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Title: INTERCALATION OF HYDROCARBONS IN POLYNUCLEOTIDES

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The binding of eleven polycyclic, aromatic hydrocarbons to the acid form of poly A has been investigated. The hydrocarbons were chosen to test a size criterion. Model building showed that some hydrocarbons could intercalate in the helix and be well protected from contact with the aqueous solvent. Experimentally it was found that those hydrocarbons which were small enough to be protected were bound to poly A. Hydrocarbons which were too large to be so protected did not bind. A size criterion for the binding of hydrocarbons to poly A therefore exists.

The flow dichroism of four poly A-hydrocarbon complexes was measured. The flow dichroism measurements were consistent with the intercalation model.

The dissociation energy of several hydrocarbons is found to occur in an order that is different from that given by the size criterion. There was no correlation between the binding of individual hydrocarbons to poly A and the energy of dissocation of individual hydrocarbons from the crystalline state.

The size criterion for poly A, together with a previously examined size criterion for DNA, and the flow dichroism data serve as unequivocal evidence that hydrocarbons intercalate in polynucleotides.

Intercalation of Hydrocarbons in Polynucleotides

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INTERCALATION OF HYDROCARBONS IN POLYNUCLEOTIDES

INTRODUCTION

A number of laboratories have established that a group of polycyclic, aromatic hydrocarbons will complex to DNA (2,5,6,7,13,20,24,25,28,30, 31,34,36). Polycyclic, aromatic hydrocarbons are practically insoluble in water; however, their solubility is greatly enhanced in DNA solutions. The first laboratories to report solubilization of hydrocarbons in DNA solution were Boyland and Green (6) and Liquori et al. (31). These workers proposed an intercalation model for the binding of aromatic hydrocarbons to DNA. In this intercalation model, the hydrocarbons slip between neighboring base pairs. The model seemed reasonable since the hydrocarbons are nearly insoluble in water and could be protected from the aqueous medium in an intercalation model. Because of its credibility, the intercalation model has been central to the theme of hydrocarbon binding to DNA. Many laboratories have worked on the intercalation problem; however, to date, no conclusive test of the model has been presented.

In 1967, it was proposed (23,24) that a size criterion might exist for the binding of aromatic hydrocarbons to DNA. The size criterion, as the name implies, is a hypothesis that the size of the hydrocarbon is a determining factor in binding to DNA. The size criterion states that only hydrocarbons bind that are protected from the aqueous medium. Therefore, hydrocarbons that intercalate and do not project into the aqueous solvent bind to DNA. Hydrocarbons too large to orient under a

base pair without protrusion from the DNA helix will not bind or will bind to a reduced extent.

Such a criterion presupposes that hydrophobic interactions not only exist, but play a dominant role in complex formation, at least to the first approximation. If, for example, polarizability forces or dispersion forces between the nucleotides and the hydrocarbons dominate binding, then the hydrocarbons exceeding the size criterion might bind to DNA.

In fact, it was found (13) that even if a small portion of a hydrocarbon protruded from the helix, the hydrocarbon would not bind to DNA. For example, in the series anthracene, 9-methyl anthracene, and 9-phenyl anthracene, the first two hydrocarbons were found to bind to DNA, but no binding of 9-phenyl anthracene was found. Model building showed that the phenyl group must extend into the medium. Furthermore, in all three complexes, model building illustrated that the anthracene moiety intercalated.

In spite of the fact that a limited number of hydrocarbons fulfill the size criterion, the criterion has not rigorously been shown to be valid. Larger hydrocarbons which do not bind also tend, in general, to have higher crystal binding energies than smaller ones. Since the hydrocarbon-DNA binding experiments measure an equilibrium between crystal and complex, it is possible that the apparent verification of the size criterion merely reflects an accidental agreement with a crystal energy progression. If this were true, the size criterion would simply be an effect of the decreased solubility of larger hydrocarbons.

In this thesis, binding of hydrocarbons to poly A will be related to a size criterion. The validity of the size criterion will be tested. The size criterion for poly A, together with a size criterion for DNA, will form strong evidence for an intercalation model.

LITERATURE REVIEW

The literature on the solubilization of polycyclic, aromatic hydrocarbons can be subdivided into three units: the early work of solubilization of hydrocarbons by purine solutions, the illustration of technical problems in DNA-hydrocarbon binding, and the attempted determination of the mode of binding to DNA.

Solubilization by Purine Solutions

In 1946, the study of the solubilization of polycyclic, aromatic hydrocarbons began with Weil-Malherbe's (39) extensive work on these hydrocarbons in aqueous purine solutions. Hydrocarbons are virtually insoluble in distilled water, less than 10^{-8}M. However, in purine solutions the solubility is appreciably increased (Table I). Table I shows the concentration of various hydrocarbons in 9.43 mM caffeine solution. This table also illustrates certain aspects of solubilization which are applicable to a number of purine solutions.

As the number of condensed rings of the hydrocarbon increases, the solubility decreases. Moreover, for a given number of rings, the solubility is greater for an angular than for a straight arrangement. In Table I, solubility of phenanthrene versus anthracene illustrates this point.

Weil-Malherbe studied temperature effects on the solubility. Duplicate experiments were carried out at 20°C and 38°C. Weil-Malherbe concluded that the solvent power of a purine does not vary greatly with the temperature.

TABLE I: Concentrations of Various Hydrocarbons in Caffeine Solution, 9.43×10^{-3}M. Data from Weil-Malherbe Study (38).

HYDROCARBON	CONCENTRATION OF HYDROCARBON (10^{-6}M)	MOLES RATIO ($C_{purine}/C_{hydrocarbon}$)
Phenanthrene	38.0	248
Anthracene	1.3	7,300
Pyrene	30.0	315
Chrysene	7.1	1,328
1,2-Benzanthracene	2.1	4,490
9,10-Dimethyl 1,2-Benzanthracene	0.31	30,600
1,2,5,6-Dibenzanthracene	0.43	20,700
20-Methylcholanthrene	0.09	104,500
Coronene	0.61	15,500

Electronegative groups, such as carbonyls, substituted on the 2,6,8-position of the purine, increase the solubility. Weil-Malherbe found aqueous 1,3,7-trimethyl-uric acid to be a better solvent than a caffeine solution. Thus guanine, which has a carbonyl in the 6-position, is a more effective solubilizing agent than adenine.

The solubilization of hydrocarbons by purines implies an interaction between the two. This interaction is strong enough so that Weil-Malherbe (38) was able to grow mixed crystals of tetramethyl-uric acid (TMU)-pyrene and TMU-3,4-benzpyrene. From diffraction studies of the crystals, Damiani et al. (15) reported a stoichiometry of 1:1 for TMU-pyrene and 2:1 for TMU-3,4-benzpyrene. They also found that TMU and pyrene were stacked plane to plane with a van der Waal's separation of 3.4 Å.

Damiani et al. (15) suggested that dipole-induced dipole interactions, together with van der Waal's forces, could account for the binding energy and for the molecular orientation. The dipole from the polar purines presumably induces a dipole in the polarizable hydrocarbon. Moreover, it was postulated (15) that stacking interactions could also account for the solubility of hydrocarbons in aqueous purine solutions.

Technical Problems Involved in DNA-Hydrocarbon Binding Studies

In 1962 Boyland and Green (6) and Liquori et al. (31) independently found that DNA solubilized polycyclic, aromatic hydrocarbons. Their

values for hydrocarbon binding and their techniques were the standards with which other workers made comparisons. However, from 1962 to 1968 these workers raised several questions. These questions came up as a consequence of disparate results from a number of laboratories: Was equilibrium established? What were the effects of salt? Was the hydrocarbon in a colloidal suspension? And what was the mode of binding? In 1968 Lesko et al. (30) and Craig (12) answered most of these questions. By and large, these queries were the results of problems in techniques of saturation binding of hydrocarbons to DNA. An examination of Boyland and Green's and Liquori's techniques will help elucidate these problems.

Boyland and Green (6) solubilized pyrene and benzpyrene by shaking solutions of DNA and crystalline hydrocarbons for one day at room temperature. The solutions were protected from the light. The suspension was centrifuged at 4500 g. The assumption was made that centrifugation effected a separation of crystals from complexed hydocarbon. Bound hydrocarbon was calculated from ultraviolet (UV) absorption spectra. The absorption of the bound hydrocarbon is red shifted 10 nm from the unbound hydrocarbon. No correction was made for the unbound hydrocarbon in solution. Except for pyrene and phenanthrene, the absorption of hydrocarbon in aqueous solutions could not be observed. Since the spectra of the bound hydrocarbon were red shifted, there was little or no contribution to these maxima from the unbound hydrocarbon.

In this early paper, Boyland and Green reported one pyrene complexed per fifty nucleotides and one benzpyrene per one-hundred and fifty

nucleotides of native DNA. In a later paper (7), they lowered the values to one pyrene per five-hundred nucleotides and one benzpyrene per one-thousand nucleotides because they found that, in the previous work, not all of the crystalline hydrocarbon had been centrifuged out of solution. They reported that centrifuging at 121,000 g for two hours was sufficient for separation; they found that centrifuging for four hours and up to 126,000 g did not decrease the value for solubilization.

Liquori's technique of obtaining equilibrium is similar to that of Boyland and Green; however, his manner of determining the amount bound is different. Liquori et al. (31) shook very fine crystals of 3,4-benzpyrene with DNA solution for one day. They also protected the solution from light, then filtered one ml of solution through sintered glass. This one ml of DNA-hydrocarbon complex was extracted three times with cyclohexane. The concentration of benzpyrene was calculated from absorbance spectra. Liquori et al. (31) reported one benzpyrene complexed per one-thousand nucleotide of native DNA.

Boyland and Green and Liquori et al. initially suggested a standard method for studying DNA-hydrocarbon complexes. This was accomplished by shaking the crystals in DNA solution. However, equilibrium was assumed to be reached by shaking for one day. Not until the experiment of Ts'o and Lu's (36) was the time required to reach saturation equilibrium known to exceed 24 hours.

Ts'o and Lu (36) equilibrated labelled hydrocarbon with DNA by dialysis. An aqueous suspension (0.005M phosphate buffer) of labelled hydrocarbon was placed outside the dialysis tubing, and DNA (3.3 mg/ml,

0.005M phosphate buffer) was inside the tubing. Ts'o and Lu reported that equilibrium was reached in 4-5 days. No increase in the amount bound was found after this time. The solubility of one benzpyrene per three-thousand nucleotides was reported (36).

