POLYCYCLIC-AROMATIC-HYDROCARBON-INDUCED ALTERATIONS

IN THE PHYSIO-CHEMICAL CHARACTERISTICS OF

ESCHERICHIA COLI DEOXYRIBONUCLEIC ACID

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By

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CHAPTER I

INTRODUCTION

Prior to 1965 the interactions of polycyclic aromatic hydrocarbons with DNA (Deoxyribonucleic acid) had been but moderately studied. The summarized results of these studies may be found in an excellent review by Brookes (5). It was concluded that, although a controversy existed, an apparent interaction occurred between DNA and certain aromatic hydrocarbon molecules and that the interaction was not an artifact of the reaction system.

The controversial aspects arose through *in vitro* studies by several investigators [Boyland and Green (4) and Giovanella, McKinney and Heidelberger (8)] and is extensively reviewed by Boyland (3) and to a lesser extent by Pullman (20). Briefly, the controversy consisted of the following: Booth and Boyland (2) found that when the aromatic hydrocarbon 3,4-benzpyrene reacted with DNA, visible spectroscopic shifts occurred and that these shifts were similar to those obtained when complexes of purines and either dibenzocarbazoles or

dibenzacridines were formed. Consistent with these data were those of Liquori *et al.* (17) which stated that the degree of interaction of the aromatic hydrocarbon with DNA was dependent upon the extent of denaturation of the DNA structure.

However, Heidelberger (11) and Giovanella *et al.* (8) demonstrated that both 3,4-benzpyrene and 1,2,5,6-dibenzanthracene were removable from DNA solutions by either centrifugation or ultrafiltration. These investigators concluded that the DNA acted to stabilize the hydrocarbon as a colloid and that no true interaction existed.

A further investigation by Boyland and Green (4) indicated that the hydrocarbons studied had a greater affinity for the polymer centrifuge tubes than for the DNA, and that when these tubes were replaced either by glass or quartz tubes the complex was stable to centrifugation.

More recently, although discrepancies still exist, there appears to be some convergence of opinion, and more unambiguous results have emerged. The findings by Ball, McCarter and Smith (1) have revealed that (1) sucrose gradient centrifugation of 3,4-benzpyrene--DNA complexes failed to separate them into component parts, (2) lower pH values enhanced the hydrocarbon binding to DNA and

(3) the absorbance loss, upon heating, at 395.0 nm correlated well with the T_m (melting temperature) of control DNA. These data add credence to the view that a moderately strong interaction occurs. Furthermore, the existence of dichroic spectra (19) for aromatic-hydrocarbon-reacted DNA also suggested a firm binding, possible by an intercalation mechanism such as proposed by Lerman (15) for acridine dyes.

Investigations by Isenberg and Baird (12) led to the conclusion that the hydrocarbons phenanthrene, pyrene, and 3,4-benzpyrene complexed with DNA but not with poly A (polyadenylate) and that the degree of complex formation varied as a function of the molecular dimensions of the hydrocarbons. In addition, spectral studies by Lesko *et al.* (16) of the visible absorption patterns of 3,4-benzpyrene in several solvent systems added support to the intercalation model. Furthermore, these investigators, using exchange techniques, concluded that neither 3,4-benzpyrene nor 1,2-benzpyrene bind to specific and identical sites on the DNA molecule.

In vivo observations by DeMaeyer-Guigard and DeMaeyer (6) that viral DNA replication was inhibited by aromatic hydrocarbons suggested a possible DNA effect similar to the action of actinomycin D.

Additional evidence by Jensen et al. (13) that 7,12-dimethyl-1,2-benzanthracene selectively damaged mammalian germinal cells which were synthesizing DNA and that the incorporation of tritiated thymidine into DNA was depressed in the presence of this hydrocarbon further suggested an effect on the DNA level. These data were extended by Loeb and Gelboin (18) with the findings that both 3,4-benzpyrene and 3-methylcholanthrene stimulated microsomal enzyme activity in rat tissue and that this stimulation was inhibited by actinomycin D. Kersten (14) demonstrated that actinomycin D interacted specifically with the guanine moiety of DNA. The specific bonding arrangement was elucidated by Hamilton (10) and was found to consist of the formation of specific hydrogen bonds between the phenoxazone ring of actinomycin D and nitrogens N_1 and N_3 of the guanine moiety. Further studies by Gelboin and Klein (7) indicated that treatment of mice with actinomycin D prior to the application of 7,12-dimethyl-1,2-benzanthracene significantly reduced the number of tumors induced by the hydrocarbon. These results indirectly indicated a hydrocarbon-DNA binding since actinomycin D reacts with DNA.

Finally, recent studies by Goshman and Heidelberger (9) have led to the isolation of hydrocarbon-bound DNA

from mouse skin which had been treated *in vivo*. These investigators studied the following hydrocarbons-- 9,10-dimethyl-1,2-benzanthracene, 1,2,5,6-dibenzanthracene, 1,2,3,4-dibenzanthracene, and 3-methylcholanthrene-- and confirmed binding to DNA by radiochemical means. They, furthermore, concluded that the binding reaction was independent of DNA replication.

In view of the information discussed above, the following proposition was investigated: if binding reactions which occur *in vitro* occur *in vivo*, as would appear to be the case, then what would be the result of subsequent DNA replication on DNA composition?

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CHAPTER II

MATERIALS AND METHODS

Hydrocarbon Purification

The aromatic hydrocarbons, 1,2-benzanthracene, 1,2,5,6-dibenzanthracene, 3-methylcholanthrene, and 3,4-benzpyrene, were obtained from Aldrich Chemical Corporation in crystalline form. Each hydrocarbon was then dissolved in a minimal volume of anhydrous diethyl ether and refluxed under reduced pressure for two hours. The solutions were then diluted with an equal volume of diethyl ether, and 10 grams of "Norite 60" were added; reflux was then continued for an additional two hours. The "Norite 60" was removed by filtration through Whatman No. 1 filter paper, the solution concentrated to 2/3 volume by distillation and the hydrocarbons precipitated at -40°C. The precipitated product was separated by decantation and redissolved in a minimal volume of diethyl ether in a 1500 ml separatory funnel. An equal volume of distilled water was added and the mixture was shaken for thirty minutes. After phase separation, hydrocarbon crystals were present at the interface and were

removed by filtration. The hydrocarbons were dried under vacuum and stored in amber bottles until used.

Ethidium Bromide Purification

Ethidium bromide was purchased from California Biochemical Corporation and was dissolved in a 70% (v/v) methyl alcohol:HOH solution. The solution was vacuum-refluxed for thirty minutes and precipitated by rapid cooling in liquid nitrogen. The precipitate was collected by filtration and three additional recrystallizations were performed in 70, 60, 50% methyl alcohol solutions, respectively. The purity of the resulting precipitate was determined by melting-point determination and by CHN analysis.

In Vivo Hydrocarbon-DNA-Reaction System

Colloidal suspensions of the appropriate hydrocarbon were prepared as follows: 10.0 mg of the hydrocarbon were dissolved in 5.0 ml of redistilled dimethylsulfoxide (Matheson, Coleman and Bell), sterilized by passing through a 0.33 μ Millipore filter, and dispersed by rapid injection of the solution into 495.0 ml of sterile 3.0% tryptic soy (TS) broth (Difco). A sterile magnetic stir bar was introduced and the mixture stirred for ten minutes at room

temperature. The hydrocarbon suspensions were innoculated by washing and decanting 1.0 ml aliquots from stock culture slants of Escherichia coli B (ATCC 11303). The innoculated cultures were incubated at 37°C. with slow stirring on an insulated magnetic stirrer.

