Yingst (unpublished U.C. thesis), was actually the hydrochloride which evidently could not be converted to the free amine. The development of the sodium hydroxide prodedure also eliminated this difficulty.

Evidence has been presented using data from analyses, molecular weight determinations, solubilities, melting points and dipole moments that the compounds prepared in this work are capable of forming dimers. A mechanism has been given to explain this phenomenon and to account for the fact that

2-glycylamino-7-acetamidofluorene has a greater tendency to exist as a dimer than the other peptide derivatives described.

A new method was reported for the preparation of 2-aminofluorene by the catalytic hydrogenation of 2-nitrofluorene. A new procedure was also described for the reduction of 2-nitrofluorene with powdered zinc. Both the yield and the melting point for 2-chloroacetamidofluorene were higher than those given in the literature. Improved procedures and/or yields were reported for phthalylglycine, phthalylglycyl chloride, phthalylphenylalanine and phthalylphenylalanyl chloride. Of the twenty reactions described in detail, twelve were completed with greater than 90% yields; an additional four, greater than 80%.

No results have yet been received regarding the carcinogenicity of any of the new compounds because of the length of time required for conducting such tests. * If the biological tests prove satisfactory, it is suggested that higher peptides of the most promising samples should then be prepared.

The unexpected phenomenon of dimerization of the peptide derivatives of 2-aminofluorene in itself would provide an interesting problem for investigation. From such a study the amount of monomer or dimer could be predicted from the conditions employed. Further work could also be done regarding

* Biological testing is being carried out by Dr. F. E. Ray, Director, Cancer Institute, University of Florida, Gainesville, Florida.

the role of water in the dimer formation.

Already reported in the literature (3) was N-(2-fluorenyl)-glycine in which the 2-position of fluorene was joined to the nitrogen of glycine, thereby providing a free carboxylic acid group. A series of peptides could be prepared from this in which the terminal group of the peptide side chain would be a carboxylic acid rather than a free amine.

Instead of a substituted fluorene, a substituted fluorenone could be utilized for a similar type of problem. Finally, the oxygen analogue of 2-aminofluorene, namely 2-hydroxyfluorene, or an aminohydroxyfluorene could provide another group of fluorene compounds which have never been tested for carcinogenic activity.

ADDENDUM

Following the completion of this thesis an attempt was made to determine the nature of the water present in the monohydrated dimer of 2-glycylamino-7-acetamidofluorene. In as much as the samples were dried to constant weight at 80° under vacuum before analysis, this drying procedure was duplicated. A quantity of the dried material was weighed and then heated at 150° under vacuum. Within two hours 1.27% of the sample was lost but decomposition became apparent. The solid turned brown as the loss in weight increased sharply and steadily and greatly exceeded the calculated loss of 2.96%.

The method was repeated on another portion of material. After drying to constant weight at 80° under vacuum, further heating was continued at 120° under vacuum. Within fourteen hours the weight lost rose slowly and leveled off at 3%, whereupon slight decomposition became noticeable. Another five hours of heating resulted in additional decomposition as the loss increased to 5.22%. The melting point of the sample used for the test was 205-206° before drying but 194-195° at the completion of heating. Because of the decomposition the recovered sample was not analyzed. Hence, drying of the monohydrated dimer of 2-glycylamino-7-acetamidofluorene seemed to indicate that removal of the water was possible, that the stability of the monohydrated dimer depended upon the presence of the water, and that removal of the water led to the decomposition of the sample.

V. SUPPLEMENT

Cancer is one of the oldest diseases known to the animal and plant kingdom. In the United States it has risen to second in the causes of death, and is found to be most frequent among people above forty years of age. To a certain extent this has been brought about by the great advances made in medical science, with the ultimate result that the average age is increasing and cancer is becoming more conspicuous. This does not mean that cancer is a completely unconquered malady. The percentages of survival among the various types of the disease have increased markedly with both a prolongation of life and even complete cures being exhibited in cases of early diagnosis (48).

Cancer was recognized as a disease even in ancient times (1500 B.C.) (49). For many centuries its treatment lay within the realm of the physician and the surgeon. Although voluminous data and theories were compiled during that time, all seemed rather fruitless in the development of a cure for the elusive disease.

A. Carcinogenesis

1. Tumors

Before proceeding further it would be advantageous to define some terms which are commonly encountered in discussions of this nature. The fundamental basis of cancer is a tumor or neoplasm, "an abnormal mass of tissue, the growth of which

exceeds and is uncoordinated with that of normal tissues, and persists in the same excessive manner after cessation of the stimuli which evoked the change" (50). A tumor arises from the cells of the host by an unknown transformation or etiology. Even though its formation remains a mystery, it seems to be very closely related to the fundamental origin of life itself, since a tumor results from an ordinary cell which has undergone a neoplastic change. Similar examples have been noted in plant life. Hence, the answer to the cancer problem may be forthcoming when the basic requirements of cell production are realized. As time progresses tumors become anaplastic, i.e. more unlike the host, since they are autonomous, uncoordinated and capricious, but they may become more like other tumors. The atypical growth may be rapid, at the expense of neighboring tissues by the process of infiltration, or it may remain dormant for indefinite periods. Even though cancerous tumors grow more rapidly than normal cells in regenerative equilibrium, they do not exceed the growth rate of normal cells which are being replaced after an injury.

Whereas normal cells are not supported in growth when transplanted to another site, tumor cells thrive under these circumstances. If the transfer takes place through the circulatory system, it is known as metastasis.

There are two classes of tumors, the first of which is the cancerous type described above; it is called malignant.

A benign or innocent tumor has typical growth; the cells retain the characteristics of the host and remain, for the most part, under the control of the organism. Its excessive growth may be temporary with the tissue eventually returning to its original size or it may achieve a static condition and retain its enlarged volume. No harm results except where the growth impinges upon some vital or sensitive center due to the abnormal expansion within a limited area. There is some conjecture that malignancies arise from benign tumors, but this has not been proven conclusively.

