THE IN VITRO INTERACTION OF 3-METHYLCHOLANTHRENE WITH DEOXYRIBONUCLEIC ACID

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CHAPTER I

INTRODUCTION

The discovery in 1961, by Lerman (12), that acridine dyes intercalate between the hydrogen-bonded nucleotide pairs in the double helix of the deoxyribonucleic acid (DNA) molecule stimulated other investigators to search for similar effects produced by other organic compounds. Several promising compounds were found among the biological alkylating agents. These comprise a diverse group of compounds which include the nitrogen mustards, certain lactones, ethyleneimines, methanesulphonates, epoxides, and diepoxides.

Brookes and Lawley (5) found that DNA, ribonucleic acid (RNA), and proteins are alkylated by several different alkylating compounds to approximately the same extent per unit of weight. On the basis of the molecular weight differences among these biological polymers, they concluded that the extent of alkylation was much greater in the DNA than in the RNA or protein. Wheeler (17) substantiated this idea when he demonstrated that enzyme function, the biological function of numerous proteins, was not inhibited by the presence of alkylating agents. The ability of

specific bacteriophages to elicit cell lysis in bacteriophage-sensitive Escherichia coli cells was inhibited by the presence of alkylating agents as was shown by Loveless and Stock (14). Brookes and Lawley (6) demonstrated that this effect resulted from specific alkylation of the bacteriophage DNA.

Lawley and Brookes (10) suggested that the probable site of alkylation is at the N-7 position of the guanine moiety in DNA. Lawley and Brookes (11) suggested that the second most likely site is the N-3 position of adenine. Furthermore, alkylation at these positions probably results in the loss of the 7-alkylguanine and/or 3-alkyladenine derivatives from the DNA molecule.

Another group of organic compounds which react with DNA is the aromatic hydrocarbons. Booth and Boyland (1) found that, when 3,4-benzpyrene reacted with DNA, spectroscopic shifts occurred and that these shifts were similar to those obtained when complexes of purines and either dibenzocarbazoles or dibenzacridines were formed. Liquori et al. (13) reported that 3,4-benzpyrene is solubilized by DNA and that the extent of solubilization depends on the degree of denaturation of the DNA structure. Under conditions which preserve the integrity of the DNA, a limited number of solubilization sites appear to be filled by the 3,4-benzpyrene. The degree of filling is one

3,4-benzpyrene molecule per ten to the third power (10^3) nucleotides.

However, Heidelberger (9) and Giovanella et al. (8) demonstrated that 3,4-benzpyrene and 1,2,5,6-dibenzanthracene were sedimented by high speed centrifugation or filtered out by Millipore filtration. They concluded that the hydrocarbons were stabilized as colloids by the DNA and that an interaction did not exist. Boyland and Green (3) repeated the sedimentation experiment with glass, quartz, and polymer centrifuge tubes. Their experiments indicated that the loss of hydrocarbon occurred only when polymer tubes were used. They suggested that the removal of hydrocarbon was due to adsorption by the polymer.

Further evidence for the interaction of DNA and aromatic hydrocarbons has been supplied by Nagata et al. (16). These investigators applied flow dichroism methods to aromatic hydrocarbons such as 3-methylcholanthrene, pyrene, 3,4-benzpyrene and tetracene. The dichroism spectra were determined by producing a velocity gradient between rotating coaxial cylinders. Monochromatic incident light was beamed into the rotating inner cylinder. The absorbed light was subsequently polarized parallel and perpendicular to the flow line by a calcite crystal. The differential dichroism spectra were obtained by determining the difference between the molar extinction coefficients of the light polarized parallel to the flow line and of that

polarized perpendicular to it. The spectra obtained by these methods presented unambiguous evidence for the interaction between hydrocarbons tested and DNA. Additional evidence led the authors to state that the hydrocarbons tested could be classified into two groups according to their mode of interaction with DNA. The first group, having a negative dichroism pattern, was assumed to be oriented parallel to the plane of the DNA nucleotides, whereas the second group, having positive dichroism patterns, was assumed to be oriented perpendicular to the DNA nucleotides.