In Vivo Ethidium Bromide-DNA-Reaction System

Purified ethidium bromide was dissolved in 3.0% tryptic soy broth to a final concentration of 20 µg/ml and the media sterilized by filtration through a 0.33 µ Millipore filter. Following filtration a sterile magnetic stir bar was added and the media innoculated and incubated as described above.

Growth Assays

Growth in hydrocarbon or ethidium bromide-treated and non-treated (control) systems was defined as an increase in absorbance (660.0 nm) as a function of time. In each instance, triplicate measurements on triplicate samples were performed and the mean value, with a standard deviation bar, plotted.

The assays were implemented by removing 5.0 ml of media for each measurement, harvesting the cells by centrifugation at 4°C. and triplicate equal volume washing with diethyl ether. The washed cells were then resuspended in 5.0 ml of distilled water and the absorbance at 660.0 nm recorded.

Each growth assay was treated as an independent measurement and innocula of 0.50 absorbance units used.

Preparation of DNA

The cells were harvested by centrifugation in the cold and extracted with 10.0 ml of peroxide-free diethyl ether until no absorbance could be detected at the appropriate wavelength. The cells were then washed once with 30.0 ml of 0.15 M NaCl containing 0.1 M EDTA (ethylene diamine tetraacetic acid), pH 8.0, and resuspended in 10.0 ml of NaCl-EDTA. The DNA was extracted by the method of Marmur (4) with the following modifications: (1) 1.0 ml of a 10.0 mg/ml solution of self-digested pronase (Sigma Chemical Company) was added immediately following the addition of 1.0 ml of a 30% solution of sodium lauryl sulfate, (2) the DNA was extracted with an equal volume of freshly-distilled, water-saturated phenol followed by two extractions with three volumes of diethyl ether, (3) the DNA was precipitated with 0.6 volumes of n-propyl alcohol and dissolved in either 0.02 M NaCl- 0.001 M Tris (2-amino-2-(Hydroxy-methyl-1,3-propanediol) buffer, pH 7.0 or 0.01 M NaCl- 0.0015 M trisodium citrate, pH 7.5.

Purification of Commercial DNA

Calf thymus DNA from Lot No. 58B-1420 was obtained from Sigma Chemical Company and dissolved to a final concentration of 2.0 mg/ml in 0.01 M NaCl-0.0015 M trisodium citrate. Complete dissolution usually required four days at 4°C., with gentle stirring. After dissolution, the DNA was extracted twice with an equal volume of freshly distilled, water-saturated phenol and the aqueous phase extracted three times with diethyl ether. The DNA was then precipitated with 0.6 volumes of n-propyl alcohol and the DNA dissolved in either NaCl-trisodium citrate or NaCl- Tris buffer.

In Vitro Hydrocarbon-DNA-Reaction System

Immediately prior to the start of the reaction, a one-half volume quantity of the appropriate buffer was rapidly injected with 5.0 ml of diethyl ether containing 2.0 mg of hydrocarbon. The buffer was then purged free of ether by bubbling with buffer-saturated nitrogen gas. The remaining one-half buffer volume, containing twice the required (w/v) DNA concentration, was then added rapidly and the mixture shaken at 4°C. for four days. The non-reacted colloidal hydrocarbon was removed by filtration through two layers of Whatman No. 1 filter paper.

In Vitro Ethidium Bromide-DNA-Reaction System

Purified-ethidium bromide-DNA reactions were prepared analogous to hydrocarbons-DNA reactions with the following exceptions: (1) a final concentration of 10.0 ug/ml of ethidium bromide was used and (2) the diethyl ether step was omitted since ethidium bromide is water soluble.

Measurement of Absorption Spectra

All spectra were measured on a Coleman-Hitachi 124 recording UV-Visible spectrophotometer and rechecked at random for photometric accuracy on a Cary 14 spectrophotometer.

Optically matched 1.00 cm quartz cuvettes were cleaned prior to each determination by a fifteen second immersion in boiling nitric acid followed by automatic rinsing in each of the following: (1) 500 ml glass distilled water, (2) 100 ml anhydrous methyl alcohol, and (3) 100 ml acetone. The cuvettes were then dried in a stream of nitrogen and stored in a vacuum desiccator until used.

Immediately prior to use, the spectrophotomer monochromator compartment was purged with dry nitrogen gas for thirty minutes and the band pass adjusted to 1.0 micron. The wavelength accuracy was determined by adjusting the

diffraction grating control to the 656.1 nm deuterium emission and the absorbance calibrated using a 10.0 transmittance filter.

The spectra of native DNA samples were determined at DNA concentrations of approximately 40 µg/ml in 0.02 M NaCl- Tris buffer at pH 7.0.

All hydrocarbon-DNA-reacted spectra were determined at DNA concentrations of approximately 400 and 800 µg/ml and at very low recorder input voltages (1.0 to 5.0 mv). On occasion the signal-to-noise ratios were high and the following method was necessary to retrieve the signal. Both the maximum and minimum incremental changes in absorbance for a minimum of five repetitive scans from 500.0 nm to 310.0 nm, at 2.0 nm intervals, were plotted as separate functions. The mean value for each interval was then calculated and a "best fit" polynominal was generated by a computer technique (see appendix). The resulting equation was then used to calculate the absorption spectrum.

Differential Spectra

The measurement of differential spectra consisted of placing an equally absorbing volume of control DNA in the sample beam of the spectrophotometer and recording the

spectrum. DNA concentrations of 500 µg/ml were used in each case with the exception of ethidium bromide. The large extinction coefficient of ethidium bromide allowed the use of 80 µg/ml DNA. In order to enhance photometric accuracy, the absorbance zero base was shifted to 0.4 absorbance units following initial absorbance balancing.

Melting-Temperature (T_m) Measurements

The apparatus for the measurement of DNA melting temperatures was designed, constructed, and calibrated by the author. The basic equipment consisted of jacketed optically matched quartz cuvettes, thermometer-well-heat exchanger, high-temperature circulator, thermal programmer, and a Coleman-Hitachi 124 recording spectrophotometer. When placed in the cuvette well, the thermometer-heat exchanger acted as a receiver for the measuring thermometer or the calibrating "Iron-Constantan" thermocouple. All calibrations were with respect to ice point references.

Actual T_m measurements consisted of diluting DNA samples with either NaCl-Tris or 0.01 M NaCl-0.0015 M Na_3 citrate buffer to a final absorbance of 0.4 units. An equal volume of buffer was placed in the reference beam of the spectrophotometer, the temperature adjusted to 25.0°C., and

the absorbance recorded. The temperature was then rapidly raised to 50.0°C. and the "Teflon" cuvette stoppers secured by twisting. Temperature increases were then either performed manually in 1.0°C. increments or linearly programmed until no further change in absorbance was observed. The data obtained from each T_m determination were then corrected for thermal expansion, converted to A_t/A_{25} (absorbance at the observed temperature relative to 25.0°C.), the minimum and maximum values found, and the T_m determined. By definition (3), the T_m value for a DNA sample corresponds to one-half the increase in total relative absorbance. Lastly, by equation number 1 of Schildkraut and Lifson (5), the guanine-cytosine content (%GC) of the DNA sample was computed. All of the above computations were performed with the computer program listed in the appendix.

$$GC = 2.44\ (T_m - 78.3 - 16.6 \log M) \qquad (1)$$

where M is the molar concentration of the cation.