Tumors may also be distinguished according to the host from which they originate. If they arise from skin or epithelial tissue and are benign, they are called papillomas; if malignant, carcinomas or epitheliomas. Sarcomas refer to malignant growths from connective tissue, bone, cartilage, muscle or fat. An adenoma is a glandular tumor (51). Since the benign neoplasm is of little importance in this paper, hereafter the term "tumor" will be used to refer to the malignant cancerous variety, unless further designated as benign.

2. Oncology

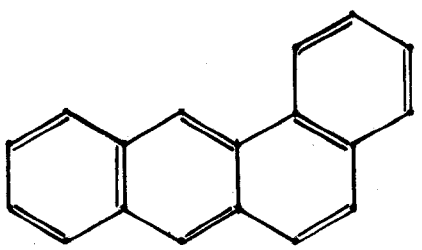
Cancer research, or oncology, did not make definite advances as an experimental science until the close of the last century. A study of animal tumors showed that transplantation into new hosts was possible. In addition it

was discovered that there was a similarity between animal tumors and those found in man, thereby providing the researcher with a greater freedom of investigation (52).

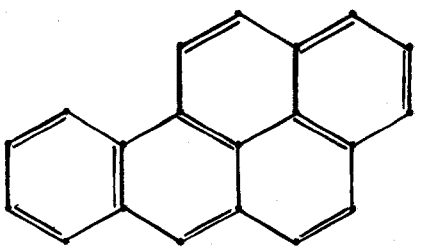
3. Induction of Cancer

Despite the fact that chemicals were recognized as being responsible for occupational cancer as early as 1775, it was not until 1915 that Yamagiwa and Ichikawa succeeded in producing artificial cancer of the skin by painting the ears of rabbits with coal tar (53). The extreme importance of this finding is readily apparent if it is realized that prior to this, only spontaneously generated tumors could be examined. Fixation of controlled conditions was difficult until intensive breeding of test animals was utilized for promoting the occurrence of cancer at a definite site; but even then the host frequently perished before much evidence could be gathered. Later, Bloch and Dreifuss concluded that the substances responsible were probably polycyclic hydrocarbon constituents of the tar, a discovery which marked the entry of organic chemistry and led to the study of both natural and synthetic carcinogens (54). The above hypothesis concerning polycyclic hydrocarbons was given further proof when it was noted that all carcinogenic tars were fluorescent in ultraviolet light and possessed similar fluorescent spectra having major bands at 4000, 4180 and 4400 Å. By comparing the spectra of tars with known polycyclic

hydrocarbons it was determined that a substituted 1,2-benzanthracene (I) would be carcinogenic (55). Finally, 3,4-benzopyrene (II) * was crystallized from coal tar as the pure hydrocarbon and was proven to be strongly carcinogenic; although other fractions of coal tar were later found to produce cancer, this was the major constituent which accounted for the carcinogenicity and fluorescence spectra of the tars and oils (56).

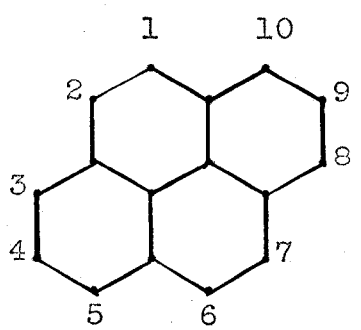


I 1,2-benzanthracene

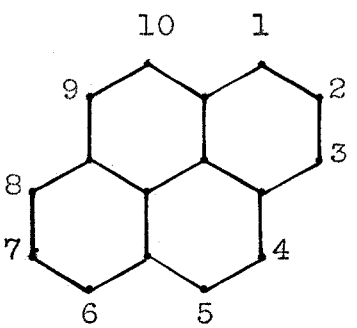


II 3,4-benzopyrene

* Most of the articles in the literature refer to 3,4-benzopyrene and other pyrene derivatives according to the pyrene numbering system III below. Since 1937 Chemical Abstracts has adopted system IV for ordinary derivatives and the alphabetical small letter system to designate benzo-derivatives; to avoid complete confusion the numbering in this paper will correspond to the Chemical Abstracts system used prior to 1937.



III



IV

Coal tar was not the only method discovered for the non-spontaneous production of cancer. Radioactive radiations, ultraviolet, x-rays, physical injuries including severe burns as well as chronic inflammations and chemical irritants (distinguished from chemical carcinogens) have been found effective. These agents are somewhat anomalous since cancer seems to involve a tumor-host relationship. Just a few of the contradictions may be cited: radioactive radiations and x-rays are also being used for the treatment of tumors; so are such chemical compounds as sex hormones, folic acid antagonists and nitrogen mustards, yet these, too can be carcinogenic. Certainly not all physical injuries, burns and inflammations lead to malignancies; some do. The same is true for chemical irritants. However, for some of the above it has been found that a pre-cancerous disposition must prevail, suggesting a heredity factor.

By means of test animals it has been found possible to breed strains which are especially susceptible or resistant to carcinogenesis. It has been noted that tumor growth may not be supported if transplanted from one species or strain to another even though the graft does take for a short period, although an external transplanation usually will succeed within the same strain. The latter procedure along with metastasis or internal transplantation serves as a criterion for malignancy (57).

For an external transplantation into the new host a subcutaneous or intramuscular injection of intact cells is required. Damaged cells, extracts or cell filtrates are ineffective. Even here, anomalies became evident when certain forms of sarcomas were able to be transmitted using cell free extracts of the tumor (58). This served as an introduction for viruses into the field of oncology.

By this time it should be apparent that induction of tumors may be brought about by physical, chemical or biological agents. These may be classified, without regard to spontaneity, as extrinsic if the agents are foreign to the host, or intrinsic if they are produced by the host or some other living organism. The distinction is not as precise as the definition implies. In practice it seems quite possible that since some of the extrinsic chemical agents have structures quite similar to those of various intrinsic agents, the former may appear carcinogenic only because the organism can transform them into intrinsic agents. Conversely the reverse process could also hold true.