Using the melting point or the thermal denaturation temperature (T_m) of DNA, Boyland and Green (2) were able to provide further evidence for the interaction of aromatic hydrocarbons and DNA. These investigators found that pyrene, 3,4-benzpyrene, anthracene, benzanthracene, and 7, 12-dimethylbenzanthracene all increased the T_m. On the basis of these observations, they concluded that the presence of the complexed hydrocarbons reduced the thermal denaturation by stabilizing the DNA.

Studies by De Santis et al. (7) of complexes formed between aromatic hydrocarbons and either tetramethyluric acid or DNA indicated the existence of similar solubility and optical properties, i.e., bathochromic shifts. If the interaction was due to the purines in the DNA, as suggested by the similarity of reaction with tetramethyluric acid,

then purines which are polymerized into DNA appear to be at least as effective as free ones.

Small angle X-ray studies by De Santis et al. (7) of crystalline complexes of 3,4-benzpyrene-tetramethyluric acid indicated that the complex consisted of alternate stacked layers of purine and hydrocarbon molecules. The complexes formed between aromatic hydrocarbons and DNA could have similar structures in which the planar hydrocarbon molecules intercalate between the nucleotide pairs of the DNA. Boyland and Green (2) have shown with molecular models that certain aromatic hydrocarbons would intercalate into the Watson and Crick model of the DNA molecule. The X-ray diffraction studies of a proflavine-DNA complex by Luzzati, Masson and Lerman (15) appear to confirm the intercalation theory. Their data indicate that there is one proflavine molecule intercalated between every four sequential base pairs. It is probable that 3,4-benzpyrene could also exist in this fashion were it not for its slight solubility in water. The solubility difference infers an intercalation of one 3,4-benzpyrene molecule per ninety base pairs of DNA (2).

The purpose of this thesis is to report the interaction of aromatic hydrocarbons with DNA and to attempt to determine the relative binding affinities. The effect of the hydrocarbons on the continuity of the DNA molecule has been studied also and discussed.

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CHAPTER II

MATERIALS AND METHODS

Marker DNA Preparation

Radioactively labeled Escherichia coli (ATCC 11303) cells were prepared according to the procedure described by Flamm et al. (1). Log phase cells were incubated with ^{32}P phosphate, specific activity of five microcuries per millimole (mc/mmole), at a final concentration of 0.01 microcuries per liter (mc/liter) in 0.5 per cent nutrient broth (Difco Corporation) for six hours at thirty-seven degrees centigrade (C). Deoxyribonucleic acid (DNA) was isolated according to the procedure of Marmur (2) with the following modifications: the log phase cells were harvested by centrifugation at 3,000 times gravity (x g) for ten minutes at four degrees centigrade and washed once with 0.85 per cent (weight to volume) saline at pH 7.0. The washed cells were resuspended in ten milliliters (ml) of saline containing 0.1 molar ethylenediaminetetraacetic acid at a pH of 8.0. Cell lysis was accomplished by incubating the suspension with 2.0 ml of a 2.0 per cent solution of sodium laryl sulfate at sixty degrees centigrade for thirty minutes. The lysate was adjusted to 1.0 molar with sodium perchlorate and

deproteinized by gentle shaking with KOH neutralized phenol (pH 7.0) saturated with saline-ethylenediaminetetraacetic acid. The nucleic acids were precipitated with two volumes of ethyl alcohol, collected on a glass rod, redissolved in 10.0 ml of 2-amino-2- (hydroxymethyl) 1,3-propanediol buffer pH 8.2, and incubated for sixty minutes at thirty-seven degrees centigrade with ribonuclease (CalBioChem) at an enzyme concentration of 100 micrograms per milliter (ug/ml). Immediately after incubation, the mixture was further deproteinized by the addition of an equal volume of chloroform:isoamyl alcohol (24:1 volume to volume) and gently shaken at room temperature for thirty minutes. The interface between the two immiscible solutions was established by centrifugation for ten minutes at 1,000 x g. The aqueous phase was removed by a large bore Pasteur pipette using gentle suction and the DNA precipitated by the addition of two volumes of 100 per cent ethyl alcohol. The precipitate was dissolved in a 0.1 molar 2-amino-2-(hydroxymethyl 1,3-propanediol (Tris) buffer, pH 8.2, and the deproteinization procedure repeated until no protein remained at the interface. After the final deproteinazation, the DNA was precipitated with 0.54 volume of isopropyl alcohol, vacuum dried, and stored at four degrees centigrade.