Spectral Analysis of Native DNA (ANDS - 1)

The method used was essentially that of Hirschman and Felsenfeld (2), which was adapted for computer solution. The techniques discussed below allowed the calculation of DNA base composition as mole fraction of adenine-thymine (AT)

base pairs. Fundamentally, the determination of the mole fraction AT pairs consisted of accurately recording the absorption spectrum of a purified DNA sample as discussed on the previous page and solving the following equation:

$$\Delta A_i = \alpha_i X + \beta_i Y + \gamma_i Z, \qquad (2)$$

which is a linear equation in three unknowns, and

$$\alpha_i = (1 - 2K_i)(\epsilon_{AA_i} + \epsilon_{GG_i}),$$
$$\beta_i = 2[K_i(\epsilon_{AA_i} + \epsilon_{GG_i}) - \epsilon_{GG_i}],$$
$$\gamma_i = \epsilon_{GG_i},$$
$$X = C(\phi^2 + \delta\phi),$$
$$Y = C\phi,$$
$$Z = C.$$

The value ΔA_i is the absorbance of the DNA sample at wavelength i, CØ is the molar nucleotide concentration in moles/liter, and Ø is the mole fraction AT pairs.

The actual solution for CØ and C required the formation of the sums $\mu_1 = \sum A_i \alpha_i$, $\mu_2 = \sum A_i \beta_i$, $\mu_3 = \sum A_i \gamma_i$ where $\alpha_i, \beta_i, \gamma_i$ were determined by "least squares fit" from pre-existing extinction coefficients for known DNAs. Also, six additional constants ($S_1 \ldots S_6$) were determined from matrix equations such as those on the following page.

$$\begin{pmatrix} \sum\alpha^2 & \sum\alpha\beta & \sum\alpha\gamma \\ \sum\alpha\beta & \sum\beta^2 & \sum\beta\gamma \\ \sum\alpha\gamma & \sum\beta\gamma & \sum\gamma^2 \end{pmatrix} \begin{pmatrix} X \\ Y \\ Z \end{pmatrix} = \begin{matrix} \mu_1 \\ \mu_2 \\ \mu_3 \end{matrix} \qquad (3)$$

where the S constants are the determinantal solutions for X, Y and Z. Both the α_i, β_i, γ_i and the S constants were stored as constant data in the computer program and retrieved for each solution. Thus, from the stored constant data and the sums μ_1, μ_2 and μ_3 equations 4, 5, and 6 were solved for molar nucleotide concentration, CØ, and mole fraction AT pairs.

$$C\phi = \mu_1 S_1 + \mu_2 S_2 + \mu_3 S_3 . \qquad (4)$$

$$C = \mu_1 S_4 + \mu_2 S_3 + \mu_3 S_5 . \qquad (5)$$

$$\phi = C\phi / \phi. \qquad (6)$$

Viscosity Measurements

All viscosity measurements were performed with a Beckman "low shear viscometer" of the Zimm and Crothers (7) design. With the exception of the magnetic field drive, this instrument is of the typical rotating cylinder viscometer design. Immediately prior to use, both the rotor and the stator were cleaned with chromic acid cleaning solution, carefully rinsed with glass distilled water, and kept wet

with buffer until use. Samples of DNA, 25 µg/ml in NaCl-Tris buffer, were carefully pipetted into the stator compartment until the rotor floated to a predetermined level. The complete viscometer was equilibrated to 25° C. and the magnetic drive started. In every case the rotor was allowed to make three revolutions before timing was started. At the end of five revolutions the time was recorded and the temperature checked. All measurements were performed in triplicate and the mean value determined.

The results were reported as average shear stress in the annulus in accordance with the following derivation:

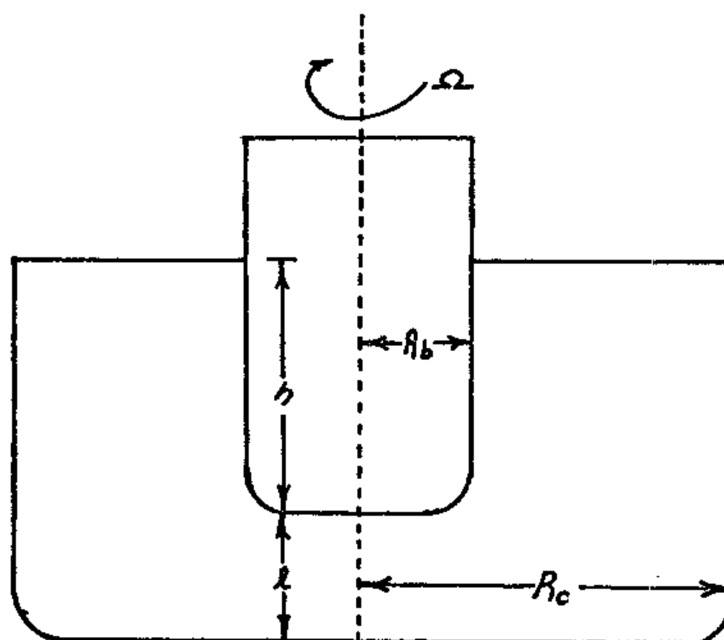

Fig. 1--A generalized rotating cylinder viscometer

In order for the following relationships of the viscometer shown in Figure 2 to hold true, certain assumptions must be met:

1. The liquid must be incompressible.
2. There must exist laminar flow.
3. The streamlines of flow with respect to the horizontal plane must be perpendicular to the axis of rotation.
4. All time derivatives must be equal to zero.
5. There must be zero relative motion between the rotor and the stator.
6. Motion must occur in two dimensions only.
7. The system must be isothermal.

Given that the cylindrical equation of continuity of Bird et al. (1) is

$$\partial\rho/\partial\theta + (1/r)[\partial(\rho r v_r)/\partial r] + (1/r)[\partial(\rho v_\phi)/\partial\phi] + \partial(\rho v_z)\partial z = 0 \quad (7)$$

where

τ = relaxation time (η/G),
θ = time (seconds),
r = radial distance measured from center,
v = velocity component,
ϕ = angular component,
p = density.

Then from the assumptions

$$(\rho/r)(dv\phi/d\phi) = 0. \quad (8)$$

Therefore, the velocity component, v_ϕ, = a constant and the equation of motion $(1/r^2)\ d(rs_{\tau\phi})/dr = 0$ (9)

integrating $\int(1/r^2)\, d\,(rs_{\tau\phi})/dt = r^2 s = C_1$ (10)

and $s_{\tau\phi}$ = s since there remains only one stress component since torque = area x radius x shear stress or, $M = 2\pi rhrs$, (11)

but from equation number ten, $c_1 = r^2 s$, therefore, division by c_1 gives

$$r^2 h = M/2\pi h, \tag{12}$$

and for any constant moment in steady state (6)

$$M = s_b 2\pi R_b^2 h = s_c 2\pi R_c^2 h$$

and $$s_b/s_c = R_c^2/R_b^2 = \epsilon^2 \tag{13}$$

which states that the stress ratio varies inversely with the square of the radii of the rotor and the stator.