In a later paper (30) from the same laboratory, Lesko et al., employing the same technique of equilibration by dialysis, found Ts'o and Lu in error. Equilibrium was reached in seven to ten days. They (30) reported the solubility of 3,4-benzpyrene in DNA as one benzpyrene per one-thousand nucleotides.

Craig and Isenberg (13) reported that saturation equilibrium between crystalline hydrocarbon and DNA was reached in four days. Shaking for periods up to two weeks did not increase the amount bound. In their study (13), saturation equilibrium was evaluated by three separate methods: 1) A simple shaking of solid hydrocarbons in DNA solution, where equilibrium was achieved in four days; 2) A zone crossing method of Lerman that shall be explained later; 3) A technique utilizing a colloidal suspension in a column. All three methods yielded similar values for hydrocarbon-DNA binding. They (13) found one benzpyrene complexed per one-thousand nucleotides.

Lesko et al. (31), and Craig and Isenberg (13) agreed in reporting that the saturation value of 3,4-benzpyrene complexed to DNA is one benzpyrene per one-thousand nucleotides.

Before Lesko et al. (31) and Craig and Isenberg's (13) papers, other workers had a number of different values for the binding of 3,4-benzpyrene to DNA.

Ball et al.(2) dissolved a number of hydrocarbons in 100 percent methanol, 500 mg/ml. They then shook 0.5 ml of methanol-hydrocarbon solution with 60 ml of DNA solution for one day. This solution was centrifuged at 105,000 g for two hours at 4°C. Ball et al. (2) reported a solubility of benzpyrene in DNA of one hydrocarbon per sixty nucleotides. This value is sixteen times greater than others reported (6,7,31,36). Ball's method of making his complex is similar to the method used for making a colloid (12). The high value of Ball et al. may have been due to the existence of a colloidal suspension of hydrocarbons in the DNA solution.

In another technique, Lerman (29) equilibrated hydrocarbon with DNA by a zone crossing procedure. A zone of caffeine-hydrocarbon on a Sephadex SE-50 column, followed by a zone of DNA will interact with a hydrocarbon when the zone of DNA passes through the caffeine-hydrocarbon zone. Lerman therefore interacted DNA with 3,4-benzpyrene and pyrene in this manner. The U.V. spectrum of the eluant was recorded Lerman found that one benzpyrene complexed to eighty nucleotides, this high value also appears anomalous when compared to others (6,7,31,36). Since caffeine interacts with both DNA and benzpyrene, it is possible that benzpyrene-caffeine complexes could have been eluted with DNA.

As mentioned before, two papers (13), (31) have resolved most of these discrepancies in binding values, and it is now assumed one benzpyrene complexes to one-thousand nucleotides. However, this value is valid only at low salt concentration.

Boyland, Green and Lui (8) reported the effects of increasing the concentration of NaCl. The amount of benzpyrene bound to DNA was

decreased three-fold as NaCl was added at concentrations ranging from 1×10^{-3}M to 5×10^{-2}. With native DNA, the higher the salt concentration the less the binding. Furthermore, if divalent cations, such as Mg^{++}, replace Na^{+}, the binding of hydrocarbon to DNA was reported to decrease by a factor of five. Therefore, binding is sensitive to both the amount and the type of salt used.

In 1964 Giovanella, McKinney, and Heidelberger (22) raised a serious challenge to the reports that hydrocarbons claimed that the samples studied by Boyland and Green did not contain true complexes at all, but were actually colloidal suspensions of the hydrocarbons, stabilized by the DNA solutions. Giovanella et al. (22) stated that a solution of benzpyrene in DNA, prepared by the method of Boyland and Green (6), lost its characteristic absorption spectrum, when it was centrifuged at 99,900 g for four hours. They claimed, therefore, that the hydrocarbon had not been in solution at all, but had been in suspension and could be pelleted by centrifugation.

In rebuttal, Boyland and Green (7) replied that the removal of benzpyrene from DNA in the experiment of Giovanella et al. (22) was not due to centrifugation, but resulted from the use of plastic centrifuge tubes. These workers pointed out that Giovanella et al. had used plastic centrifuge tubes and that the hydrocarbon could be removed by absorption to the plastic tubes. Moreover, the ultraviolet spectrum of benzpyrene dissolved in DNA and in purine solution were both red shifted, unlike the spectra of benzpyrene in cyclohexane. Furthermore, the fluorescence of benzpyrene was quenched by DNA (6) but was not quenched when benzpyrene was in colloidal suspension.

Boyland and Green (7) concluded that the hydrocarbons were bound to the DNA. Subsequent studies have supported their conclusions (12,13,15,20,24,25,28,30,31,36). However, there were still more technical problems to be resolved. Beside absorbing to plastic, hydrocarbons absorb to filters.

Insco (23) found that, in filtering an aqueous phenanthrene solution, Whatman No. 1 paper absorbed only 7 percent of the hydrocarbon. Other commonly used filters absorbed considerably more. Glass frit, sintered-glass, and millipore filters absorbed 50 percent of the phenanthrene in solution. In 1964, Ts'o and Lu (36) published a low value for binding, one benzpyrene to three-thousand nucleotides. They claimed this was the result of absorption of hydrocarbons to the sintered-glass filter.

By now, it is evident that many technical problems thwarted researchers in this field. As Craig and Isenberg (13) pointed out, in the study of hydrocarbon-DNA binding, technical considerations assume a critical importance. Only small amounts of hydrocarbon bind to DNA. For example, if separation of solid and solubile hydrocarbon by filtration is not carried out well, even a small crystal of hydrocarbon left in the solution will cause the value for the amount of hydrocarbon solubilized to be too high. If the removal of solid hydrocarbon is imperfect, so that only 1.0 μg of 3,4-benzpyrene crystal is present, an error of 100 percent would be made with laboratory proceedures commonly used by Liquori's group, Ts'o' group or Isenberg's group. Moreover, as discussed above, the use of proper filters (23) and the avoidance of plastic centrifuge tubes (7) are also critical.

Extreme care must be exercised if order of magnitude variations are to be avoided.

What is the mode of binding? Undoubtly this was the question in the minds of all the groups that have worked on this problem.

Nagata et al. (34) oriented DNA-hydrocarbon complexes by flow gradients. If the orientation of the DNA was known, then the orientation of the hydrocarbon could be related to DNA's orientation in a flow gradient. To orient the DNA, a velocity gradient is produced with rotating coaxial cylinders. The gap between inner and outer cylinder is 0.05 cm. The inner cylinder rotates. This produces a velocity gradient that aligns the DNA. The light is polarized parallel or perpendicular to the flow. The differential dichroism, $\Delta\epsilon$, is defined as the difference between the molar extinction coefficients of the light polarized parallel to the flow line, $\epsilon_{\parallel}$, and the perpendicular to it, $\epsilon_{\perp}$; that is, $\Delta\epsilon = \epsilon_{\parallel} - \epsilon_{\perp}$. From the value of $\Delta\epsilon$, the direction of the transition moment can be related to the direction of the flow. In DNA the transition moment of the $\pi \longrightarrow \pi^*$ transition is in the plane of the bases. Therefore, DNA, when oriented, would have a negative $\Delta\epsilon$. In fact, by experiment the value of $\Delta\epsilon/\epsilon$ for calf thymus DNA, λ = 260, was found to be -0.41 to 0.47 (34). The range of $\Delta\epsilon/\epsilon$ is more than the variation due to experimental error. Nagata et al. conjectured that the variation was due to the inequality of chain lengths of calf thymus DNA. For any given flow gradient, the orientation increases with increasing molecular weight. Thus, if small segments of DNA were present, the $\Delta\epsilon/\epsilon$ value could be lower because of partial orientation of some DNA strands.

As in the case with DNA, aromatic hydrocarbons also have a plane of symmetry. Moreover, the transition moment of the $\pi \longrightarrow \pi^*$ transition is in the plane of the aromatic hydrocarbon. Therefore, if a hydrocarbon were intercalated, the $\Delta\epsilon/\epsilon$ value should be the same as the $\Delta\epsilon/\epsilon$ for DNA. Nagata et al. found that the $\Delta\epsilon/\epsilon$ value for phenanthrene, pyrene, and 3,4-benzpyrene varied from -0.33 to -0.47. However, the value for 20-methylcholanthrene, tetracene and pentacene was +0.74 to 0.84. Unfortunately, the results could only be interpreted in a qualitative manner. Nagata
which showed a negative dichroism are different from those which gave a positive one. Moreover, for the hydrocarbons that showed a negative dichroism, it was reasonable to conclude that they were oriented perpendicular to the flow, an orientation consistant with the intercalation model.

Several parts of the paper by Nagata et al. needed further examination since Isenberg et al. (25) did not find 20-methylcholanthrene, tetracene or pentacene soluble in DNA solution. The spectrum of tetracene, that Nagata presented in his paper, is not similar to any standard spectra of tetracene. Moreover,
Nagata, et al. (34) used no salt with their DNA, and there is a question as to whether it was native. If the DNA was denatured, then any hydrocarbon could bind wherever there was an oily pocket in the loops of the DNA. The hydrocarbons that had a positive value of $\Delta\epsilon/\epsilon$ could not be intercalated. Even for the hydrocarbons that had a negative dichroism, the orientation could not be determined. The dichroism values for DNA varied from -0.41 to -.047, whereas, the

values for the hydrocarbons varied from -.033 to -.047. The only conclusions that could be drawn from this work is that there is a possibility that some of the hydrocarbons that bind to DNA may intercalare.

Green and McCarter (20) investigated flow orientation of DNA similar to the work of Nagata et al. (34), but used fluorescence instead of absorption. Several laboratories (6,24,25,34) had found the flyorencence of pyrene and 3,4-benzpyrere quenched. However, Green and McCarter (20) by using wide slits were able to see the fluroescence of the complexed hydrocarbons. Green (21) found the fluorescence depended on pH. The intensity of fluorescence was decreased as the pH was lowered from 7.5 to 5. Therefore, by using pH 7.0, Green and McCarter (18) reported quenching of 3,4-benzpyrene to be only "3 fold". However, the problem again is that the binding of hydrocarbons to DNA is low; thus, the intensity is weak. Furthermore, the fluorescence is quenched, and the intensity is further reduced. With such low intensity, the noise becomes a significant part of the signal. Slits, consequently, are widened, and the resolution of vibrational bands is diminished.