Commercial DNA Preparations

Highly polymerized preparations of calf thymus DNA were obtained from California Biochemical Foundation. These samples were certified to contain less than 0.1 per cent arginine, 0.1 per cent chlorine and to have nitrogen/phosphorous ratio of 1.73. All samples were from lot number 50208 and were "A" grade preparations.

Except when in use, all DNA samples were stored over silica gel desiccant at 4.0 degrees centigrade in light-shielded containers.

Aromatic Hydrocarbons

The aromatic hydrocarbon, 3-methylcholanthrene, was obtained from Eastman Chemical Company and the (^{14}C) 3-methylcholanthrene from New England Nuclear Corporation. The radioactive label was in the number six carbon position of the cholanthrene ring of the (^{14}C) 3-methylcholanthrene. Radiochromatograms on silica gel "G" developed with both diethyl ether and n-hexane solvents indicated a purity of ninety-eight per cent, a total radioactivity of 0.10 millicuries, and a specific activity of 2.71 millicuries/millimole. The radioactivity assays were carried out by New England Nuclear Corporation.

Ultraviolet Spectrophotometry

All samples were analyzed in a Beckman DB (double beam) spectrophotometer equipped with either an internal (Beckman)

or external recorder. All DNA samples studied were at a concentration of 10.0 ug/ml, and the figures presented in the results of this paper are direct tracings of the recorder. In all cases, 1.0 centimeter ultraviolet-far ultraviolet matched quartz cuvettes were used.

Radioactivity Determinations

All radioactivity measurements were made in a Picker Nuclear Corporation flow detector using methane as the quenching gas and a gold foil window. Digital readout was through a Labscaler III module.

One milliliter of each sample was plated into concentric ring planchets, dried with infra-red heat, and counted ten times. In order to correct for background, radiation counts were made prior to, during (at 5 planchet intervals), and following each counting sequence. All computations of counts per minute (cpm) corrected for background, mean and standard deviation for each sample, and time incorporation of the radioactive label were accomplished using an IBM 1620 computer (North Texas State University Computer Center). The Fortran programs used in each computation are on file in the Computer Center, and the derived data are stored on IBM punch cards for future reference.

Aromatic hydrocarbon-DNA Reaction System

Reaction mixtures of DNA and either (^{14}C) 3-methylcholanthrene or 3-methylcholanthrene were prepared by

dissolving 2.0 milligrams of the hydrocarbon in 10.0 ml of analytical grade diethyl ether. The solutions were cooled to approximately ten degrees centigrade and rapidly injected through a twenty-seven gauge needle into 25.0 ml of 0.015 molar saline containing 0.0015 molar sodium citrate. The diethyl ether was removed by purging the reaction vessel with nitrogen for thirty minutes. Sufficient DNA was added to the hydrocarbon suspension and the reaction mixture stirred gently at room temperature in a light-shielded vessel.

A reaction system control was prepared simultaneously with the hydrocarbon-DNA system and consisted of identical procedures and materials with the exception of the hydrocarbon or DNA addition.

Reaction System Evaluation Parameters

In order to determine the extent of binding and the relative affinity of the hydrocarbon for the DNA, several test situations were established. The relative specific activity (cpm/amount of material) for forty-eight hours for 100 ug samples was established under conditions of low gravity field strength (18,000 x g for one hour), high gravity field strength (105,000 x g for one hour), diethyl ether extraction, and Millipore filtration (100 millimicron filters). For each of the parameters tested, 10 ml aliquots were withdrawn from the reaction vessels of the