Where there exists a velocity gradient, dv/dr, then

$$dv/dr = \omega + r\, d\omega/dr. \tag{14}$$

From equations eleven and fourteen,

$$-r\, d\omega/dr = f(M/2\pi r^2 h) = f(s). \tag{15}$$

Differentiation of fifteen and substitution for M gives

$$dr/r = -ds/2s \tag{16}$$

and $$d\omega = (1/2)\, f(s)\, ds/s. \tag{17}$$

Integrating equation sixteen from the rotor to the stator, $s = s_b$, $s = s_c$ and from $\omega = 0$ to Ω (angular velocity)

$$\Omega = (1/2) \int_{s_b}^{s_c} f(s)\, ds/s. \tag{18}$$

Since no relative motion occurs between the rotor and the stator, the shear stress at the surface of the stator is zero and equation eighteen may be rearranged to give

$$\Omega = (1/2) \int_{s_b}^{0} f(s)\, ds/s \tag{19}$$

which is then differentiated to give

$$f(s_b) = -2d\Omega/d \ \ln s_b, \tag{20}$$

and

1. $f(s_b)$ is found by plotting Ω against $\ln s_b$, and
2. validity is established only for solutions having no yield value.

Finally, for a viscometer of narrow rotor to stator separation and from equation thirteen, the following approximation may be made:

$$\langle s \rangle = (R_c^2 + R_b^2)/R_c^2 - R_b^2 \tag{21}$$

where $\langle s \rangle$ is the mean rate of shear.

For the experiments discussed below was equal to the speed of the rotor in revolutions per second and $R_c = 0.65$ cm and $R_b = 0.50$ cm.

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CHAPTER III

RESULTS

The results obtained for the four separate hydrocarbons (1,2-benzanthracene, 1,2,5,6-dibenzanthracene, 3-methylcholanthrene, and 3,4-benzpyrene) and ethidium bromide under *in vivo* growth conditions are given in Figure 2 (See appendix II for Figs. 2, 3, 4). These data represent the results of four separate experiments in triplicate sets. Although not presented, the results of statistical analysis indicate the data to be within the tolerances of the experimental method. Also, since there exists very close agreement between the family of curves for both the dimethylsulfoxide and the dimethylsulfoxide-control, it may be concluded that dimethylsulfoxide exhibits little or no effect on *Escherichia coli* B growth in the concentrations used. The observed effects may, therefore, be attributed to either the hydrocarbons or the ethidium bromide.

The data obtained for DNA-hydrocarbon binding under *in vitro* conditions, Figure 3, is in agreement with the findings of Liquori *et al*. (2) for 3,4-benzpyrene. Furthermore, it

may be seen that although little binding occurs for 1,2-benzanthracene there occurs an increase for 1,2,5,6-dibenzanthracene, 3-methylcholanthrene and 3,4-benzpyrene.

Unless otherwise stated all _in vitro_ data presented are for _Escherichia coli_ B.

In addition to DNA binding there is an observed shift, Table I, toward longer wavelengths at the major absorption bands of the hydrocarbons when compared to spectra obtained in cyclohexane.

TABLE I

ULTRAVIOLET ABSORPTION PEAKS OF AROMATIC HYDROCARBONS

Hydrocarbon: Solvent System	Wavelength (nm)		
1,2-benzanthracene: cyclohexane	342	358	388
1,2-benzanthracene: DNA	350	363	393
1,2,5,6-dibenzanthracene: cyclohexane	320	333	348
1,2,5,6-dibenzanthracene: DNA	331	340	360
3-methylcholanthrene: cyclohexane	+	372	401

TABLE I Continued

Hydrocarbon: Solvent System	Wavelength (nm)		
3-methylcholanthrene: DNA	+	380	412
3,4-benzpyrene: cyclohexane	348	363	385
3,4-benzpyrene: DNA	355	372	395

+No absorption.

Also, Figure 4 indicates the existence of an isobestic point at 510 nm for ethidium bromide reacted DNA; whereas, no isobesticity was detected in hydrocarbon reacted DNAs.

The use of differential spectrophotometry (1) allows the nullification of any contribution to the spectral pattern which the solvating molecule may make. It also, under carefully controlled conditions, enhances photometric precision in addition to the gain in accuracy. When both ethidium bromide and hydrocarbon reacted-DNAs were examined differentially, with respect to DNA, Table II, it was found that a shift towards longer wavelengths still existed, but that these shifts were somewhat less than those observed from simple absorption spectra.

TABLE II

DIFFERENTIAL ULTRAVIOLET ABSORPTION PEAKS FOR ETHIDIUM BROMIDE AND AROMATIC HYDROCARBONS

Compound: Solvent System	Wavelength (nm)		
Ethidium bromide: NaCl-Tris	+	+	475
Ethidium bromide: DNA	+	+	496
1,2-benzanthracene: cyclohexane	342	358	388
1,2-benzanthracene: DNA	345	360	392
1,2,5,6-dibenzanthracene: cyclohexane	320	333	348
1,2,5,6-dibenzanthracene: DNA	326	339	354
3-methylcholanthrene: cyclohexane	+	372	401
3-methylcholanthrene: DNA	+	377	410
3,4-benzpyrene: cyclohexane	348	363	385
3,4-benzpyrene: DNA	354	370	383

+No absorption.

Both simple absorption spectra and differential spectra were obtained for DNAs extracted from cells grown in the presence of both ethidium bromide and polycyclic hydrocarbons. While DNA preparations from ethidium bromide-grown cells

showed slight amounts of bound dye, it was impossible to obtain bound DNA from hydrocarbon-grown cells.

The results of *in vitro* thermal denaturation, T_m, and rate of shear, $\langle s \rangle$, studies are summarized in Table III and indicate that increases in both T_m and $\langle s \rangle$ occur following binding. These increases are the greatest for ethidium bromide and the least for 1,2-benzanthracene.

TABLE III

T_m and $\langle s \rangle$ VALUES FOR *IN VITRO* AROMATIC HYDROCARBON AND ETHIDIUM BROMIDE REACTED DNAs IN 0.02 M NaCl-Tris

Sample	No. of Observations	T_m(°C.)	$\langle s \rangle$ dynes/cm^2
Control	10	75 0.3	382
Ethidium Bromide	7	87	597
1,2-benz-anthracene	5	76	396
1,2,5,6-di-benzanthracene	7	77	411
3-methyl-cholanthrene	5	77.9	425
3,4-benzpyrene	9	79	440

The analysis, however, of *in vivo* prepared DNAs for %GC by T_m measurements, mole fraction AT pairs by ANDS - 1, and viscosity indicate the following: (1) a decrease in the %GC concentration, (2) an increase in the mole fraction AT pairs, and (3) a decrease in the $\langle s \rangle$. These observations are summarized in Table IV.