Nevertheless, Green and McCarter obserbed the polarization and effects of flow orientation on fluorescence for 3,4-benzpyrene and anthracene. They found that the hydrocarbons when complexed had a high polarization. When caffeine was added to DNA-hydrocarbon complex, the hydrocarbon was released from the DNA, and the polarization went down. Green and McCarter found this result for both benzpyrene and anthracene complexes. For orientation of flow, Green and McCarter

passed the hydrocarbon complexes through a 1 mm tube. The intensity of the polarized fluorescence was measured. Unfortunately, again, only qualitative results are obtained from flow-induced changes in intensity. Moreover, there is a variation in results. In five separate experiments, the increased intensity of flow over that of stationary solution ranged from 36 percent increase to 52 percent increase. Green and McCarter could only conclude that, in general, their results were consistent with the intercalation model.

Up to this point, mostly indirect evidence had existed for the intercalation model. The intercalation hypothesis was reasonable. Workers had noted a 10 nm red shift; the fluorescence was quenched; some hydorcarbons, when oriented in a flow field, had a dichroism that might suggest intercalation. However, no one had shown rigorously that hydrocarbons do intercalate.

In 1967, Isenberg et al. (25) suggested that molecular size might be a criterion for the solubilization of hydrocarbons by DNA. They found that phenanthrene, pyrene and 3,4-benzpyrene bound to DNA, and tetracene, pentacene and 20-methylcholanthrene did not bind. Using Corey-Pauling-Koltun space filling models, they suggested that the three smaller hydrocarbons intercalated in the DNA and were protected from the water, whereas, the larger three molecules, if they intercalated, could do so only in such a way that a substantial part of the hydrocarbon molecule was exposed to the aqueous solvent.

Craig and Isenberg (13) tested a number of predictions of the size criterion for DNA. They built, from Corey-Pauling-Koltun space filling models, a short segment of DNA and all the hydrocarbons they tested.

They predicted, on the basis of the size criterion, whether a hydrocarbon would bind to DNA. All predictions were verified. However, since only a limited number of hydrocarbons bound to DNA, they pointed out, that the size criterion could be accidental. Saturation binding experiments measured an equilibrium between DNA-hydrocarbon complexes and crystalline hydrocarbon. It was possible that larger hydrocarbons would have bound to DNA if the crystal lattice did not have such strong interactions. If this were the case, the size criterion would be just a solubility artifact. On the other hand, if the size criterion were verified, this would be strong evidence to substantiate the model of intercalation.

In this thesis, strong evidence will be given for the hypothesis hydrocarbons intercalate in polynucleotides. To this end, a new size criterion, that is appropriate to poly A rather than DNA, will be tested. In addition the flow dichroism of four poly A-hydrocarbon complexes will be correlated to the size criterion. The fluorescence polarization of the complex will be shown. And, finally, the determination of the vapor pressure of several hydrocarbons will show that the size criterion cannot be a solubility artifact.

MATERIALS AND METHODS

Binding of Hydrocarbon to Poly A

Highly polymerized poly A, K salt, lot 157B-1840, from Sigma Chemical Co., was further purified by phenol extraction. Fifty ml of water saturated phenol was added to 250 ml of poly-A solution (4 mg/ml, 0.001M NaCl, 0.001M cacodylate buffer). The aqueous phase was decanted off. Poly A is made from RNA polymerase. Therefore, care was taken when decanting off the water so that no protein impurities were obtained at the interface. The aqueous phase was dialyzed against 1.1M NaCl in 0.01M cacodylate buffer, pH 7.1, for six days. The extended long time in dialysing was to insure removal of all phenol.

Fractions were taken according to the method of Eisenberg and Felsenfeld (17). At $5^{o}C$ 4 mg/ml poly A solution in 1.1M NaCl is completely soluble. When the temperature is raised to $12^{o}C$, high molecular weight poly A precipitates. If raised to $14^{o}C$, smaller strands precipitate. In this way, poly A can be fractionated according to size.

Molecular weights were determined by sedimentation equilibrium, using the meniscus depletion method of Yphantis (37). Poly A was at a concentration of $3.2 \times 10^{-4}M$, 0.5M NaCl and 0.01M cacodylate buffer. Temperature was kept at $20.01^{o}C$. $\overline{V} = 0.55$ was used (17). The molecular weight calculation was facilitated by a program written by Dr. R. Dyson (private communications). The heterogeneity of the sample can be seen by the lack of linearity in Figure 1. The fraction used to study hydrocarbon

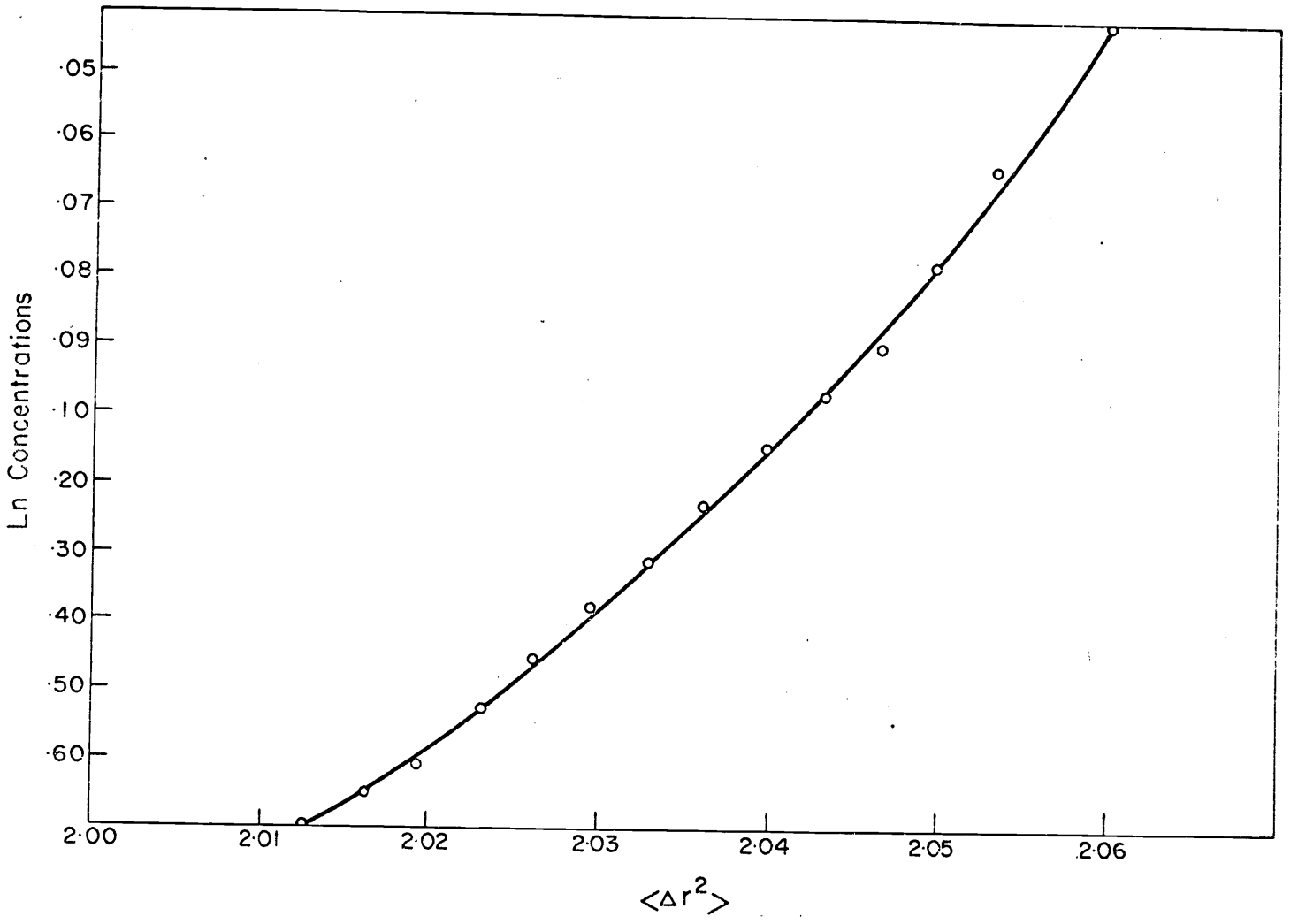

FIGURE 1: Ln concentrations vs Δr^2 for poly A used in binding studies. The curvature of the plot indicates heterogenity of poly A strands.

binding had a weight average degree of polymerization of 6100. The poly A solutions were dialyzed against 0.001M NaCl, 0.01M acetate buffer, pH 5.0. A small amount of gelatinous material formed during dialysis. This was removed by centrifugation at 1000 g for twenty minutes. The preparations were concentrated, by flash evaporation, to 1.45×10^{-2}M(P). They were then dialyzed against 0.001M NaCl, 0.01M acetate buffer, pH 5.0 for two days. pH titrations, using circular dichroism measurements (9) to monitor helix formation, showed our stock solution to be double stranded (Figure 2). These measurements were taken on a Durrum-Jasco circular dichroism spectrometer.

All hydrocarbons were commercial products. They were sublimed at least once, and purity was checked by melting point determination and by comparison of U.V. spectra. In each case the purity was greater than 99 percent when checked with standard curves (1,3) and standard melting point values.

Proflavin dihydrochloride from the National Aniline Division, Allied Chemical Corporation, New York, was precipitated as the base from a 1:1 water ethanol mixture using 0.1 normal sodium hydroxide. The precipitate was filtered, washed and dried before recrystallization from a 5:1 water ethanol mixture. The proflavin solution was prepared from the dry purified base by adding the calculated amount of dilute hydrochloric acid to neutralize the base. The molar extinction coefficient used was 4.1×10^{4} at 444 nm (19).