hydrocarbon-DNA mixture, the hydrocarbon-less, and the DNA-less controls. The determination of low gravity field strengths consisted of centrifugation of the samples in the 856 rotor of the International HR-1 centrifuge at twenty-five degrees centigrade for one hour at 18,000 x g, removal of 8.0 ml of supernatant, plating in 1.0 ml aliquots, and determining the amount of radioactivity. The effect of high gravity field strengths were evaluated in the 50 "Ti" rotor of a Spinco Model L ultracentrifuge. Prior to centrifugation, the rotor and rotor chamber were equilibrated to twenty-five degrees centigrade for at least two hours. The 10.0 ml aliquots from the appropriate reaction vessels were placed in cellulose nitrate tubes, capped, and accelerated to 50,000 rpm (105,000 x g). The samples were centrifuged at this rpm for one hour, the centrifuge decelerated without braking, and the radioactivity of the centrifugate was determined. Extraction of samples against diethyl ether required the mixing of 10.0 ml of diethyl ether with 10.0 ml of sample and shaking at room temperature for one hour. The mixtures were centrifuged at 1,000 x g for ten minutes to form the interface, and 8.0 ml was withdrawn from both the diethyl ether and the aqueous phase. Aliquots from both phases were plated in 1.0 ml volumes, and the radioactivity determined. The effect of Millipore filtration was evaluated in a Micro-millipore assembly using 100 millimicron filters. Samples

were withdrawn and filtered under positive pressure, and the radioactivity determined.

In each of these cases, 2.0 ml of the total 10.0 ml aliquot remained after each test. Of this remaining 2.0 ml, 1.0 ml was removed, and the ultraviolet absorption spectrum of this sample was obtained by using an instrument blank of saline-citrate in each case.

Equilibrium Sedimentation Analysis

The degree of polymerization and molecular continuity of the hydrocarbon-DNA and control mixtures remaining at the end of the forty-eight hour incubation period was determined by equilibrium sedimentation in an SW- 50 rotor of a Model L ultracentrifuge. Cesium chloride (CsCl) was recrystallized from a distilled water solution by the dropwise addition of absolute ethyl alcohol. The precipitate was evaporated to dryness in vacuo, and 65.0 grams were dissolved in 35.0 ml of 0.02 molar Tris buffer at pH 8.0. To 9.0 ml of the CsCl solution was added 0.3 ml of a 50 ug/ml solution of ^{32}p-labeled Escherichia coli DNA, the solution mixed thoroughly and divided equally into three screw cap vials. To the first vial was added 0.1 ml of a 50 ug/ml solution of hydrocarbon-reacted-DNA, and to the second and third vials was added 0.1 ml of a DNA-less hydrocarbon suspension and 0.1 ml of a 50 ug/ml solution of a hydrocarbon-less DNA solution respectively.

All three vials were then adjusted with crystalline CsCl to a refractive index of 1.4020 which is equivalent to a density of 1.718 grams/cubic centimeter (3). The contents of the vials were then transferred to separate cellulose nitrate tubes, and the tubes were filled to within 1.5 millimeters of the top with paraffin oil. The rotor was assembled and allowed to accelerate automatically to 44,700 revolutions per minute. At the end of twenty-four hours of operation, the centrifuge was decelerated without braking, the tubes removed, and two-drop fractions were collected by puncturing the bottom of the tube with a twenty-seven gauge needle and allowing the solution to drip out. From each two-drop fraction, 0.1 ml was removed with a micropipette, diluted to 2.0 ml with saline-citrate, and the ultraviolet absorbance determined at 260 millimicrons. The remaining solution in each drop fraction was diluted to 1.1 ml with distilled water, and the radioactivity determined.

The procedure was repeated a second time and two-drop fractions obtained. In this case, however, the ultraviolet absorbance and the refractive index of the fractions were obtained. All equilibrium sedimentation runs were repeated in triplicate under identical conditions and averages of the three runs determined.

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CHAPTER III

RESULTS

The extent of binding of the (^{14}C) 3-methylcholanthrene to DNA varies as a function of the evaluation procedure used to determine binding capacity.

TABLE I

THE EXTENT OF BINDING OF (^{14}C) 3-METHYLCHOLANTHRENE TO DNA

Evaluation Parameter	cpm/100ug DNA/48 hours
18,000 x g for one hour	250
105,000 x g for 90 minutes	240
100 mu Millipore filtration	195
Diethyl ether extraction	102

The data presented in Table I are mean values for each of five separate experiments, each carried out under conditions as nearly identical as possible. The numerical information from each of the five experiments was grouped, and the mean values are reported in Table I. The standard deviations for all the information calculated did not exceed 2.3.

The ultraviolet absorption spectrum for unreacted DNA is shown in Figure 1. In each native sample studied, the spectrum presented the classical 230 millimicron minimum and 260 millimicron maximum absorption profiles.