TABLE IV

THE *IN VIVO* EFFECTS OF AROMATIC HYDROCARBONS AND ETHIDIUM BROMIDE ON THE CHARACTERISTICS OF *ESCHERICHIA COLI* DNA

Sample	No. of observations	%GC	Mole Fraction AT Pairs	$\langle s \rangle$ dynes/cm^2
Control	10	48	51	390
Ethidium bromide	8	41	59	253.5
1,2-benzanthracene	8	48	52	387
1,2,5,6-dibenzanthracene	6	47.5	53	382
3-methylcholanthrene	5	46.8	55	384
3,4-benzpyrene	13	45.6	56	271

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CHAPTER IV

DISCUSSION

The first suggestion for an interaction between polycyclic aromatic hydrocarbons and DNA was made in 1953 by Booth and Boyland (1). In a later study Boyland and Green (2) proposed an intercalation mechanism similar to that proposed by Lerman (11). There remained, however, a controversy between Giovanella *et al.* (5) and Heidelberger (8) with respect to the methods used and the interpretation of the results.

In an attempt to resolve the difficulties, Boyland and Green (2) and Nagata *et al.* (14) performed additional studies using different techniques and appeared to establish the binding effect. Additional evidence was supplied later by Isenberg and Baird (10) and by Lesko and his co-workers (12).

The data presented in Figure 3 of this paper further support the interaction proposition. It would also seem possible to rate the degree of interaction as follows: 1,2-benzanthracene $<$ 3-methylcholanthrene $<$ 1,2,5,6-dibenzanthracene $<$ 3,4-benzpyrene.

In view of the spectral shifts towards longer wavelengths (Tables I and II), the possibility of a "charge transfer" type mechanism, as developed by Pullman and Pullman (15) and Hoffman and Ladik (9), should be considered. It is noted, however, that spectral shifts alone do not constitute proof for a "charge transfer" mechanism but merely suggest it as a possibility.

The observations in Figure 4, that ethidium bromide-reacted-DNA contains an isobestic point, confirm the findings of Waring (16). The existence of an isobestic point suggests that the observed spectra are a result of two forms of ethidium bromide, i.e., a free and a bound form. The lack of isobesticity for hydrocarbon-reacted DNAs implies that either the spectral contribution is due to a bound form or that any free hydrocarbon lies below the limit of instrumental capability. In view of the extremely low aqueous solubilities of the hydrocarbons studied, the latter may well be the case. Nevertheless, it would appear that there exists an interaction between polycyclic aromatic hydrocarbons and DNA and that this interaction is amenable to spectrophotometric investigation.

Viscosity determinations also present data confirming hydrocarbon binding to DNA. The results summarized in

Table III indicate an increase in the mean rate of shear, $\langle s \rangle$, for both hydrocarbon and ethidium bromide reacted DNAs. These data would indicate that following binding there is an overall increase in the rigidity of the DNA molecule which would therefore indirectly lend support to the intercalation concept (11).

Obviously, information obtained *in vitro* from isolated systems cannot necessarily be extrapolated to *in vivo* systems without extensive investigation. Using isotopic methods, Brookes and Lawley (3) and, more recently, Goshman and Heidelberger (6) have been able to isolate hydrocarbon-labeled DNA from the skin of higher animals.

It was first suspected that if certain hydrocarbons would react *in vivo* with DNA then perhaps one of the manifestations of the reaction would be an alteration in growth kinetics. The data presented in Figure 2 indicate that some effect is observed but that the magnitude of the effect is small. That this statement is valid is especially relevant when the data are compared to that of ethidium bromide. It would appear that ethidium bromide has an immediate suppressive effect on the growth of *Escherichia coli* B and that this effect is of several orders of magnitude greater than that of hydrocarbons.

The most interesting results obtained in this study are those summarized in Table IV. These data indicate that the DNA isolated from cells grown in the presence of certain polycyclic hydrocarbons is altered with respect to its nucleotide composition. This alteration is reflected in the decrease in the %GC value and a concomitant increase in the mole fraction AT pairs. At this point it is important to note that there exists an exact correlation between the extent of hydrocarbon bound *in vitro* and the decrease in %GC value *in vivo*. That is, the magnitude of the %GC decrease varied directly with the hydrocarbon present in the growth medium in the order 1,2-benzanthracene < 3-methylcholanthrene < 1,2,5,6-dibenzanthracene < 3,4-benzpyrene. As would be expected, the increase in mole fraction AT pairs was consistent with the same series.

Although somewhat disappointing, the mean rate of shear $\langle s \rangle$ data for *in vivo* systems reflect differences when compared to control values. The most important results are, however, that an increase in $\langle s \rangle$ exists for *in vitro* systems and a decrease exists for *in vivo* systems. As stated above, there would appear to be a loss in molecular rigidity, and this loss is greater than control values for *in vivo* systems.

The data obtained for *in vivo* ethidium bromide reactions agree qualitatively with the effects of hydrocarbons on DNA. Quantitatively, however, the difference is one of magnitude.

In view of the information discussed above, it would appear that a discrete relationship exists between certain polycyclic aromatic hydrocarbons, ethidium bromide, and DNA. Under *in vivo* conditions these compounds are able to influence the replication of the DNA. Furthermore, these influences are permuted towards increases in AT pair concentrations. Since neither hydrocarbon nor ethidium bromide-bound DNA was isolated from *in vivo* systems to any appreciable extent, it is suggested that perhaps weak binding forces were destroyed during extraction and the DNA-bound compounds removed. It is also possible that, since protein binding is known to occur (13) and to be quite extensive, dilution of the hydrocarbon by protein binding occurred as a function of incubation time.

Since there occurs an increase in AT pair concentration, it is suggested that perhaps GC pairs are attacked preferentially by the hydrocarbons or at least are more subject to their reactivity. This view is reinforced by the findings of Gelboin and Klein (4) that tumor induction is substantially inhibited in mice by pre-treatment with actinomycin D, which

has been shown to react with guanine residues in DNA (7). Regardless of the binding site, there remains the possibility that during replication of hydrocarbon-bound DNA, the template induces a replication or copy error by presenting a topographical configuration which more closely resembles an AT pair than a GC pair and an error is copied in.

Whatever the mechanism involved may be, there appears to be a definite DNA alteration, and this alteration results in a decrease in GC pairs and an increase in AT pairs.

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APPENDIX I

The below-listed programs contain the necessary steps for (1) computation of the necessary generator equations for spectral curve fitting, (2) calculation of T_m and %GC values and (3) computing the mole fraction AT base pairs for any given DNA molecule.