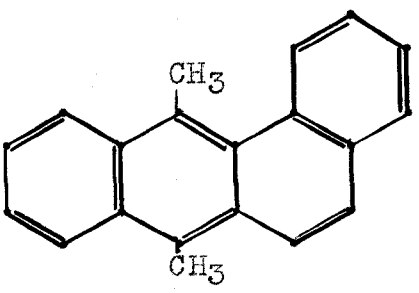
The principal problem of chemical induction of cancer involves a search by purely chemical methods for the changes that can be brought about by the introduction of known chemical structures, and from which might be achieved some realization of the balance of forces and the properties controlling the normal cell growth which is disturbed by

the carcinogen.

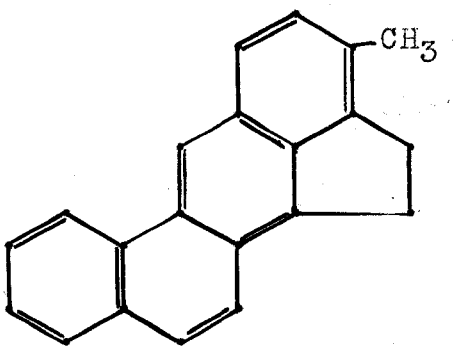
In 1951 there were listed 1329 chemical substances which had been tested as neoplastic agents (59). These tests were conducted in order to obtain some insight into the type of structure inherent in the carcinogens, and from which some generalizations could be derived regarding their mode of action. In the light of difficulties previously mentioned it should not be considered unreasonable for the agents to differ among families, genera or species, or to produce different kinds of tumors, even within the same strain. The activity was found to depend on a multitude of conditions such as dosage, media, length of administration, strain, species, sex, age, site of application, and even the amount and kind of nutrition associated with the test animal. Hence, when a substance has been termed carcinogenic, the conditions should also be defined in order to convey the maximum significance. That this could be done satisfactorily was proven when the two main schools of cancer research, the London group of Cook, Kennaway, Hieger, et al operating from the 1920's to the 1940's, and the Boston group of Fieser, Shear, Andervont, et al operating from the 1930's to the 1950's, were able to corroborate in almost all details, but only when the conditions were rigorously stipulated.

The original hypothesis regarding the carcinogenicity of substituted 1,2-benzanthracenes (I), and which led to 3,4-benzopyrene (II), also was responsible for the discovery

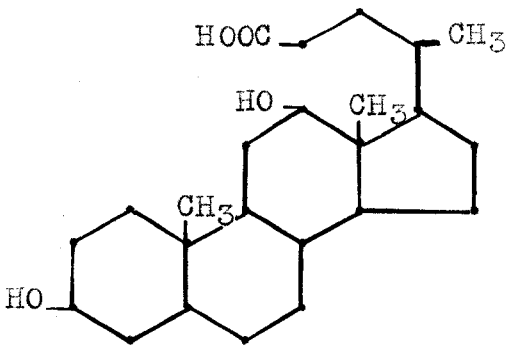
of 9,10-dimethyl-1,2-benzanthracene (V) and 20-methyl-cholanthrene (VI), two of the most powerful and rapidly acting carcinogens (60). The latter is one of the most widely used and was originally prepared from desoxycholic acid (VII); this immediately heralded the entrance of bile acids, sex hormones and sterols into the project due to the similarity of structures.



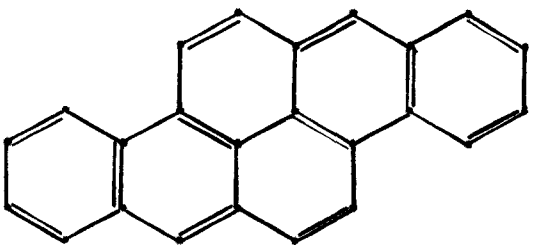
V 9,10-dimethyl-1,2-benz-anthracene



VI 20-methylcholanthrene



VII desoxycholic acid



VIII 3,4,8,9-dibenzopyrene

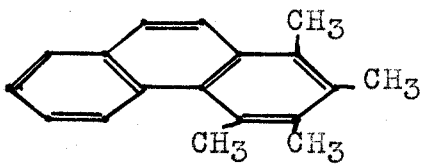
To go into detail regarding all the compounds prepared and tested would be useless since one reference has already been given which covers 1329 substances. In general it might be stated that the efforts regarding the polycyclic

hydrocarbons involved the isolation or synthesis of all the possible polycyclic compounds which were expected to exhibit carcinogenic activity. This group varied in size between 1,2-benzanthracene (I) * and a dibenzopyrene such as VIII. In addition some of the aromatic rings in the series were replaced by five membered and heterocyclic rings with retention of activity.

a) Classes of Chemical Carcinogens

As a result of the extensive surveys it was found possible to divide the chemical carcinogens into four main groups about which some generalization could be made. The first to be discovered was a class of aromatic polycyclic hydrocarbons which included the original coal tar fractions as well as related compounds such as the sterols. Even the polycyclic aromatic heterocycles are sometimes placed in this division. The azo compounds, common to the dye industry, form another group, and related to these are the aromatic amines some of which are found as dyestuff

* Later, it was discovered that substituted benzophenanthrenes also were potent carcinogens. Even 1,2,3,4-tetramethylphenanthrene (IX) showed weak activity, this being the simplest carcinogenic polycyclic hydrocarbon.



IX 1,2,3,4-tetramethylphenanthrene

intermediates. The final class consists of the amino stilbenes in which the ethylenic linkage has been substituted for the azo moiety. There are numerous other chemical agents which can initiate the formation of a malignancy. However, thus far it has been relatively impossible to make many definite conclusions regarding them. Each of the above groups will be discussed in greater detail in subsequent sections.

b) Administration vs. Type of Tumor

Various methods are utilized in the administration of these agents depending upon the type of tumor desired and the quantity of material available for testing.

Carcinoma or skin tumors were originally formed by painting an area with a 0.3% solution of the hydrocarbon in benzene (patch test). This type was frequently encountered as an occupational disease where prolonged contact with coal tar, oil or soot was common. The tumor formation may occur after several months or even years, depending upon the concentration and length of time of application.