Approximately 3 mg of crystalline hydrocarbon were added to 12 ml of stock poly A. Samples were shaken at 4°C for at least two weeks. To remove remaining crystalline hydrocarbon, the samples were

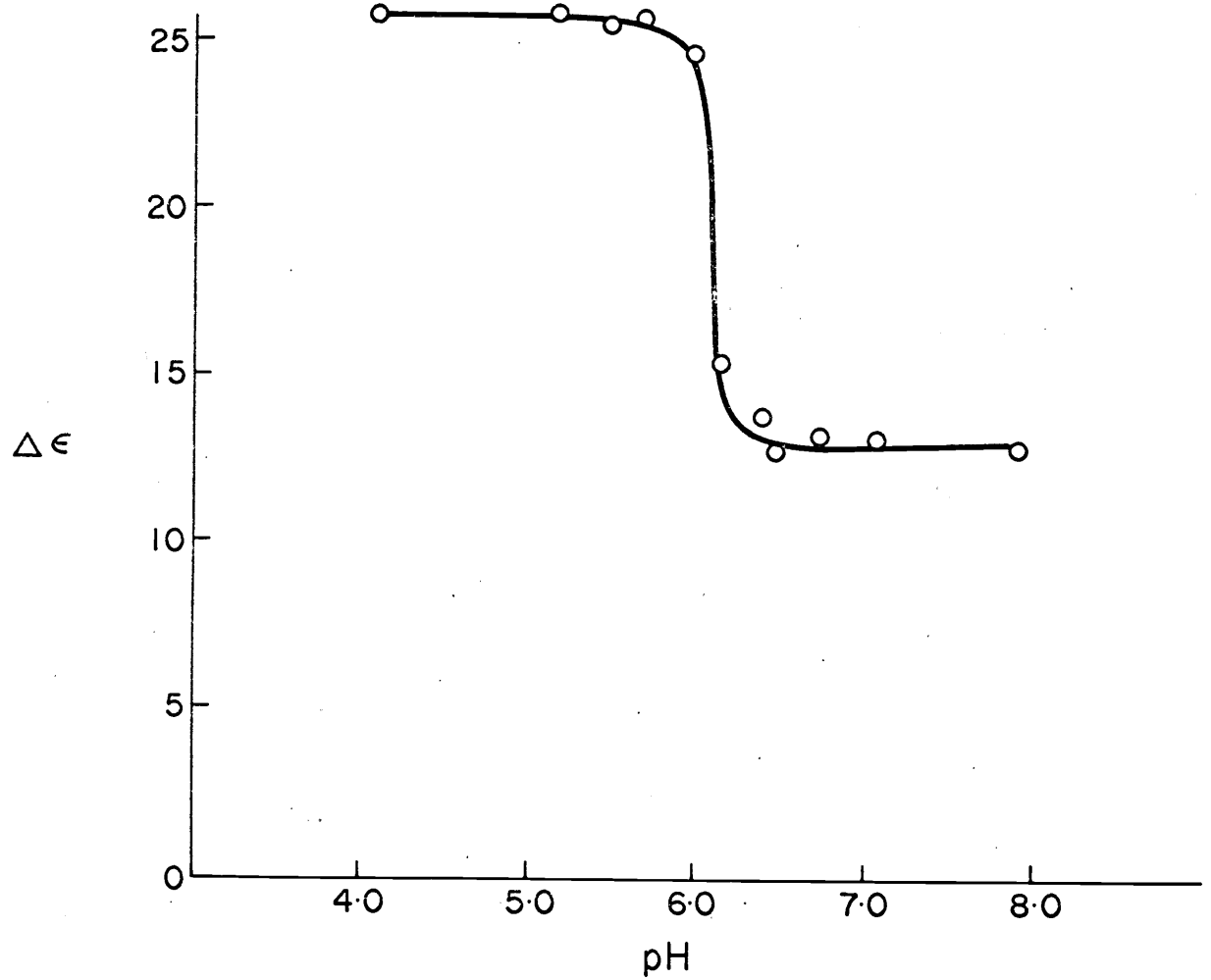

FIGURE 2: $\Delta\epsilon = \epsilon_L - \epsilon_R$ vs pH for poly A fraction used in binding studies.

then centrifuged at 34,800 g for two hours. Absorbance spectra of the supernatants were measured on a Cary Model 14 spectrophotometer, using 10 cm sample cells.

Seven ml aliquots of the supernatant were then pipetted into 100 ml beakers and diluted with water to 40 ml. After filtering these solutions through Whatman No.1 paper, the filtrate was extracted three times with 6 ml portions of cyclohexane. The extracts were combined and evaporated to 7 ml. Absorbance spectra were then recorded.

The anisotropy and intensity of excitation and emission were measured on an instrument built by S. L. Baird, Jr. and I. Isenberg. This instrument is described in a paper by Evett and Isenberg (16). The anisotropy, μ, was measured as $\frac{3}{2}$ (E-B)/(E+2B), and the intensity of the emission as $\frac{1}{3}$ (E+2B); where E and B represent emission polarized parallel and perpendicular to the polarization of the exciting light, respectively.

Fluorescence samples were prepared in the same manner as the absorption samples. The poly A concentration was 1.45×10^{-2}M, 0.001M NaCl, 0.01M acetate buffer pH 5.0; the salmon sperm DNA concentration was 1.12×10^{-2}M, 0.001M NaCl, 0.003M cacodylate buffer pH 6.8. Crystalline hydrocarbon and polynucleotides were shaken for at least two weeks at 4°C. Crystals were removed by centrifuging at 34,800 g for at least two hours. The absorption spectra was taken. If this agreed with the values already determined from absorption and extraction techniques, the sample was not further centrifuged, and the fluorescence was measured.

Flow Dichroism Apparatus

Callis and Davidson (10) described a new flow dichroism apparatus. Previously, most flow dichroism had been done with two rotating cylinders. Polarized light excites the sample which is oriented by the flow due to a rotating cylinder. The typical optical path for rotating cylinders is 0.05 cm. Thus, if a sample has a low molar extinction coefficient or is very dilute, the rotating cylinder type of apparatus is not effective. The Callis and Davidson's apparatus has a light path of 10 cm. Since our solutions are dilute in hydrocarbon, this apparatus with a path length two-hundred times longer than customary ones, was especially suited for our needs.

The basic design of the cell is presented in Figure 3. The flow cell is made of two blocks of titanium, 10cm x 1.2cm x 2.8cm. A pair of spacers separate the two blocks, thus forming a channel 10cm long, 1.4cm high, and 0.01cm wide, the width being determined by the spacers. Quartz windows are at both ends of the assembly so that light may pass through the cell in the direction of flow. The solution is held in two 10 ml syringes at both ends of the cell. These syringes are used to push the solution back and forth through the cell. The cell and the 10 ml syringes are held rigidly in a holder which fits into the cell compartment of a spectrophotometer that was also built.

Uniform flow is produced by the driving assembly which sits on the cell holder. The driving assembly consists of a motor which works a worm gear. This worm gear provides translational motion to the syringes, and thus, gives reproducible volume flow rates.

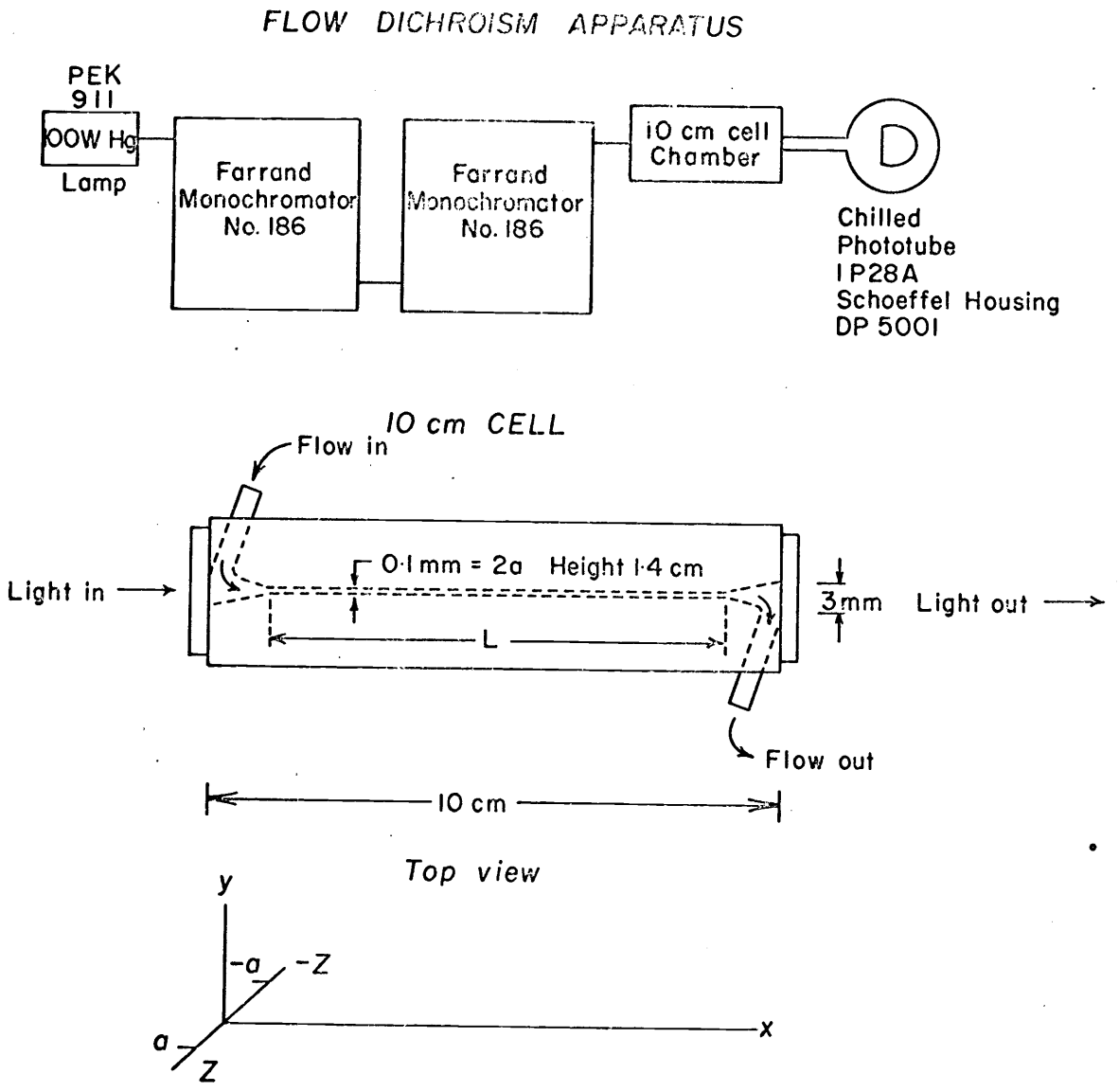

FIGURE 3: Flow Dichroism Apparatus.