All of these listed programs were designed to be used on IBM (International Business Machines) 1620, 20k computer, equipped with either AFIT or PDQ "FORTRAN" with format language.

```
C       POLYNOMIAL CURVE FIT FOR UP TO  5TH ORDER--PDP FORTRAN
C      SS3 -- ON TO PRINT BACK SOLUTIONS AFTER EACH FIT
       DIMENSION X(250), Y(250),              A(7,7),SUMX(15), SUMY(7)
     1 READ 10, N, TOL, LAST
    10 FORMAT (I3,E10.4,I2)
       DO 40 I=1,N
       READ 11, X(I) , Y(I)
    11 FORMAT (2E10.4)
    40 CONTINUE
    70 SUMX(1)=N
       SUMX(2)=0.
       SUMX(3)=0.
       SUMY(1)=0.
       SUMY(2)=0.
       DO 90 I=1,N
       SUMX(2)=SUMX(2)+       X(I)
       SUMX(3)=SUMX(3)+       X(I)*X(I)
       SUMY(1)=SUMY(1)+       Y(I)
    90 SUMY(2)=SUMY(2)+       X(I)*Y(I)
       NORD=1
    91 L = NORD + 1
       KK=L+1
       DO 101 I=1,L
       DO 100 J=1,L
       IK=J-1+I
   100 A(I,J)=SUMX(IK)
   101 A(I,KK)=SUMY(I)
       DO 140 I=1,L
       A(KK,I)=-1.
       KKK=I+1
       DO 110 J=KKK,KK
   110 A(KK,J)=0.
       C=1./A(1,I)
       DO 120 II=2,KK
       DO 120 J=KKK,KK
   120 A(II,J)=A(II,J)-A(1,J)*A(II,I)*C
       DO 140 II=1,L
       DO 140 J=KKK,KK
   140 A(II,J)=A(II+1,J)
       S2=0.
       DO 160 J=1,N
       SI=A(1,KK)
       DO 150 I=1,NORD
   150 S1=SI+A(I+1,KK)*X(J)**I
   160 S2=S2+(S1-Y(J))*(S1-Y(J))
       B=N-L
       S2 = (S2/B)**.5
   163 CONTROL 971
       PRINT 204
       PRINT 205, NORD, TOL, S2, N
       PRINT 206
       DO 164 I=1,L
       J=I-1
   164 PRINT 205, J, A(I,KK)
       IF(SENSE SWITCH 3)167,165
   165 IF(NORD-LAST)166,167,166
   166 IF(S2-TOL)167,167,171
   167 PRINT 208
       DO 169 I=1,N
```

```
      S1=A(1,KK)
      DO 168 J=1,NORD
  168 S1=S1+A(J+1,KK)*X(I)**J
      S3=Y(I)-S1
  169 PRINT 209, X(I),Y(I),S1,S3
      IF(NORD-LAST)170,1,1
  170 IF(S2-TOL)1,1,171
  171 NORD=NORD+1
      J=2*NORD
      SUMX(J)=0.
      SUMX(J+1)=0.
      SUMY(NORD+1)=0.
      DO 172 I=1,N
      SUMX(J)=SUMX(J)+X(I)**(J-1)
      SUMX(J+1)=SUMX(J+1)+X(I)**J
  172 SUMY(NORD+1)=SUMY(NORD+1)+Y(I)*X(I)**NORD
      GO TO 91
  204 FORMAT ( /4H ORD,10X,3HTOL,13X,3HDEV,4X,3HOBS/)
  205 FORMAT ( I3,2E16.8,I4)
  206 FORMAT( / 8H  COEFF. /)
  208 FORMAT ( /6X, 1HX, 17X, 1HY, 17X, 2HYC, 15X, 4HDIFF/)
  209 FORMAT ( E14.8,4X,E14.8,4X,E14.8,4X,E14.8)
      END
```

53.9

25	1.0000
26	1.0003
27	1.0005
28	1.0008
29	1.0011
30	1.0014
31	1.0017
32	1.0020
33	1.0024
34	1.0027
35	1.0030
36	1.0034
37	1.0037
38	1.0041
39	1.0045
40	1.0049
41	1.0053
42	1.0057
43	1.0061
44	1.0065
45	1.0069
46	1.0073
47	1.0078
48	1.0082
49	1.0087
50	1.0091
51	1.0096
52	1.0100
53	1.0105
54	1.0110
55	1.0115
56	1.0120
57	1.0125
58	1.0131
59	1.0135
60	1.0141
61	1.0146
62	1.0152
63	1.0157
64	1.0162
65	1.0168
66	1.0174
67	1.0180
68	1.0185
69	1.0191
70	1.0197
71	1.0203
72	1.0209
73	1.0215
74	1.0221
75	1.0228
76	1.0234
77	1.0240
78	1.0247
79	1.0253
80	1.0260
81	1.0266
82	1.0273

```
   83  1.0280
   84  1.0287
   85  1.0293
   86  1.0300
   87  1.0308
   88  1.0314
   89  1.0321
   90  1.0329
   91  1.0336
   92  1.0343
   93  1.0351
   94  1.0358
   95  1.0365
   96  1.0373
   97  1.0380
   98  1.0388
   99  1.0396
  100  1.0404
  101  1.0411
  102  1.0419
  103  1.0426
  104  1.0433
  105  1.0441
99999 99999
```

```
      DIMENSION T(82),VT(82),A(81),AP(81),ATA25(81),XT(81)
      READ,XX
C     XX - VARIABLE 1 IN GC FORMULA
      DO 100 I=1,81
      T(I)=0
  100 VT(I)=0
      DO 1 I=1,82
      READ,T(I),VT(I)
      IF(T(I)-99999.)1,200,1
    1 CONTINUE
  200 I=0
      DO 1000 L=1,81
      XT(L)=0
      A(L)=0
      AP(L)=0
 1000 ATA25(L)=0
    2 I=I+1
      READ,TI,AI
      IF(TI-99999.)6,5,6
    6 DO 3 L=1,81
      IF(TI-T(L))3,4,3
    3 CONTINUE
      STOP
    4 A(I)=AI
      XT(I)=TI
      AP(I)=A(I)*VT(L)
      ATA25(I)=AP(I)/AP(1)
      GO TO 2
    5 I=I-1
      IF(SENSE SWITCH 1)500,501
C     SS1 - ON READ IN MIN AND MAX
C             OFF READ IN INTERVAL CHANGE AND STARTING POINT
  500 READ,TX1,XMIN,TX2,XMAX
      DO 502 L=1,81
      IF(TX1-T(L))502,503,502
  502 CONTINUE
      STOP
  503 XMIN=XMIN*VT(L)/AP(1)
      DO 504 L=1,81
      IF(TX2-T(L))504,505,504
  504 CONTINUE
      STOP
  505 XMAX=XMAX*VT(L)/AP(1)
      GO TO 510
  501 READ, ZXY,I99
C     ZXY - INTERVAL CHANGE
C     I99 - INTERVAL TO START COMPARISON AT - 1.
      DO 7 L=I99,I
      DIFF=ABS(ATA25(L)-ATA25(L-1))
      IF(DIFF-ZXY )7,8,8
    7 CONTINUE
      STOP
    8 XMIN=ATA25(L-1)
      DO 9 JJ=1,I
      J=I-JJ+1
      DIFF=ABS(ATA25(J)-ATA25(J-1))
      IF(DIFF-ZXY )9,9,10
    9 CONTINUE
      STOP
```

```
 10 XMAX=ATA25(J)
510 XMID=(XMAX-XMIN)/2.+XMIN
    DO 201 LI=1,81
    IF(XMID-ATA25(LI))203,202,201
201 CONTINUE
    STOP
202 TEMP=XT(LI)
    GO TO 205
203 VX=ATA25(LI)-ATA25(LI-1)
    TEMP=XT(LI-1)+(XMID-ATA25(LI-1))/VX*(XT(LI)-XT(LI-1))
205 PER=(AP(I)-AP(1))/AP(I)*100.
    GC=(TEMP-XX)*2.44
    PRINT20
 20 FORMAT(10X,4HTEMP,15X,1HA,15X,2HAP,10X,7HAPT/A25/)
    DO 21 II=1,I
 21 PRINT22,XT(II),A(II),AP(II),ATA25(II)
 22 FORMAT(F14.0,F16.4,F17.4,F16.4/)
    PRINT 25,XMIN,XMAX
 25 FORMAT(/5X,3HMIN,F7.4/,5X,3HMAX,F7.4/)
    PRINT23
 23 FORMAT(5X,8HMIDPOINT/)
    PRINT24,XMID,TEMP
 24 FORMAT(5X,7HAPT/A25,F7.4,/5X,4HTEMP,F7.2/)
    PRINT240,PER,GC
240 FORMAT(5X,2HPC,F8.2,/5X,4HGC =,F10.4)
    CGTL=CUM(971.1)
    GO TO 200
    END
```

```
C       ANALYSIS OF NATIVE DNA SPECTRA
        DIMENSION S(5),A(12),B(12),G(12),X(12)
        DO 2 I=1,12
      2 READ 1,A(I),B(I),G(I)
      1 FORMAT(3F10.0)
        DO 6 I=1,5
        READ 7,S(I)
      6 S(I)=S(I)*1.E-07
      7 FORMAT(F7.4)
     10 DO 3 I=1,12
      3 READ 4,X(I)
      4 FORMAT(F10.3)
        XM1=0
        XM2=0
        XM3=0
        DO 5 I=1,12
        XM1=XM1+A(I)*X(I)
        XM2=XM2+B(I)*X(I)
      5 XM3=XM3+G(I)*X(I)
        CO=XM1*S(1)+XM2*S(2)+XM3*S(3)
        C=XM1*S(4)+XM2*S(3)+XM3*S(5)
        O=CO/C
        CONTROL 971
        DO 20 I=1,12
     20 PRINT 21,X(I)
     21 FORMAT(25X,F10.4)
        PRINT8,XM1,XM2,XM3,CO,C,O
      8 FORMAT(5X,4HM1 =,F10.2/,5X,4HM2 =,F10.2/,5X,4HM3 =,F10.2/
       15X,4HCO =,E14.7/,5X,3HC =,E14.7/,5X,3HO =,E14.7)
        GO TO 10
        END
```