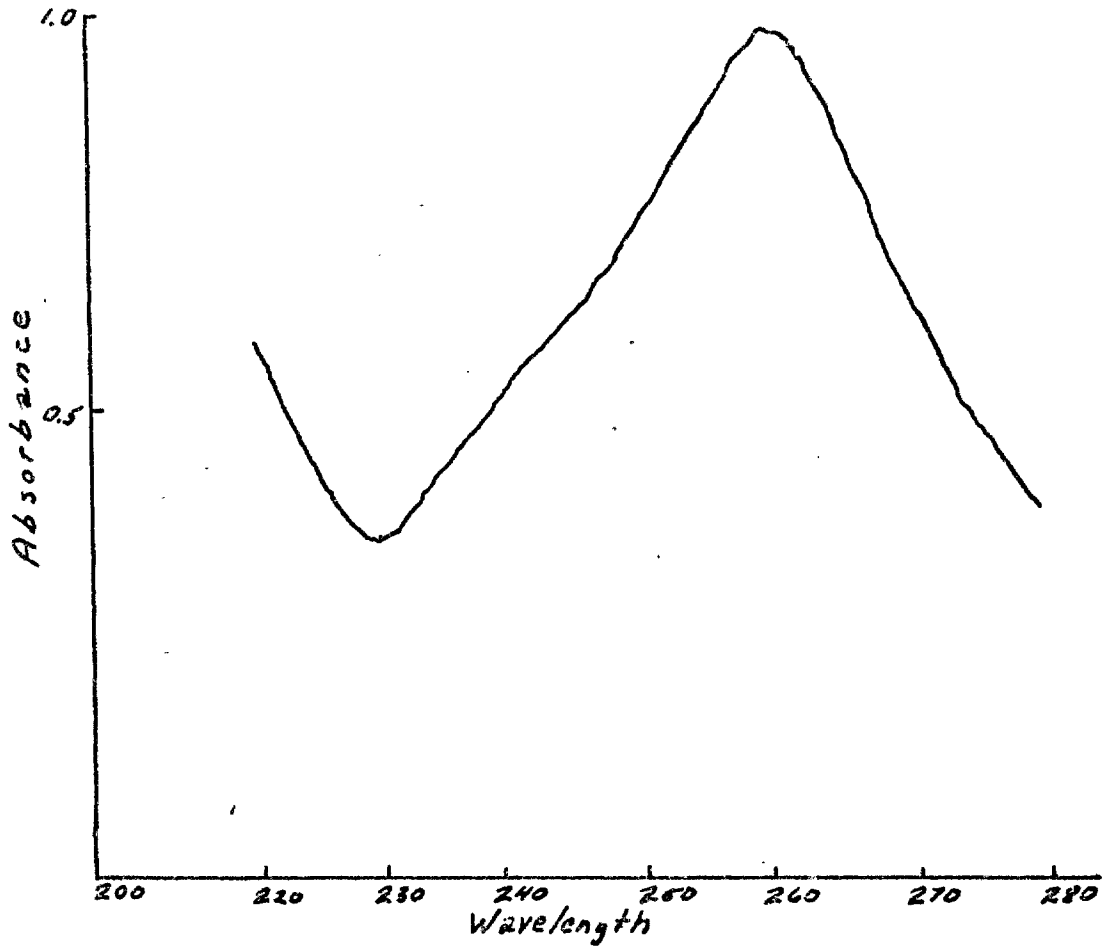

Fig. 1--The ultraviolet absorption spectrum for un-reacted DNA.

The sharp slopes seen in the native DNA absorption spectrum indicate a very small degree of bound residual protein. This evidence is entirely consistent with that reported by the producer, CalBioChem Corporation. The lack of band spread at both the absorption minimum and maximum is indicative of the retention of the highly polymerized configuration by the DNA in solution.

Reacted samples of 3-methylcholanthrene-DNA showed a shift in the ultraviolet absorption spectra toward the visible end of the spectrum (Figure 2).