The inconsistencies of this method were realized and subcutaneous injections of the material in lard, sesame oil or tricaprylin were instituted to achieve a greater uniformity. Even this was not entirely satisfactory due to solvent effects. Some workers utilized the injection of glycerol moistened crystals or the implantation of pellets

containing the carcinogen in cholesterol or paraffin, especially to induce brain or stomach tumors. A silk thread soaked in the agent prior to being passed through the organ also was used.

Subcutaneous injections give rise to sarcomas of the fibrous tissue which usually commence as inflammations on or under the skin with proliferation occurring about the fifty-fifth day at or near the site of the injection.

If an intravenous injection is employed, the blood stream may carry the substance throughout the host. Depending upon the type of agent or susceptibility of the host, the tumor may be formed in almost any organ of the body. Formation usually occurs after several months but in less than one year.

A fourth experimental method involves the oral administration of the agent mixed with the food. To overcome the loss of material through digestive disintegration and discharge, larger quantities are necessary, usually a sample weighing 0.05% of the total food supply. Although this may be used for the production of gastric tumors, the agent may still be useful for neoplastic production after assimilation into the blood stream. The carcinogen and host determine the site of action.

c) Tumor Formation in Body Organs

The lung seems to be the most susceptible organ for tumor production. The carcinogen can be transported through

the blood stream or else inspired directly into the pulmonary system. The higher incidence of lung cancer among industrial employees and residents exposed to smoke filled areas has been attributed to this fact.

Liver tumors are rather common but are induced mostly by the azo-carcinogens. The mechanism is probably more complex than that involving the aromatic polycyclic hydrocarbons. Only rarely will an azo compound produce a tumor at or near the site of application; hence, intravenous or oral administration is used.

Mammary tissue is quite sensitive to neoplastic agents, especially when female sex hormones (intrinsic agents) are used as carcinogens either by direct application to the site or by injection elsewhere. Aromatic polycyclic hydrocarbons are also effective, although the normal stimulation of the mamma by sex hormones may provide a pre-cancerous condition. This type of tumor formation has considerable dependence upon heredity factors.

Gastric neoplasms arise after injection or oral administration of the carcinogen, 20-methylcholanthrene being frequently used for this purpose.

d) Conditions Affecting Carcinogenesis

A brief comment was made regarding some of the conditions which may affect the carcinogenic activity of a substance.

Although it was assumed that sex does not affect the results, this was eventually disproven or a least seriously

questioned after a series of carefully controlled experiments with only minimum quantities of carcinogen (61). Prior to this no distinction could be noted due to the excess material employed.

Age was found to be of importance by a number of investigators, but the data was conflicting.

Heredity is of utmost importance since it has been proven that special strains could be developed which would be susceptible, resistant or at any stage intermediate between the two. Pure strains of mice were inbred by employing brother-sister mating for at least twenty generations.

Although the rabbit was the first animal used for the artificial production of epitheliomas, it is not generally as active as other species.

Mice are most widely used since they are quite responsive, have a short life span and a high reproductive rate.

Rats are less sensitive to subcutaneous and skin tumor formation.

Most other animals are prone to develop one kind of malignancy and are used only for that purpose.

One example is recorded involving human beings, but this was discontinued when a benign growth appeared (62). Information about human cancer is obtained from industrial data on occupational diseases, particularly where the workers are exposed to coal, oil, smoke, soot or dyestuffs.

It has been recognized that the diet is important for

carcinogenesis, perhaps not during the period of initiation, but during development. Evidence is conflicting but it has been agreed that a restricted calory diet hinders cancer formation.

The influence of the solvent was recognized as being an important factor in carcinogenesis. It was apparent that it could either affect the host or alter the carcinogen. The outcome also varied depending upon whether a volatile or non-volatile solvent was used. It was not known if the differences among solvents resulted from an inhibition by one, a promotion by the other, or both.

The solvent inhibition observed with animal fats over vegetable fats was thought to be associated with the fact that homologous fats would be more apt to foster elimination of the carcinogen. Later, it was shown that phospholipids in the animal fat caused the inhibition by acting as an anti-oxidant (63). Absence of the phospholipids prevented the inhibition. This theory was based upon an oxidative metabolism of the carcinogen.

This chemical explanation was challenged by Strait et al (64) who presented a physical theory which depended upon the distribution of the carcinogen between solvent and interstitial fluid. The activity was found to vary as the distribution coefficient. The greater the distribution of the agent into the cellular fluid, the greater the carcinogenicity.

e) Anti-Carcinogenesis and Co-Carcinogenesis

Material capable of inhibiting or promoting carcinogenesis has been defined more precisely as an anti-carcinogen or a co-carcinogen. An anti-carcinogen is a substance which, when added to a carcinogen during application, inhibits carcinogenesis and therefore increases the latent period (time required for a tumor to be noticeable). This definition is accepted loosely to include the calory deficient diet as being anti-carcinogenic. A co-carcinogen enhances the incidence of tumors and provides a shorter latent period.

It was discovered that certain inhibitors were excreted as mercapturic acids and were noticed to cause a local depletion of the sulfur content when applied to the skin. From this it was concluded that the carcinogen must react with sulfhydril groups. The action of the inhibitor may serve to preferentially remove these active groups before the carcinogen can attack.

In the same way it was found that the addition of inhibitors containing sulfhydril groups would react with the carcinogen and prevent carcinogenesis (65). Although inhibition was noted, it differed from the first type since the carcinogens were not excreted as mercapturic acids (66).

The question of how a non-carcinogen could affect carcinogenesis was answered when proof was given that there are two stages connected with cancer production, a pre-carcinogenic and an epi-carcinogenic period (67). The first period commences after application of the agent with the

formation of latent cancer cells. This is promoted by either the co-carcinogen or additional quantities of the carcinogen. Numerous chemical irritants are capable of acting as co-carcinogens. Two main conclusions were drawn: latent cancer cells are formed after only one application of carcinogen; co-carcinogens or additional carcinogens will develop the latent tumor cells. It was found in a series of experiments using mice that the latent cells may exist as such for twenty weeks without losing their ability to be transformed into the epi-carcinogenic stage by co-carcinogens. It was also discovered that some carcinogens were good initiators, but poor promoters, whereas some non-carcinogens served as excellent promoters.

f) Carcinogenic Index

Some attempt to establish a carcinogenic index had been made by Iball who suggested a relation between the proportion of epithelial tumors arising in the animals tested and the average time (induction period) necessary for their appearance (68). A long latent period was generally found to correspond to a minimum incidence of tumors (Table I).