The spectrophotometer consists of a PEK 100 watt mercury lamp and PEK power supply, two Farrand monochromators and a 1P28A photomultiplier tube that is chilled in a Schoeffel housing. The signal from the photomultiplier tube is fed into a Heath log-linear current module, DU-20-29, that changes the current from the photomultiplier tube into a voltage that is proportional to an absorbance signal. This absorbance is read out on a Heath recorder, DU-20. Double monochromators were used to reduce stray light, and a frosted photomultiplier tube minimized the fluctuation due to light striking different parts of the cathode. Moreover, by filling the syringe plungers with India ink, stray light was significantly reduced.

The linearity of the instrument was checked against the Cary 14. Dyes at various optical densities were compared on both the Cary 14 and the flow dichroism apparatus. The optical densities are accurate to within $\pm$ 0.005 O.D. units. The change in optical density between air versus sample is linear over a range of three O.D. units. Scattered light was reduced to less than 0.2 percent of the transmitted light. Figure 3 illustrates the design and coordinates of the flow cell. In laminar, viscous flow, the velocity vector, u , is along the x direction. Its magnitude varies from a maximum value in the middle of the channel to zero along the walls. This follows the relationship (4):

$$U(Z) = (P/2L\eta)\ (a^2 - Z^2)$$

Where P is pressure in dynes/cm^2, η is viscosity, 2a is the width of the cell.

The average velocity is:

$$\bar{u} = \int_o^a u\,dz \Big/ \int_o^a dz = Pa^2/3L\eta$$

The volume rate of flow is the average velocity times the area and is a parameter that can be measured.

$$V = 2ah\,\bar{u}$$

The average sheer stress (i.e., du/dz average) can be expressed as:

$$\bar{G} = 3\,V/4ha^2$$

The average sheer stress is just a linear function of volume rate of flow.

Callis and Davidson (10) calculated the distance down the flow path that the entering fluid must flow before a parabolic flow pattern was formed. They found for 0.01 cm spacers that the flow pattern formed in 0.3 cm; therefore, the true length of 10 cm was only shorter by about 3 percent.

If A_f and A_s represent the absorbance of the flowing and the stationary solutions, the parameter measured is

$$\frac{\Delta A}{A} = \frac{A_f - A_s}{A_s}$$

The absorbance, A, is measured on the Cary 14. The "ΔA" measured on the flow dichroism unit, is the absorbance during flow minus absorbance stationary. For molecules that absorb in a plane perpendicular to a helical axis, the value is positive, that is, the absorbance increases during flow. The change in absorbance is due to two effects: The asymmetry of the molecule that will line up in the velocity gradient an the anisotropy of the molecule.

$\Delta A/A$ is a function of both average sheer gradient and molecular weight. If the molecular weight is kept constant, the dichroism changes only with sheer gradient (Figures 30, 31, 32).

Poly A used for the flow dichroism study was handled in the same manner as that for the absorption and extraction. Poly A (1.45×10^{-2}M) was mixed with crystalline hydrocarbon for at least two weeks. The complex was centrifuged for two hours. Approximately 10 ml of solution was needed to fill the syringe. Argon was slowly bubbled through the solution for one-half hour. The solution was then placed in a vacuum and degassed for two hours. Both the deoxygenating and the degassing help eliminate air bubbles during flow since bubbles were an annoying source of noise. Care was also exercised to minimize dust particles since they caused scattering artifacts.

Vapor Pressure of 9-Phenyl Anthracene

The author went to the University of Washington to Dr. N. Gregory's laboratory to measure the vapor pressure of 9-phenyl anthracene. A description of Dr. Gregory's apparatus follows (Figure 4): The quartz diffusion cell is a cylindrical tube 5 cm long and 1.5 cm outside diameter. It has two holes of nearly equal area on opposite sides of the cell. These holes are 0.5 cm from the end. The cell is attached to a tungsten wire 55 cm long. The entire assembly is surrounded by a 10 cm Pyrex tube. During measurements, this tube is kept in a high vacuum. The pressure within the tube, monitored continuously with an ionzation gage, was kept below 10^{-5} mm. The

VAPOR PRESSURE APPARATUS

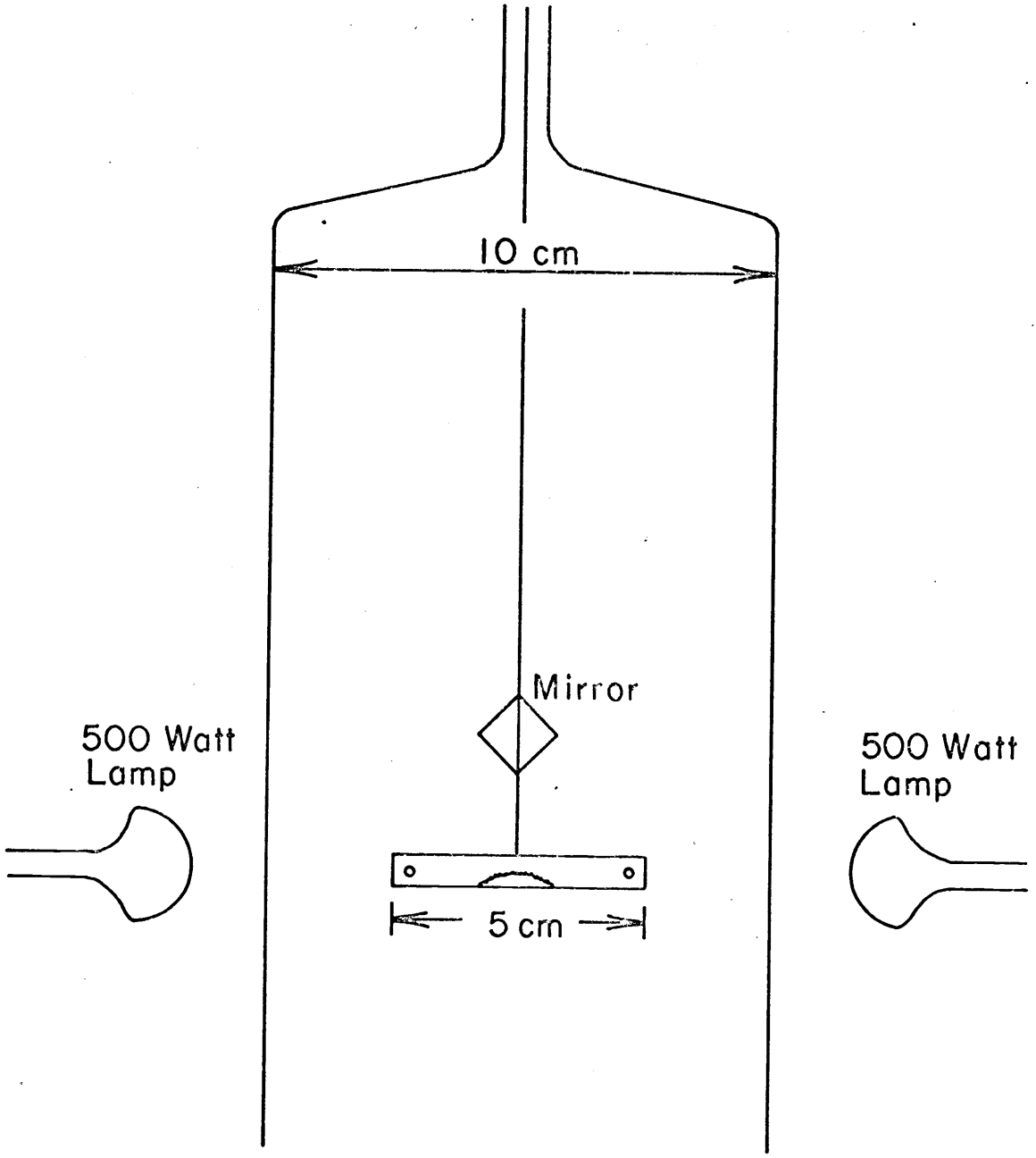

FIGURE 4: Vapor Pressure Apparatus.

vapors from the hydrocarbon crystal escaping from the cell, produced a torque on the tungsten wire. The angle of twist was measured with a telescope and scale assembly. The scaler is reflected from a small mirror on the tungsten wire. Scale deflections were read within 0.05 cm, corresponding to an uncertainty of the torque angle of 5×10^{-4} radians. The angle of twist corresponds to the vapor pressure, $p = k\theta$, where k is a constant for a particular tungsten wire and cell assembly. To measure the torsion constant, Dr. Gregory has a set of calibration compounds of known vapor pressure. One such compound is zinc. Its vapor pressure is known with good accuracy, and the effusion temperature range is reasonably close to that of interest for the hydrocarbon. Since two cells were used in this experiment, there are two constants: one corresponding to a cell with larger orifices for more crude determination, the other corresponding to a cell for finer values. The torsion constant for the cell with larger orifices was 2.10×10^{-2} mm/radian; the other constant was 8.19×10^{-3} mm/radian.

Since the vapor pressure is plotted as a function of temperature, the vapor pressure is measured at various temperatures. The cell was heated by direct radiation from three 500 watt projection bulbs, and temperature recorded from a thermocouple inside the cell. As the temperature of the samples was raised, degassing was followed by observing the angular deflection of the cell. Vapor pressures were calculated from the minimum torsion deflection reached at a given temperature on heating.

9-Phenyl anthracene was prepared by subliming it twice. It was protected from light and stored in a freezer. Purity was checked by melting point and determinations by comparison with absorption spectra from standard curves (1,3). Its purity was greater than 99.5 percent.

RESULTS

Model Building Experiments

Figure 5 is a scale drawing for poly A and DNA (27,35). In poly A, the long axis of the paired adenine bases is between C_2-C_2 on the adenines. The phosphodiester backbone is located to the side of the bases, that is, to the side of the long axis. In DNA, the phosphodiester backbone is at either end of the bases. Hydrocarbons that intercalate are thus longer and narrower than those that intercalate in DNA.

Space filling Corey-Pauling-Koltun models were built and each hydrocarbon was tested to establish a size criterion for poly A. Figures 6-18 show stick models of these complexes. Although stick models are useful for illustrative purposes and are easily understood in the figures, the author feels that space-filling models are necessary to examine the steric hinderances that are involved in the size criterion. He recommends that anyone working with these hydrocarbons build space filling models.