-2026	-656	3952
-1889	-1251	5031
-1390	-1917	6338
43	-2830	7480
-319	-1807	7616
-608	-1141	7307
2515	-3379	7052
871	-1409	5740
-386	-154	4587
1159	-1558	3938
1797	-2424	3164
1197	-2099	2188

0.9329
2.0631
0.6198
0.2792
0.2124

APPENDIX II

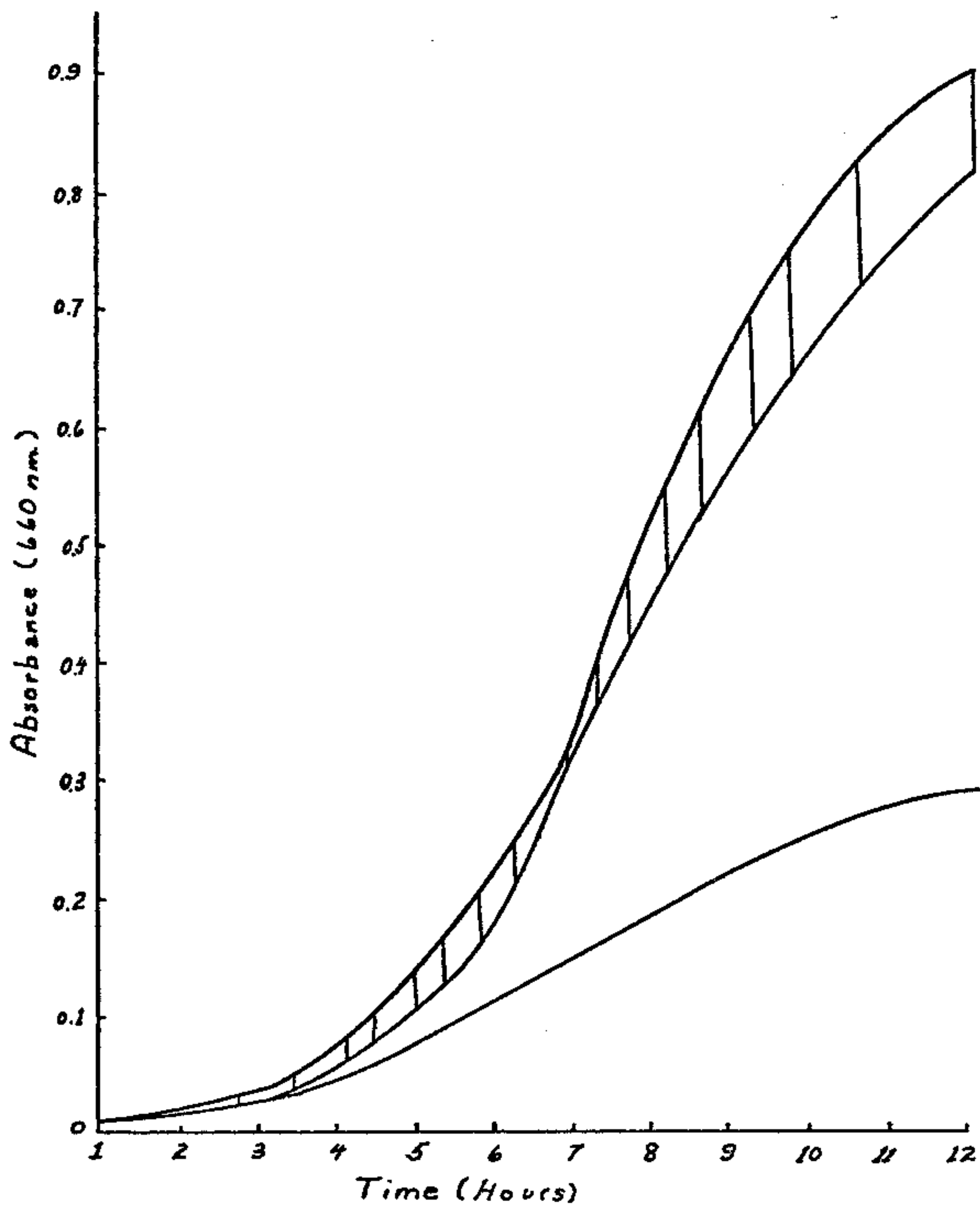

Fig. 2--The effect on the growth of Escherichia coli B of aromatic hydrocarbons; 1,2-benzanthracene, 1,2,5,6-dibenzanthracene, 3-methylcholanthrene and 3,4-benzpyrene (111) and ethidium bromide.