A more complicated set of equations was devised by Berenblum (69) for ordinary carcinogens or very weak carcinogens as given below. G is the grade of activity, W is the latent period in weeks.

$$G = 16 - 6.5 (\log W)$$

$$G = 16 - 6.5 (\log 2W)$$

Table I. Carcinogenic Compounds Arranged in Decending Order of Potency (**68**). This table has been based on the formation of benign and malignant tumors. Only the latter type is regarded as cancer according to the generally accepted definition.

Compound	No. of mice alive when first tumor appeared.	No. of tumors	% of tumors (A)	Papillomas	Epitheliomas	Av. latent per. (B)	Index (A/B x 100)
1. 9,10-Dimethyl-1,2-benzanthracene	20	13	65	6	7	43	151
2. 20-Methylcholanthrene	18	18	100	1	17	99	101
3. 20-Methylcholanthrene	8	5	62.5	0	5	151	41
4. 20-Methylcholanthrene (2+3)	26	23	88.5	1	22	109	80
5. 3,4-Benzopyrene (from pitch)	10	10	100	2	8	127	79
6. 3,4-Benzopyrene (synthetic)	9	7	78	2	5	109	72
7. 3,4-Benzopyrene (5+6)	19	17	89.5	4	13	119	75
8. Cholanthrene	49	28	57	5	23	112	51
9. 5,6-Cyclopenteno-1,2-benzanthracene	14	13	93	1	12	194	48
10. 2-Methyl-3,4-benzophenanthrene	16	12	75	5	7	155	48
11. 10-Methyl-1,2-benzanthracene	18	12	66.5	2	10	147	45
12. 5,6-Dimethyl-1,2-benzanthracene	19	16	84	0	16	220	38
13. 6-isoPropyl-1,2-benzanthracene	15	11	73.5	1	10	204	36
14. 3,4,5,6-Dibenzocarbazole	19	9	47.5	4	5	143	33
15. 3,4,8,9-Dibenzopyrene	17	10	59	0	10	205	29
16. 5-Methyl-1,2-benzanthracene	8	7	87.5	2	5	317	28
17. 5-Ethyl-1,2-benzanthracene	9	7	77.5	2	5	285	27
18. 1,2,5,6-Dibenzanthracene	65	41	63	8	33	239	26
19. 3,4-Benzophenanthrene	18	12	67	5	7	387	17
20. 1,2,5,6-Dibenzocarbazole	9	4	44.5	1	3	263	17
21. 5-n-Propyl-1,2-benzanthracene	20	6	30	3	3	192	16
22. 3,4,5,6-Dibenzacridine	28	11	39.3	2	9	357	11
23. 3'-Methyl-1,2,5,6-dibenzanthracene	25	7	28	1	6	325	9
24. 1,2,5,6-Dibenzacridine	25	6	24	2	4	350	7
Totals		305		60	245		

A third method of comparison was used in which dosage amounts were varied to obtain the maximum incidence of tumors and the shortest induction period (70). Although important information was obtained, the relative order corresponded to that of Iball.

4. Theories of Chemical Carcinogenesis

The discovery of a chemical method for the induction of cancer made possible the controlled formation of neoplasms. In addition it provided a means of studying tumors and the biological changes occurring. The direct results of such studies involved the postulation of numerous mechanisms to account for the action of the carcinogens. The appearance of many explanations merely emphasized the complexity of the problem and the difficulty in correlating all of the variables which were found to influence the experiments.

a) Epidermal Carcinogenesis

The most easily formed and probably the least complex tumor results from patch tests or subcutaneous injections, after which the neoplasm appears at or near the site of application. The initiating action of the carcinogen has been characterized as an irreversible change brought about by a small quantity of agent. The incidence is directly proportional to the amount of agent and follows the "all or none" response, i.e. the number of tumors is constant after one application regardless of the length of the latent period (67). This is not unequivocally true because there has been found a maximum

latent period, varying with the conditions, but during which the promoting action must be commenced in order to obtain the highest tumor incidence. This implies that there is a competitive reaction within the cells during the initial period. After reaching a maximum latent period the normal cells are able to overcome the foreign agent unless a promoter supplies some additional unknown quantity to enable the malignant cells to proliferate.

The way in which a specific carcinogen initiates the malignant action is still suppositional. An aromatic polycyclic hydrocarbon, if painted on the skin, must be absorbed through the cell membrane. A particular size or shape seems to be required, but it is not known if this is due to the barrier of the cell membrane or a constituent within the cell or is required for a selective absorption on some surface. It has been noted that planarity of the compound is essential for carcinogenicity and it has been suggested that this is necessary to permit diffusion through the cell membrane, after which the normal permeability of the cell is hindered and a neoplastic change occurs (71).

The variations encountered with the different solvents employed for injections have received both physical and chemical explanations (cf. page 69). Of greater importance is the fact that two schools of thought are represented. The postulation of phospholipids as anti-oxidants suggests that an oxidation or metabolism is required for the transformation

of the substance applied into an active carcinogen. Others maintain that no such alteration is necessary.

Observations have been made after patch tests or subcutaneous injections, and it was noted that there was a general thickening of the skin, inflammation, and an increase in connective tissue. This was followed by damage to the epithelium and degeneration of the cells, but then by reparative regeneration. This would correspond to the initial or pre-neoplastic stage of carcinogenesis (cf. page 70) which could be promoted by non-carcinogenic irritants, additional carcinogens, wounds, injuries, burns or even an increase in the caloric intake. The promotional effect, although indefinite, may be due to a stimulation of mitosis.

b) Carcinogenic Phenanthrene Derivatives

Extremely popular from 1940-1947 was an attempt on the part of the French oncologists to provide an electronic index for carcinogenic agents. Polycyclic hydrocarbons were examined as derivatives of phenanthrene with the 9,10-positions labeled as the K-region (72). The increased reactivity of the 9,10-olefin was well recognized by organic chemists, but by means of the resonance or valence bond method numerical values were placed on the carbon atoms of carcinogens, particularly in the K-region. The calculations were based upon a consideration of bond order, charge density and free valencies and marked the first application of numerical estimates to

chemical compounds for describing their potencies as carcinogens. The results appeared encouraging, but the number of exceptions substantiated the claims that although it may still prove its importance when coordinated with additional theories, as such it cannot be utilized alone.