The stick figures will illustrate the orientation of all the hydrocarbons tested. In these figures, the open circles represent the hydrocarbons and the darkened circles belong to poly A base pairs and backbones. Figure 6 presents a model of anthracene and poly A. Anthracene is small enough to assume a range of orientations under the poly A base pair (Figure 6). Not only can it move between C_2-C_2 of the base pair, but it can move back and forth between the

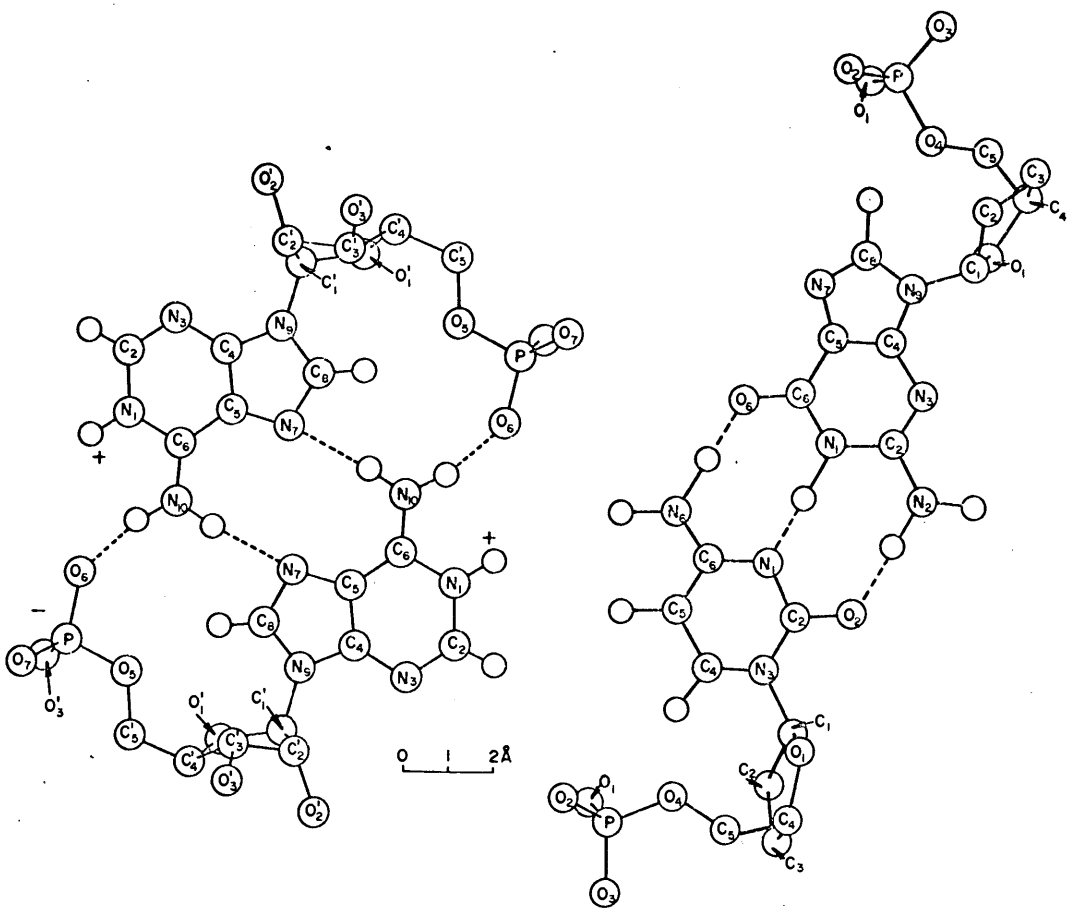

FIGURE 5: Cross section of double stranded poly A (35) and G-C base pair of DNA (27).

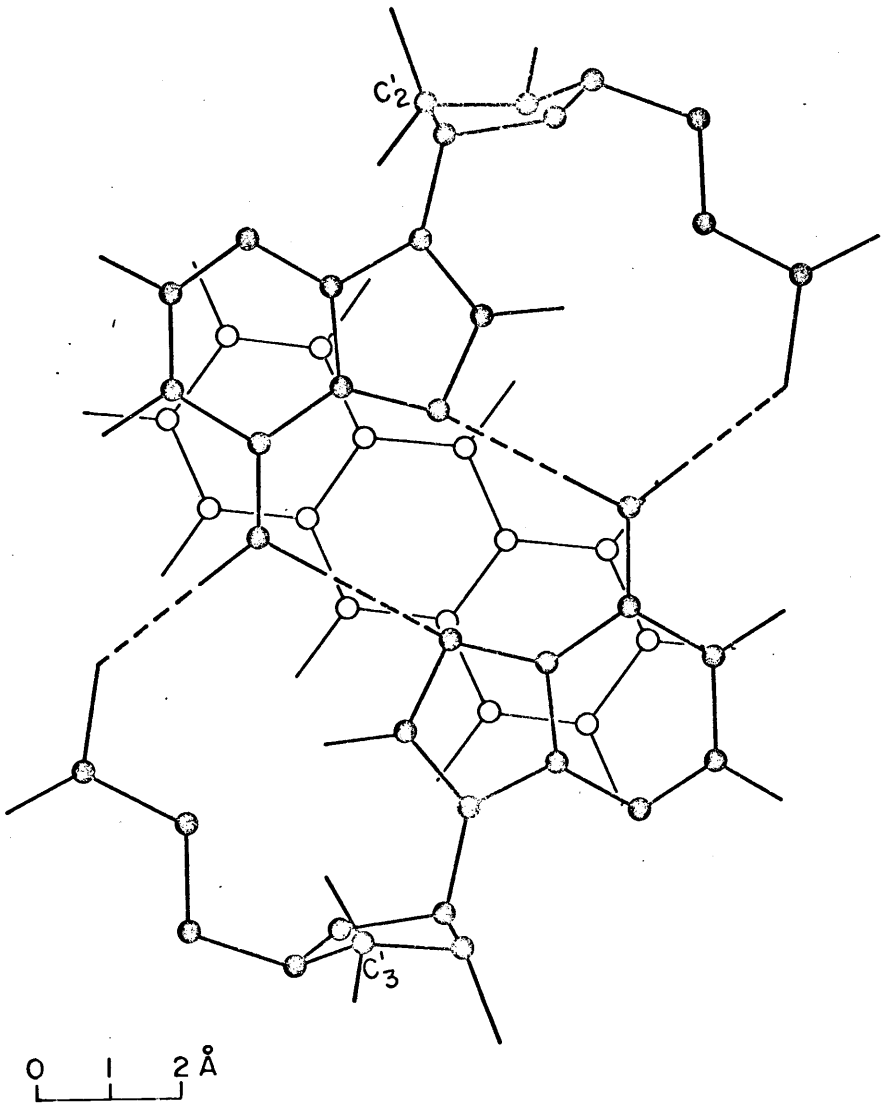

FIGURE 6: Model of Anthracene - poly A in which the Hydrocarbon is shielded from contact with the medium.

phosphodiester backbone and still be completely shielded from the aqueous solvent. Obviously, the prediction would be that anthracene would bind to poly A.

Figure 7 illustrates a model of 1,2-benzanthracene and poly A. Benzanthracene is also small enough to assume a range of orientations under the poly A base pair; even though, it is about 15 percent longer than anthracene and 20 percent wider. Benzanthracene was predicted to bind to poly A.

Figure 8 depicts a stick model of 1,2,5,6-dibenzanthracene and poly A. 1,2,5,6-Dibenzanthracene is the critical size that can just fit under a poly A base pair and still be protected from the aqueous solvent. This figure shows that if the hydrocarbon were any longer or wider, it would protrude from the base pair. Thus, the hydrocarbon's orientation is fixed by the size criterion alone. Moreover, since the hydrocarbon is symmetrical, it can only assume one orientation. The prediction was that 1,2,5,6-dibenzanthracene would bind to poly A.

Figure 9 represents the intercalation of tetracene. This hydrocarbon is nearly the same critical length of 1,2,5,6,-dibenzanthracene. There is some freedom to move between the C_2-C_2 of the base pair. This movement can cover about 1.5 Å. In these model studies, a movement of two angstroms is considered the limits of resolution; therefore, the orientation of tetracene could be considered fixed within the accuracy of the model building experiments. Again, binding was predicted.

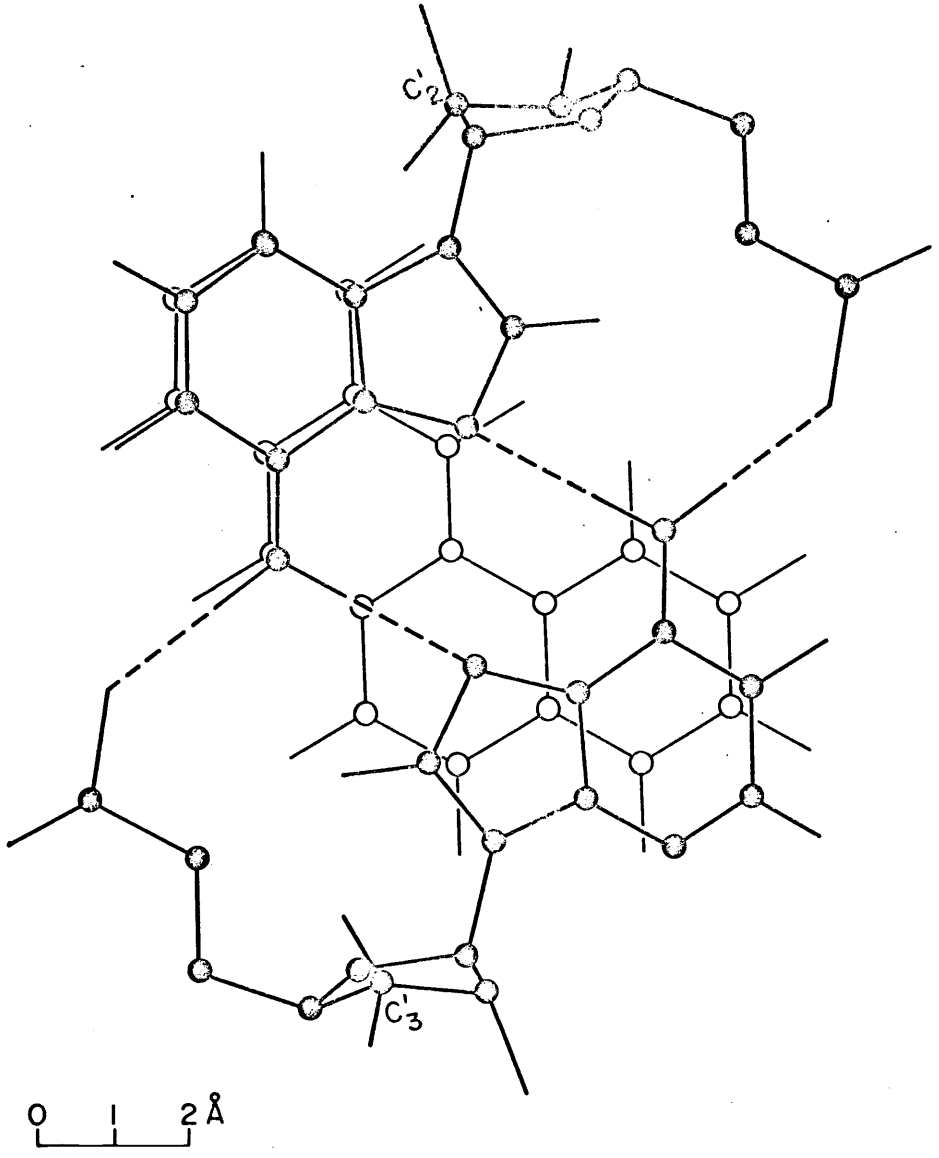

FIGURE 7: Model of 1,2-Benzanthracene - poly A in which the hydrocarbon is maximally shielded from contact with the medium.