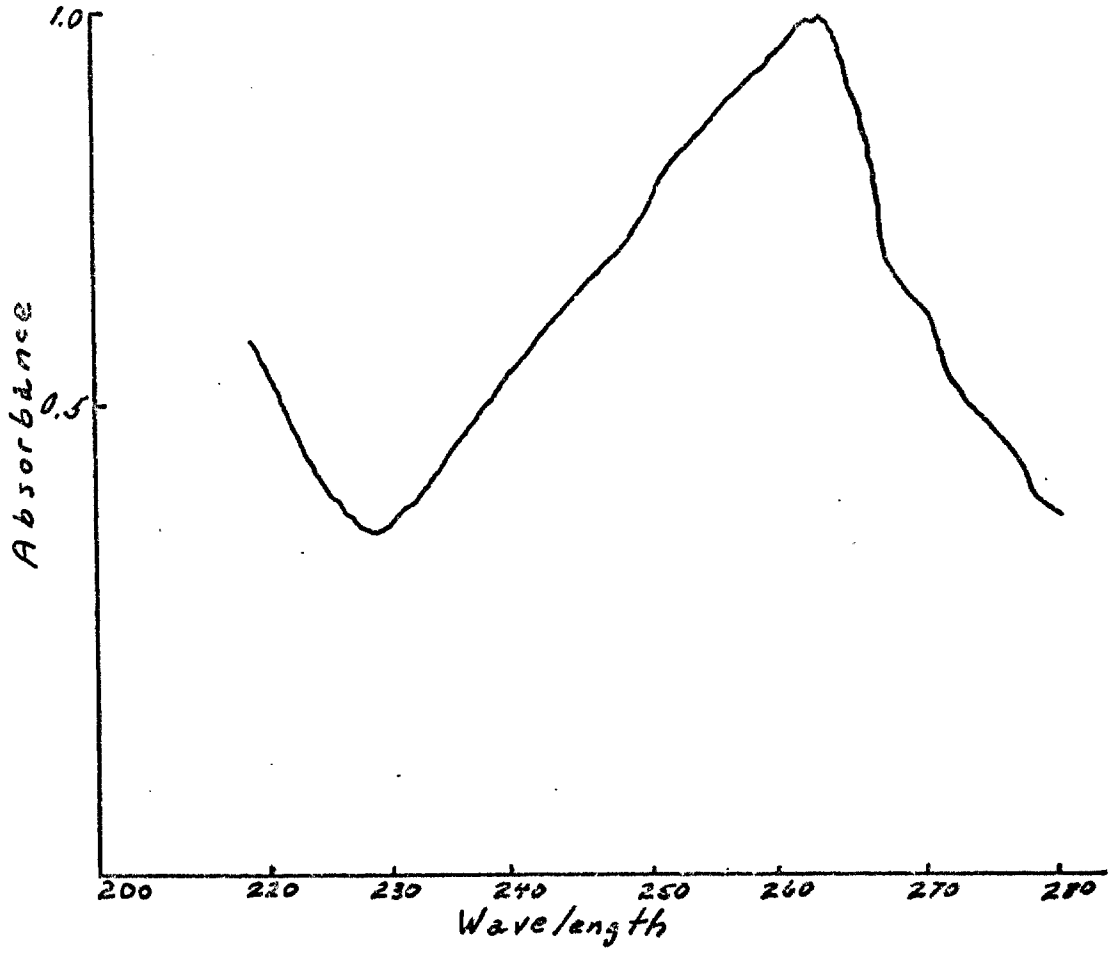

Fig. 2--The ultraviolet absorption spectrum for 3-methylcholanthrene reacted DNA after centrifugation at 18,000 x g for one hour.

No hyperchromicity or band spread was observed at either the absorption maximum or minimum. This indicated that the 3-methylcholanthrene did not influence the degree of polymerization of the molecule and that a high degree of polymerization was retained.

It can be seen from Table II that the degree of shift of the absorption maximum varies as a function of the experimental procedure used to evaluate the firmness of binding.

TABLE II

THE SHIFT IN ULTRAVIOLET ABSORPTION MAXIMUM OF DNA- (^{14}C) 3-METHYLCHOLANTHRENE COMPLEXES

Evaluation Parameter	Ultraviolet Absorption Shifts (mu)
18,000 x g for one hour	268
105,000 x g for 90 minutes	268
100 mu Millipore filtration	265
Diethyl ether extraction	263

The data presented in Table II represent mean values for each of five separate determinations. In no case was the absorption shift found to deviate more than plus or minus 1.0 millimicrons.