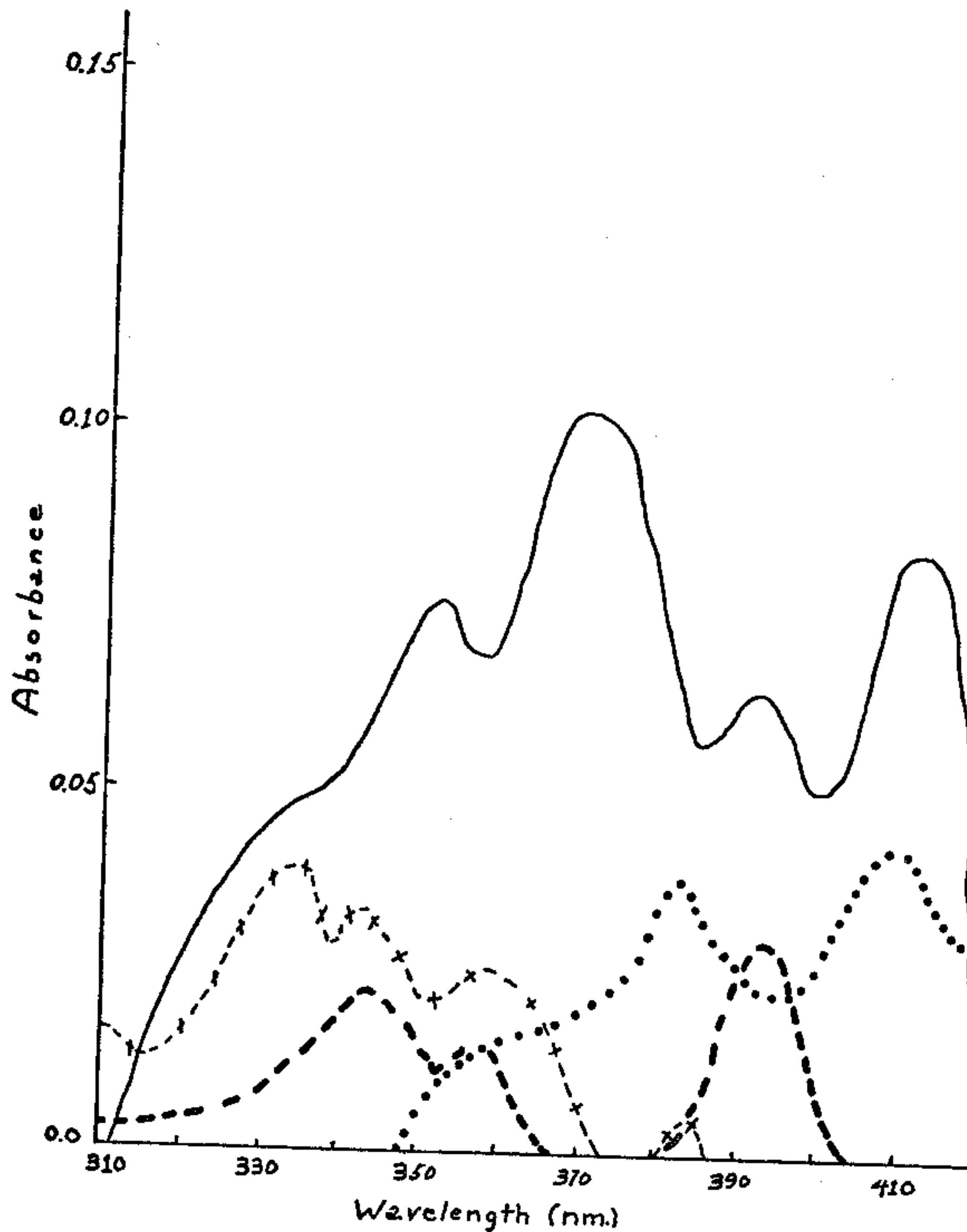

Fig. 3--The absorption spectra of <u>in vitro</u> polycyclic hydrocarbon reacted DNA, 1,2-benzanthracene (---), 1,2,5,6-dibenzanthracene (///), 3-methylcholanthrene (...) and 3,4-benzpyrene (___).

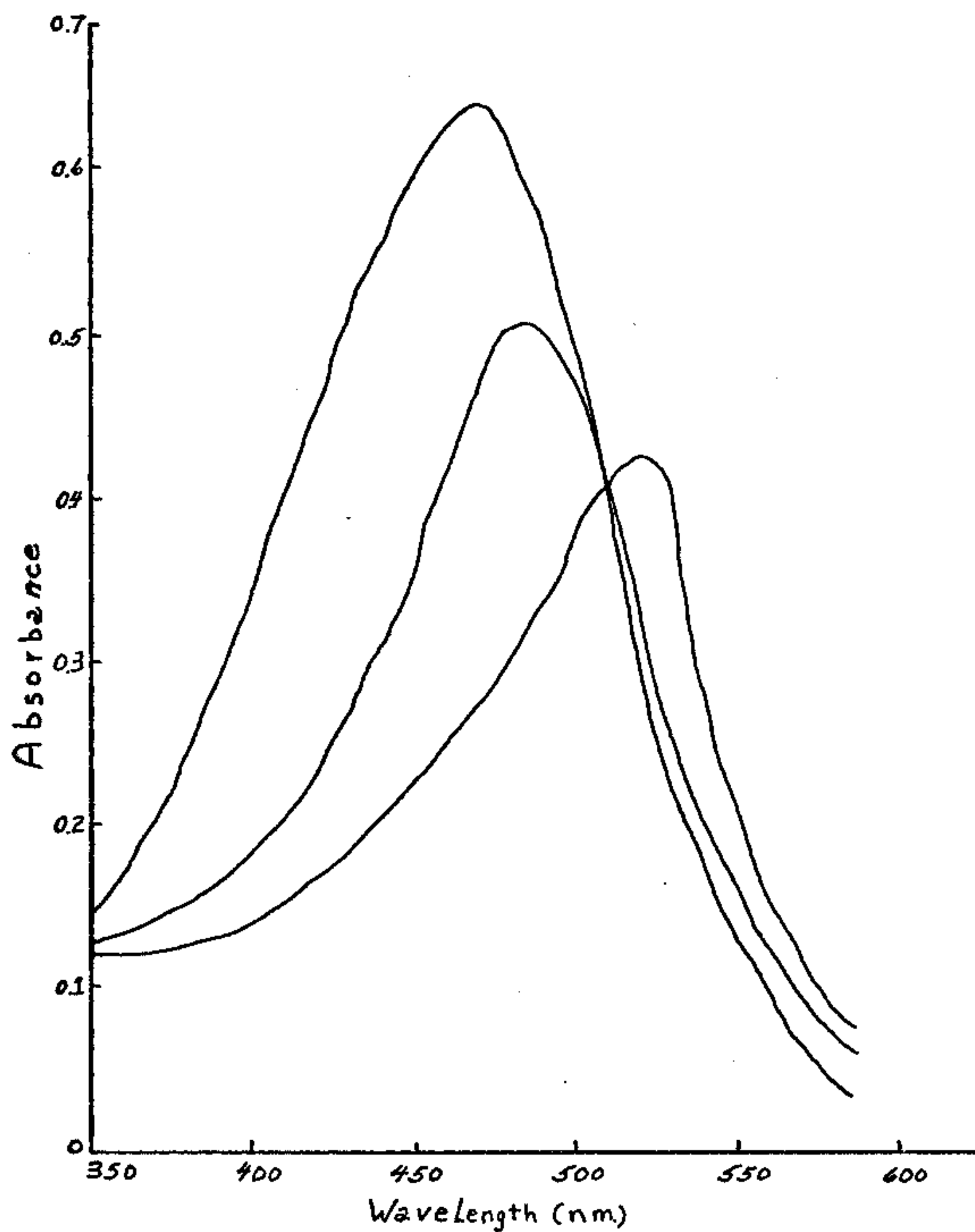

Fig. 4--The absorption spectrum of ethidium bromide and ethidium bromide reacted DNA at different DNA concentrations.

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