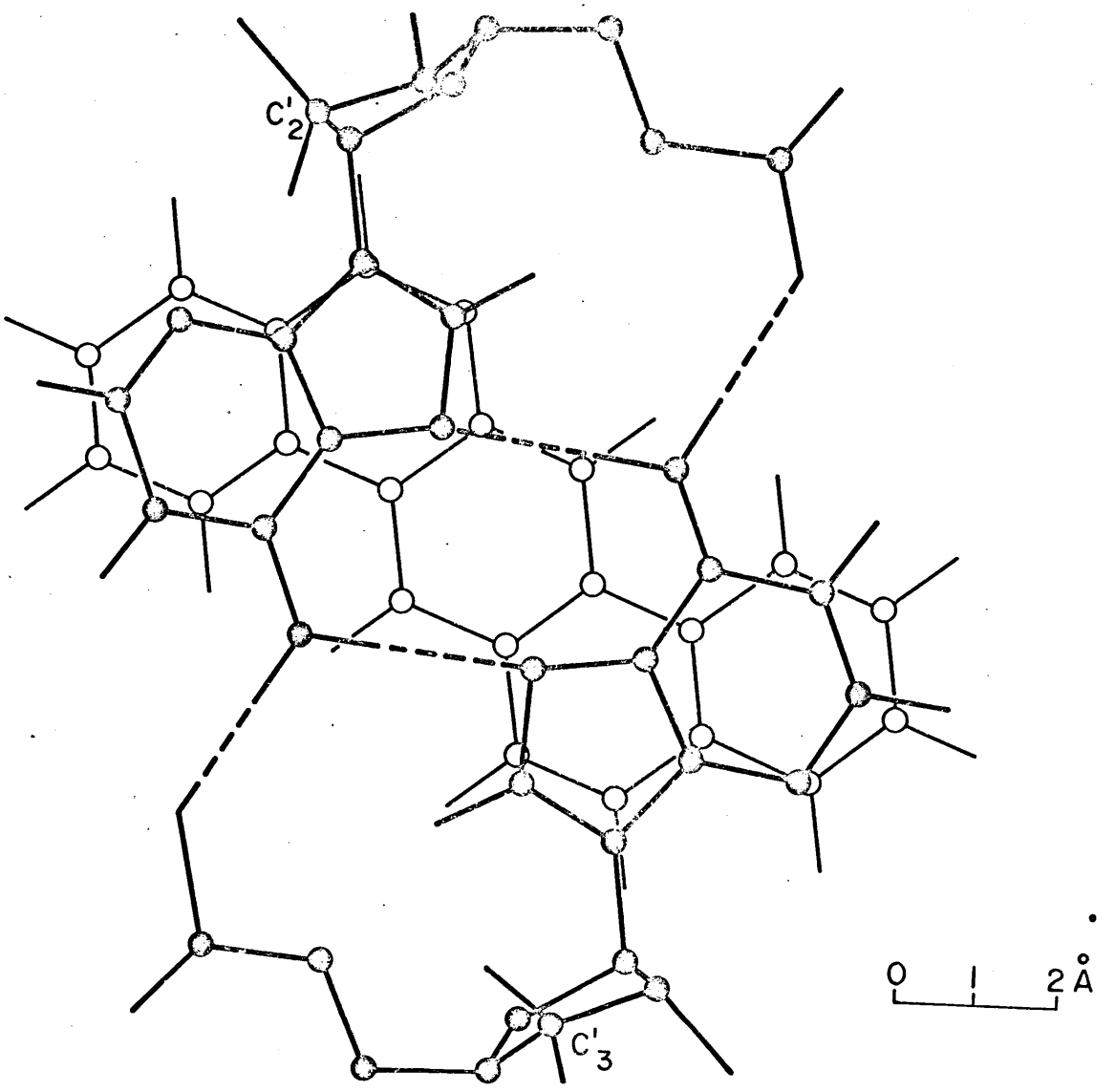

FIGURE 8: Model of 1,2,5,6-Dibenzanthracene - poly A in which the hydrocarbon is maximally shielded from contact with the medium.

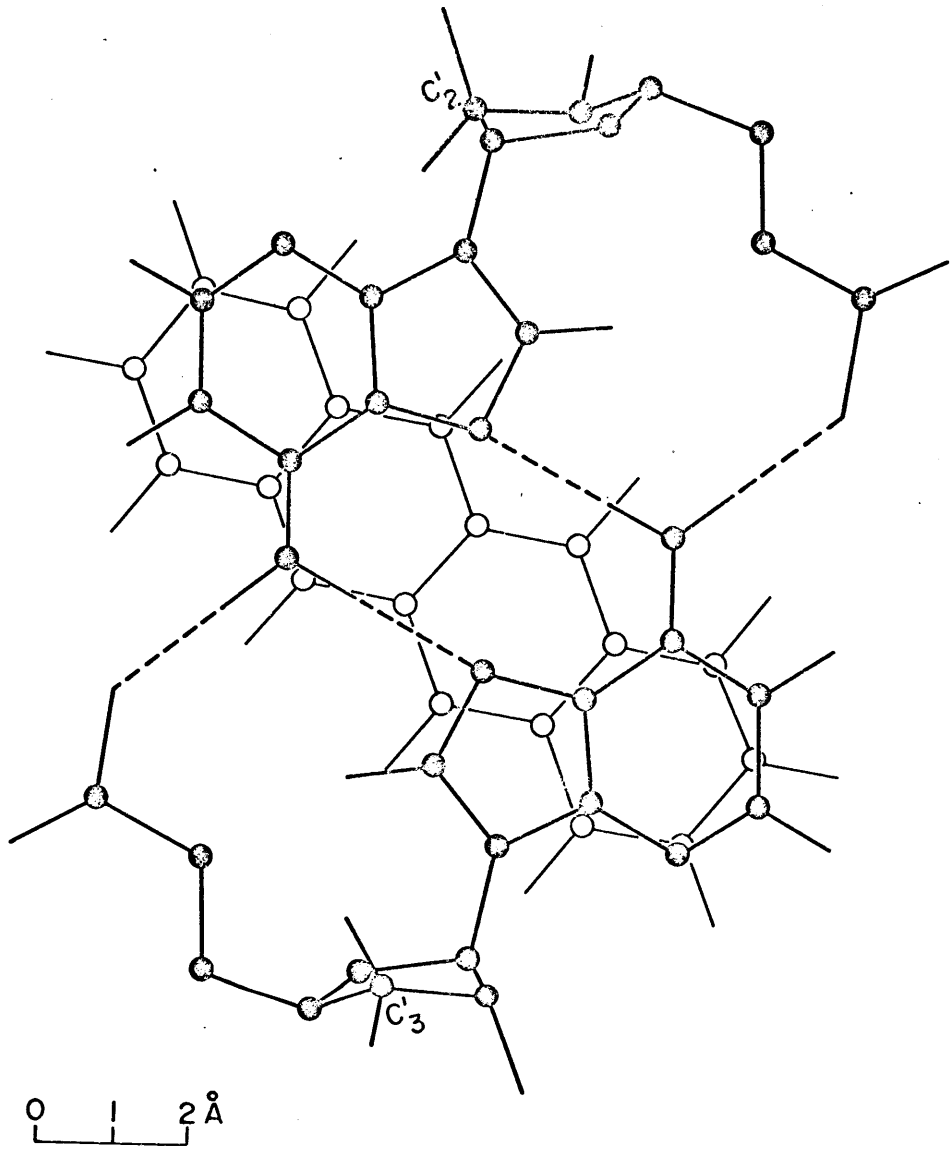

FIGURE 9: Model of Tetracene - poly A in which the hydrocarbon is maximally shielded from contact with the medium.

The next two hydrocarbons were predicted not to bind. Figure 10 illustrates a presumed model of 1,2,3,4-dibenzanthracene and poly A. This hydrocarbon has been intercalated and inserted as far as possible into the helix. Further insertion is prevented by steric hindrance between the hydrogens of the hydrocarbon and the ribose C_2' hydrogen on one chain and the C_3' hydrogen of the other. As a result, the hydrocarbon extends into the medium. Because of the protrusion of 1,2,3,4-dibenzanthracene, no binding was predicted.

Figure 11 portrays a presumed intercalation of pentacene in poly A. Pentacene is too long to fit under the base pair. Nearly one benzyl ring protrudes into the medium. Whereas, anthracene and tetracene would fit; pentacene is just too long.

In this first group of hydrocarbons, the length was increased from anthracene to pentacene, and the width varied only slightly. A critical length was determined by model building, and hydrocarbons longer than this length were predicted not to bind.

Figures 6, 12 and 13 make up a second group of hydrocarbons. These hydrocarbons all have an anthracene group as a basic unit; however, there are different substitutions on the 9-position. These groups substituted on the 9-position increase the width of the hydrocarbons; however, the length stays constant.

Figure 12 shows a methyl group on the 9-position. 9-Methyl anthracene can intercalate in poly A and be protected from the aqueous solvent.