A similar viewpoint was taken by another group who considered all the aromatic polycyclic hydrocarbons tested as derivatives of phenanthrene. The potent examples were found to be substituted by methyl groups or condensed benzene rings in the 1,2,3,4-positions (73). At least the partial metabolism of the active phenanthrene derivatives involves the formation of a hydroxyl group at a position adjacent to the reactive double bond. This situation would be required if an initial complex arose from a reaction between the 9,10-olefin and the substrate. Credence was extended to the above theory when a benzopyrene and an azo compound were isolated as protein complexes after administration (74). In the case of the inactive compounds this hydroxyl group was formed at the reactive position. The isolation of hydroxy compounds has been taken as proof that a metabolism is necessary before the carcinogen is formed, a premise also suggested after the inhibition of cancer by phospholipids was observed (cf. page 69).

c) Carcinogenic Influence on Environment

Most of the theories depend upon a particular class of carcinogens as a basis. A very good generalized explanation

has been offered which thus far has withstood serious rebuttal for over a decade (75). It was discovered that both spontaneous and transplanted tumors exhibited a restrained growth when exposed to carcinogenic agents. With but a few exceptions the non-carcinogens and poisons did not display this property. The carcinogen may act as a growth inhibitor, the effect of which is to alter the environment of the cell, necessitating an irreversible heredity transformation, and thus the mutation of cell characteristics. If correct, this would parallel the irreversible change which is common in bacteria during environmental variations. Just as with bacteria some cells would be completely destroyed, but the surviving ones would adjust themselves to the new environment. Hence, tumors are not found exactly at the point of application of the carcinogen, where the concentration is lethal, but slightly removed, where the cells are just able to survive. Although this explanation seems satisfactory, it is incomplete. Many substances cause injury and environmental changes without producing cancer. Therefore, additional information must be obtained to determine the reasons for the differences in growth inhibition. It has already been suggested that the carcinogens react with a protein constituent containing a sulfydril group (cf. page 70). If this were an enzyme necessary for growth, the altered enzyme could then bring about a mutation. The nature of the cell change is still speculative as to whether it is a true somatic mutation, i.e.

a change in the nuclear genes, or a cytoplasmic mutation resulting from differences in the plasmagenes. The filterable Rous chicken sarcoma virus may be an example of a mutated plasmagene (cf. page 61).

It should now be reiterated that although much has been learned from the numerous theories proposed to explain carcinogenesis, none has proven entirely satisfactory due to the vast amount of uncorrelated data which has accumulated.

d) Possible Relation to Spontaneous Carcinogenesis

The in vitro formation of methylcholanthrene from desoxycholic acid as well as the discovery of the carcinogenicity of sex hormones are just two examples which indicated a possible relationship between chemical and spontaneous carcinogenesis (cf. pages 63 and 67). There was immediately recognized a similarity between the carbon skeletons of the aromatic polycyclic hydrocarbons and the natural products found in the body. Numerous experiments have proven that biological organisms are capable of performing complicated chemical reactions with relative ease.

It was discovered that injections of extracts of human organs from people who had died from cancer as well as from other diseases were capable of inducing sarcomas (76). Although the incidence rate was low, the lengthy latent periods were presumed to indicate that an in vivo chemical transformation was taking place. The fact that patch tests failed to show activity substantiated these views.

Since the first artificial carcinogenic tars were prepared by heating natural substances to high temperatures, it was suggested that the temperatures to which some cooked foods are submitted would be sufficient to form carcinogens. Both heated vegetable and animal fats were found to corroborate such an assumption (77).

Sex hormones have supplied additional data for the theories of spontaneous carcinogenicity (cf. page 67). In as much as mammary tumors could be developed by the local or distant application of estrogens or other carcinogens, a pre-cancerous condition of mammary tissue may exist. That this was due to the hormones themselves seemed probable when it was noted that male mice injected with female hormones could then develop mammary tumors in the usual manner. The inbreeding of female strains of mice (cf. page 68) which were resistant to mammary tumor formation despite normal estrogenic development indicated that the pre-cancerous condition is not wholly dependent upon sex hormones. The administration of androgens (male hormones) to inhibit mammary development prevented mammary tumor formation (78). These developments served to increase the importance of heredity, but only when the mammary organs were developed.

Even though a vague correlation has been shown to exist between spontaneous carcinogenesis and chemical carcinogenesis from aromatic polycyclic hydrocarbons, it tends to overshadow the many other types of carcinogenic agents which have no

similarity to the aromatic polycyclic hydrocarbons. The isolation of the latter from organs subject to spontaneous cancer would be the only means of proving the suggested correlation. Even if the correlation did exist, the amounts of material may be so slight as to be non-isolable. Therefore, the prospects for such a finding seem remote, and so the search for a relationship between the chemical and spontaneous induction of cancer must continue.

5. Aromatic Polycyclic Hydrocarbons

This class of carcinogenic agents has already received considerable description. Although the emphasis was placed upon the study of the metabolic products to determine the mechanism of action, there is no assurance that the metabolism is responsible for carcinogenesis. It is possible that only a trace of material is required to initiate the malignancy, and the elimination products which are isolated arise from a detoxication of the excess.

The fate of aromatic polycyclic hydrocarbons was initially followed by utilizing the fluorescence spectra (cf. page 58). The first important discoveries resulted from experiments with 3,4-benzopyrene injected intraperitoneally or subcutaneously (79). The material was metabolized to the extent of 99% and the rate of disappearance was found to be directly proportional to the amount remaining at the site of injection. However, the concentration in the blood remained low and constant so that the disappearance must depend upon the rate

of diffusion through the cells.