In order to insure that the molecular continuity of the DNA was retained after reaction with 3-methylcholanthrene, sedimentation equilibrium studies were carried out. Native DNA was found to have a density of 1.727 grams per cubic centimeter (Figure 3), whereas DNA, reacted with

3-methylcholanthrene, had a density of 1.730 grams per cubic centimeter, regardless of the evaluation parameter tested. This lack of variance probably is due to the inability of the instrument to resolve fine differences in densities.

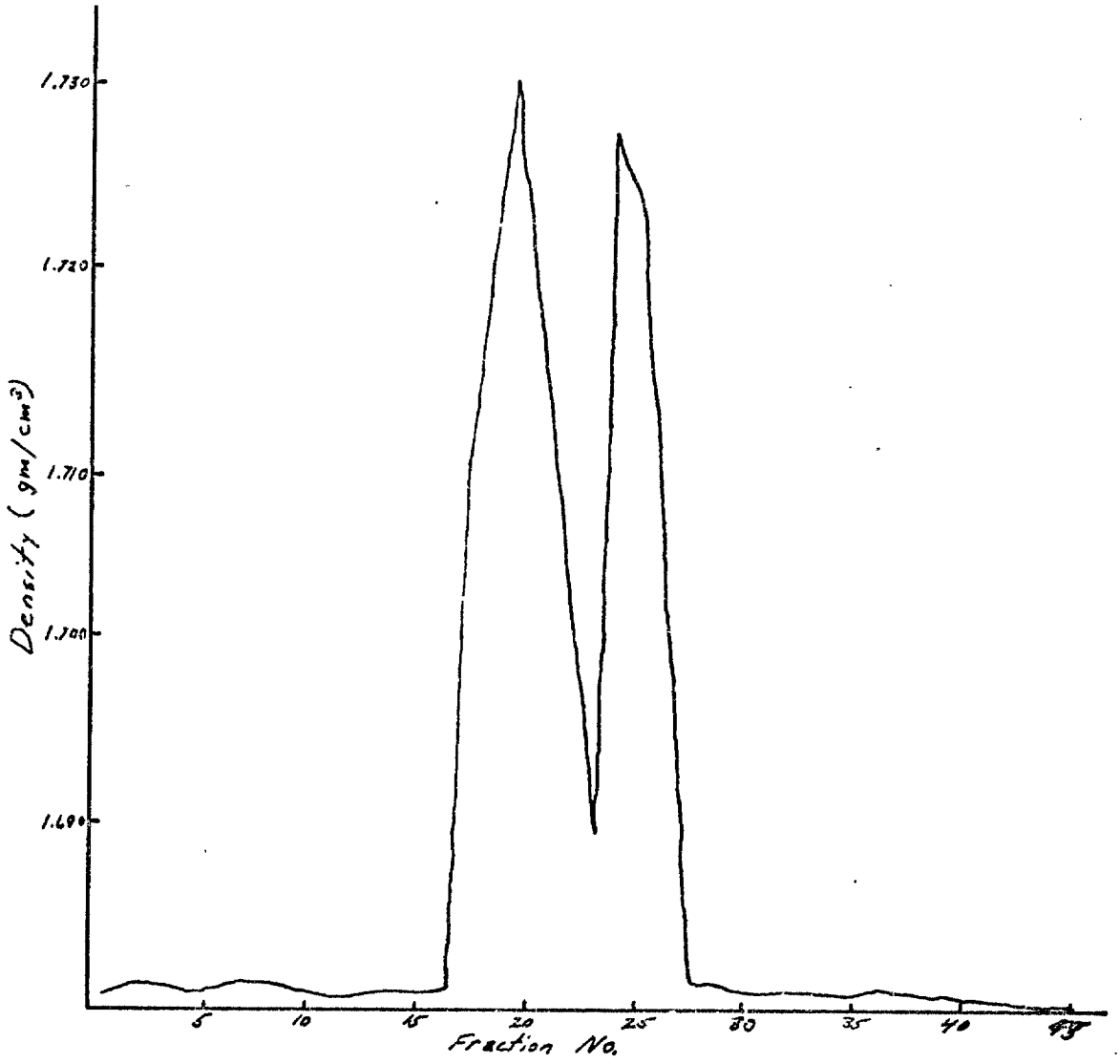

Fig. 3--The densities of un-reacted and 3-methylcholanthrene reacted DNA in CsCl solution.