The chemical reactions involved became more fully known as the mixtures of metabolic products were able to be divided into various components. Of particular importance was the application of chromatography to the separation of complex mixtures. The possible relation of carcinogenesis to detoxication was indicated when the hydrocarbons were isolated as the glucuronides of hydroxy and vicinal dihydroxy compounds. Mechanisms were then presented to show by means of the different substances isolated that both carcinogenesis and detoxication reactions could commence and terminate with the same compounds but proceed through different intermediates (80).

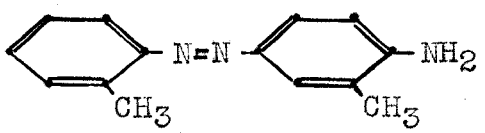
Other theories of action were presented, but like the above, were not conclusive. The most recent innovation utilized test materials which were labeled with radioactive atoms which could be traced quantitatively through the body. From this approach some new discoveries may be forthcoming, not only in the study of aromatic polycyclic hydrocarbons, but other carcinogenic agents as well (81).

6. Azo Compounds

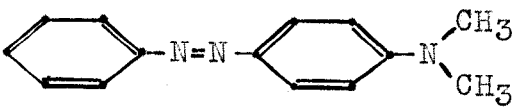
The incorporation of azo dyes into medicinals to facilitate the healing of wounds was found to produce cancerous growths as early as 1906 (82). It was not until 25 years later that a thorough investigation of this type of carcinogen was begun after it was noted that o-aminoazotoluene (X) could cause tumors of the liver (83). This was followed by the

discovery of the even more potent butter yellow (XI) (84).

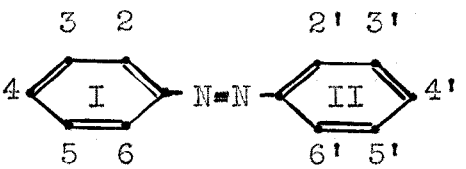
Because of the relatively simple structure attempts were made immediately to designate the characteristics essential for carcinogenic azo compounds, and to corroborate these by preparing the desired derivatives. Conversely, the polycyclic hydrocarbons were examined extensively before the essential characteristics were determined. The results are tabulated below and conform to the numbering as in XII.



X o-aminoazotoluene



XI butter yellow
4'-dimethylaminoazobenzene



XII azobenzene

1') As such XII is inactive. Usually an amino group must appear at the 4'-position (required for comments 2'-6'). Replacement by a hydroxy group lessens the activity. A few examples are known containing methyl groups in various positions but these possess decreased activity.

2') Substitution of a carbon atom for a nitrogen or a naphthyl group for a phenyl is accompanied by loss of activity, but the corresponding ethylenic derivative is potent.

3') The hydrogens on the amino group may be replaced by

one or two methyl groups or a methyl and an ethyl group, but not by two ethyl groups.

4') A methyl group in ring I increases the activity slightly if in the 2- or 4-position, greatly if in the 3-position. However, the 3,5-dimethyl derivative is inactive.

5') Apparently any group in the 3-position enhances carcinogenicity.

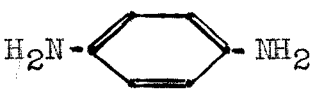
6') Additional substitution in ring II is not well understood.

Because of the relative ease of isolation and identification of the metabolic products, much information was obtained about azo carcinogens. Although various types of tumors could be obtained under the proper conditions, the principal site affected was the liver, the main organ of metabolism in the body. This probably indicates a more complex mechanism of carcinogenesis since the agent must be transmitted from the site of application. Like the hydrocarbons, there was a direct relation between the quantity administered and the number of tumors arising, and although it was possible to breed resistant or susceptible strains of animals, the males were more impervious to the azo carcinogens (85).

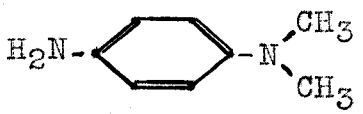
The influence of the diet was recognized immediately since the agent was administered orally. Rice proved beneficial for tumor formation, but riboflavin and casein were inhibitors (86). It was then noted from experiments using dimethyl-aminoazobenzene as the carcinogen that there was a marked

reduction of riboflavin and co-enzyme I in the liver. Subsequently, it was shown that the metabolism of this type of carcinogen involved a rupture of the azo linkage to form amines. The decreased concentration of riboflavin and co-enzyme I in the malignant liver would occur if these two substances were required for the splitting of the azo linkage (87). The 4'-amino groups were reversibly methylated or demethylated as the monomethyl and dimethyl derivatives respectively, but only irreversibly demethylated to the free amine. Detoxication occurred by the in vivo formation of a hydroxy compound, acetylation of the amine or both.

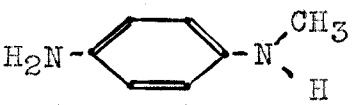
An investigation of the effects of the metabolic products of dimethylaminoazobenzene on a fermentation system led to a possible theory of action (88). Such products as XIII, XIV and XV were shown to inhibit a fermentation which also



XIII p-phenyl-enediamine



XIV N,N-dimethyl-p-phenylenediamine

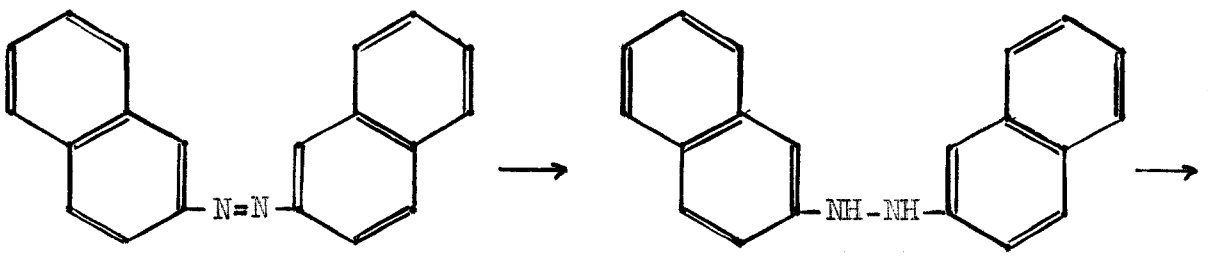


XV methyl-p-phenyl-enediamine

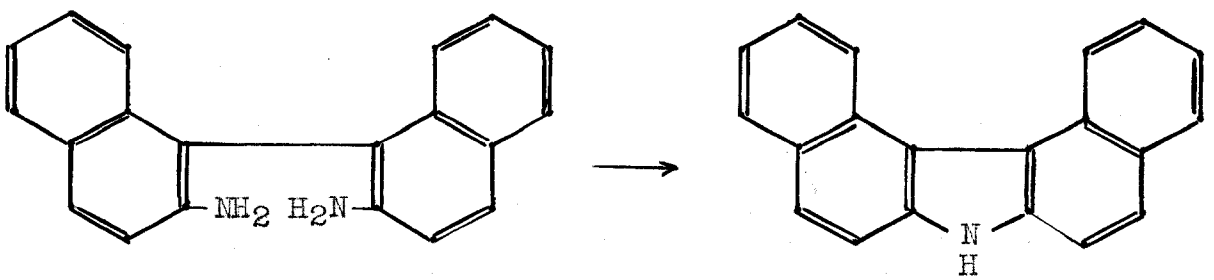
required co-enzyme I, but the addition of an excess of co-enzyme I prevented the inhibition. The enzyme was thought to produce the actual inhibitors by oxidizing the metabolic products which then competed with co-enzyme I for a key enzyme probably containing a sulfhydril group. The addition of excess co-enzyme I could permit the competition to favor the co-enzyme. Various enzymes were shown to be deactivated by

the oxidized split products of azo compounds, and this was accepted as additional evidence that a similar mechanism could explain the neoplastic mutations occurring in cells (89). The deactivation of essential enzymes may produce an environmental change analogous to that suggested for the polycyclic hydrocarbons.

An attempt was made to correlate the azo compounds and aromatic heterocycles after it was noted that 2,2'-azonaphthalene (XVI) and 3,4,5,6-dibenzocarbozole (XVII) were able to induce liver tumors and also were more reactive among female mice (90). An in vivo transformation was postulated to account for the preparation of the heterocyclic derivative from the former.



XVI



XVII

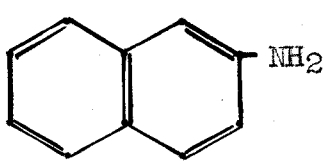
Intermediates of a type similar to diaminobinaphthyl, when oxidized, also were shown to be inhibitory toward certain

enzyme systems (91).

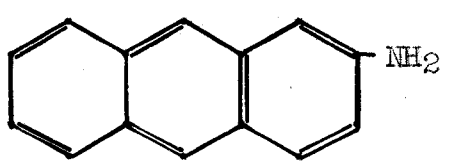
A final theory has been offered to explain why some of the azo metabolities were ineffective when administered as such. It suggested that the metabolized azo compounds were merely detoxication products, the original azo compounds being the carcinogens. From the livers of mice fed with dimethylaminoazobenzene was isolated a complex which contained a protein and the original agent and likewise depended upon the diet for its concentration. The removal of this protein may have caused an environmental change which was followed by the formation of a malignant cell, with the number of tumors being proportional to the concentration of bound azo compound (92).

7. Aromatic Amines, Aminostilbenes and Additional Carcinogens

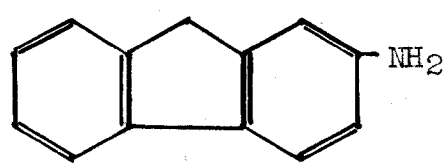
Although the aromatic amines have an aromatic polycyclic structure, they were classified separately because of their metabolic differences and the types of tumors produced. The three parent substances in this group are the 2-amino derivatives of naphthalene (93), anthracene (94) and fluorene (XVIII, XIX, XX). In each case the amino group in the 2-position was required for carcinogenicity. In addition, these substances were unique in being able to produce many



XVIII 2-naphthyl-amine



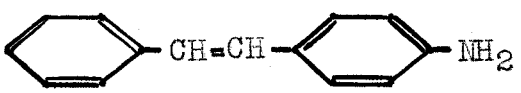
XIX 2-anthramine



XX 2-aminofluorene

types of tumors.

The excretion of 2-naphthylamine occurred either as the 2-amino- or acetylamino-1- or 6-hydroxy derivative (95). A distinction was suggested similar to that proposed for the aromatic polycyclic hydrocarbons to indicate the possibility of either carcinogenesis or detoxication. Since so little is known about the aromatic amines, as yet no theories have been offered which have much foundation in fact. The only outstanding feature noted thus far was that 2-anthramine and 2-aminofluorene were analogous except for a variation in the center ring. Since this could also be a heterocyclic ring containing sulfur or oxygen or could be removed entirely to form a biphenyl derivative, no definite conclusions exist regarding what is necessary (96). The biphenyl type seems quite closely related to the aminoazo derivatives mentioned previously and to the aminostilbene carcinogens which will be described (XXI). These were discovered as growth inhibitory



XXI p-aminostilbene

substances but were predicted to be carcinogenic according to the theory of Haddow (cf. page 77) (97). Although administered orally or subcutaneously they were capable of inducing wide varieties of tumors and in general resembled the aromatic amines in metabolism. They likewise were excreted as acetylated or non-acetylated amines and possessed in addition

a hydroxy group, but there was no evidence to indicate the rupture of the ethylenic linkage.

It should be evident that most of the theories of action for each of the four types of chemical carcinogens are inadequate as such. To attempt to devise mechanisms for all the other apparently non-related carcinogenic agents, not only organic but inorganic, appears extremely more difficult (cf. pages 61 and 62). The only theory common to various types of carcinogens was that of Haddow (cf. pages 77 and 87) in which the agent was described as a growth inhibitor which promoted a neoplastic mutation. However, there was no proof for a specific chemical reaction which could cause the inhibition. Needless to say, the mutations involved are even more incomprehensible.

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