The extrapolation of the density values was determined by the method by Schildkraut, Marmur, and Doty (2) and indicated a per cent guanine:cytosine (%GC) content of the native DNA as sixty-eight per cent and seventy-two per cent for the reacted DNA. However, since the reaction was carried out under in vitro conditions, the increase in density could not be attributed to an increase in the percentage of guanine and cytosine but is interpreted as an actual increase in weight due to the binding of the 3-methylcholanthrene to the DNA molecule. Furthermore, it is unlikely that reaction of the DNA with the 3-methylcholanthrene leads to a loss in either guanine or cytosine, as suggested by Brookes and Lawley (1) for alkylated derivatives, since this would lead to a decrease in density rather than an increase as was indicated in this study.

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CHAPTER IV

DISCUSSION

The formation of complexes between polycyclic hydrocarbons and DNA was suggested by Booth and Boyland (1). Similar studies by Liquori et al. (5) confirmed the possibility of complex formation. Both studies suggested that the binding was weak and non-specific.

The results of this study regarding the reaction between solutions of DNA and (^{14}C) 3-methylcholanthrene and 3-methylcholanthrene indicate two forms of hydrocarbon binding; one weak and one firm.

Of the two forms of binding, the weak effect probably is due to non-specific surface adsorption of the hydrocarbon to the DNA molecule or to the production of a non-sedimenting colloidal hydrocarbon particle (as indicated by the loss in relative specific activity following Millipore filtration).

The second, or firm binding form, indicates that the affinity of the hydrocarbon for the DNA is greater than the affinity of the hydrocarbon for diethyl ether.

Results of studies by Weil-Malherbe (7) on the solubilization of hydrocarbons by solutions of DNA suggested

a possible intercalation of the hydrocarbon molecules between alternate nucleotide pairs. The binding of planar acridine molecules to DNA, as described by Lerman (4), more fully defines the nature of the interaction. Lerman concluded that, of the possible modes of binding, only intercalation of the acridine molecule in a plane perpendicular to the axis of the helix fully explained the experimental evidence. Moreover, the possibility of displacement of a base pair was excluded. The sedimentation equilibrium data presented above are compatible with this hypothesis. The variation of the %GC content from sixty-eight per cent in the un-reacted state to seventy-two per cent in the reacted state obviously could not occur under in vitro conditions. This leads to the conclusion that the hydrocarbon was bound to the DNA molecule and that no displacement or replacement occurred. The firmness, with respect to diethyl ether extractibility further indicates that the binding is in the interior of the DNA molecule rather than purely on the surface.

It is unusual that such large, non-polar, highly insoluble hydrocarbons should bind to the DNA molecule. Boyland and Green (2) state that polarization or van der Waal's forces may be involved in the binding. The existence of ultraviolet absorption spectrum changes strongly indicate charge transfer effects. The charge transfer theory, as developed by Pullman and Pullman (6) and Hoffman and Ladik (3), shows that the highest filled quantum level of the

hydrocarbon-DNA complex would occur when an electron is transferred from the DNA to the hydrocarbon, and this formation would be accompanied by a shift in the ultraviolet spectrum toward the visible end.

Therefore, it is possible that the formation of a firmly bound DNA - 3-methylcholanthrene complex is a result of the intercalation of the hydrocarbon into the interior of the DNA molecule by a charge transfer mechanism.

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CHAPTER V

SUMMARY

The aromatic hydrocarbon 3-methylcholanthrene was found to react, under in vitro conditions, with DNA and to form a stable complex. This complex was stable to both low and high speed centrifugation and only partially stable to diethyl ether extraction and Millipore filtration. The complex showed an ultraviolet absorbance shift toward the visible spectrum and showed no hyperchromicity. Sedimentation equilibrium studies indicated that the molecular continuity of the DNA molecule is maintained following formation of the complex and that the complex is due to a charge transfer mechanism.

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