In Figure 13, a phenyl group is placed on the 9-position. 9-Phenyl anthracene is too fat to intercalate in poly A. Again,

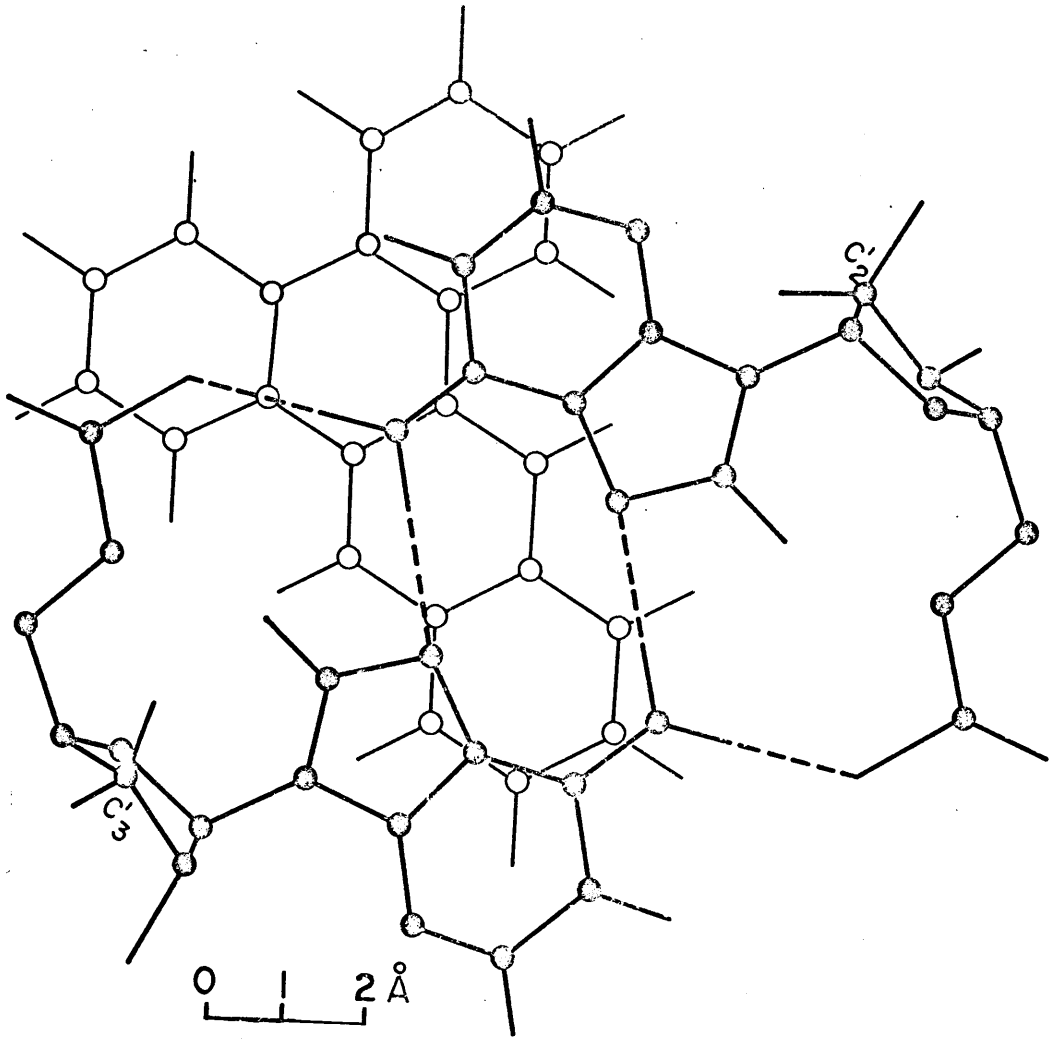

FIGURE 10: Presumed model of 1,2,3,4-Dibenzanthracene - poly A.

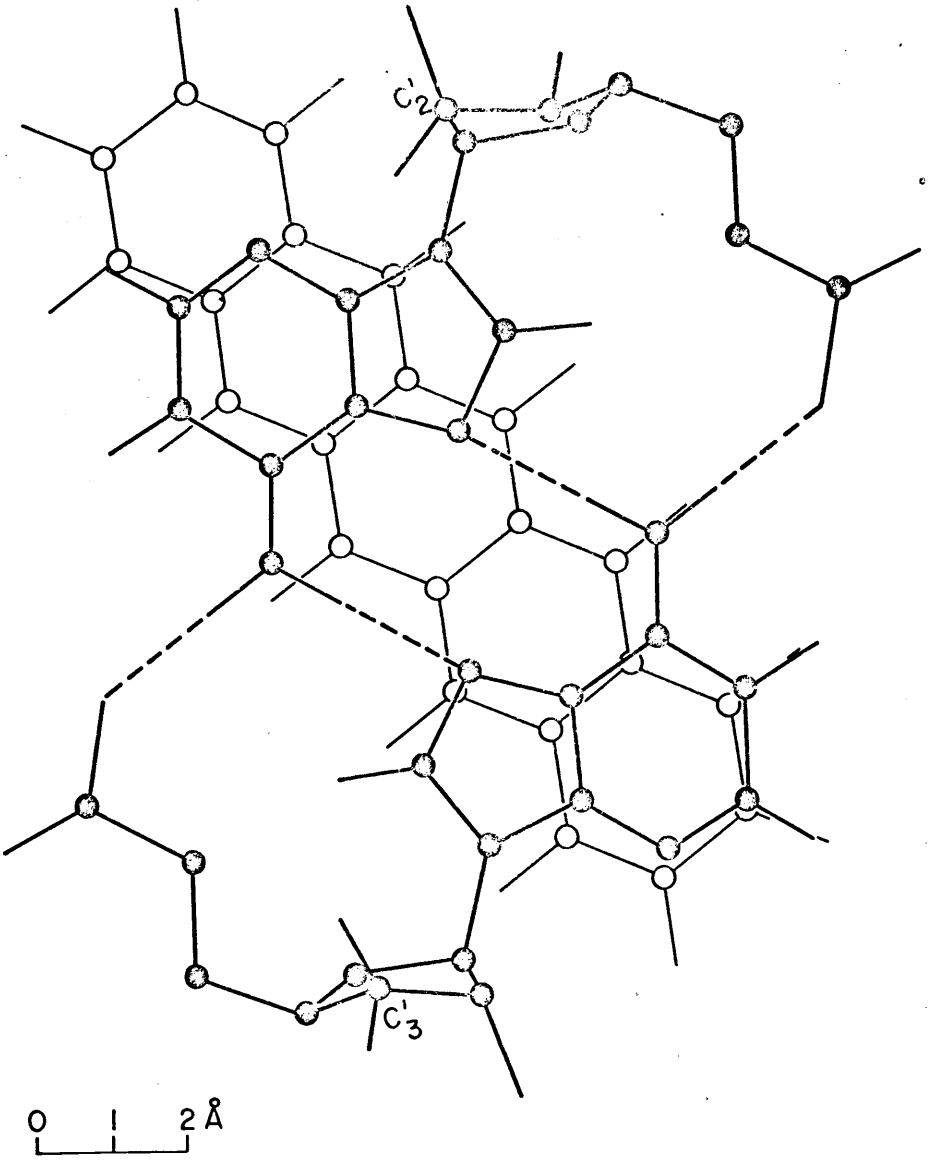

FIGURE 11: Presumed Model of Pentacene - poly A.

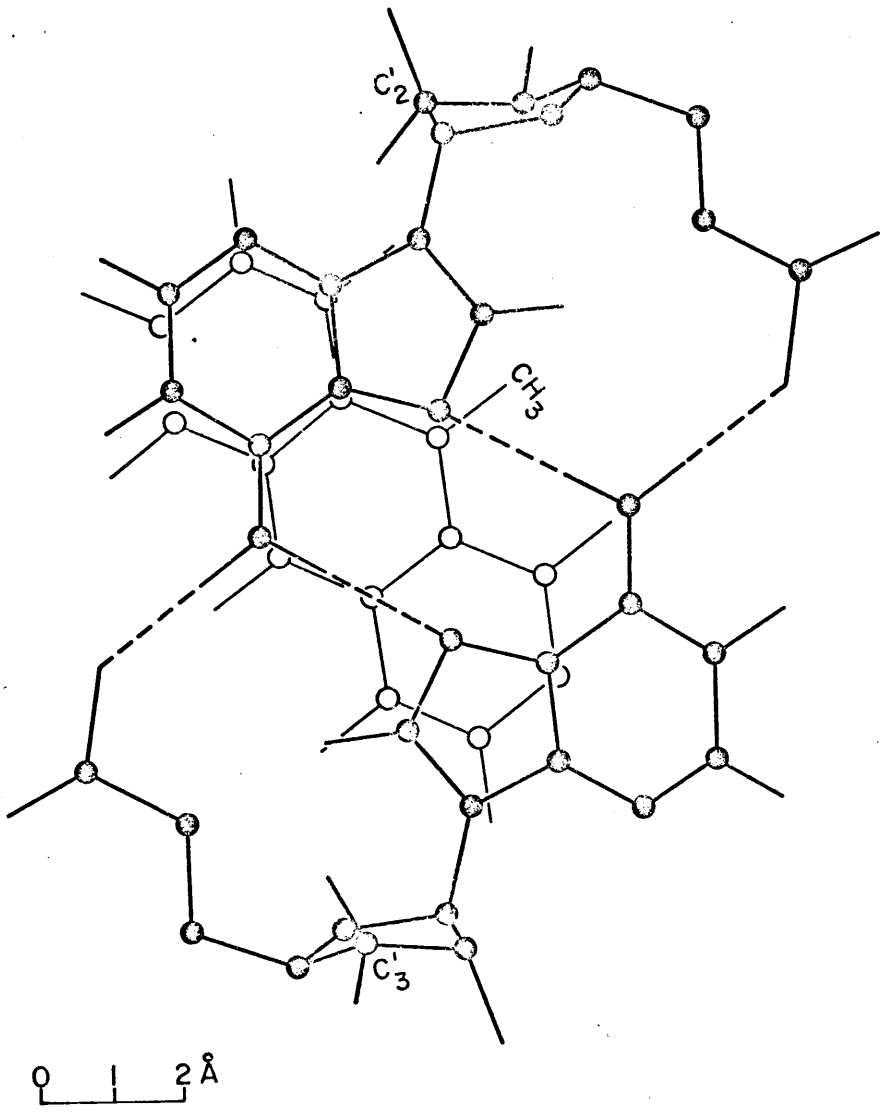

FIGURE 12: Model of 9-Methylanthracene - poly A in which the hydrocarbon shielded from contact with the medium.

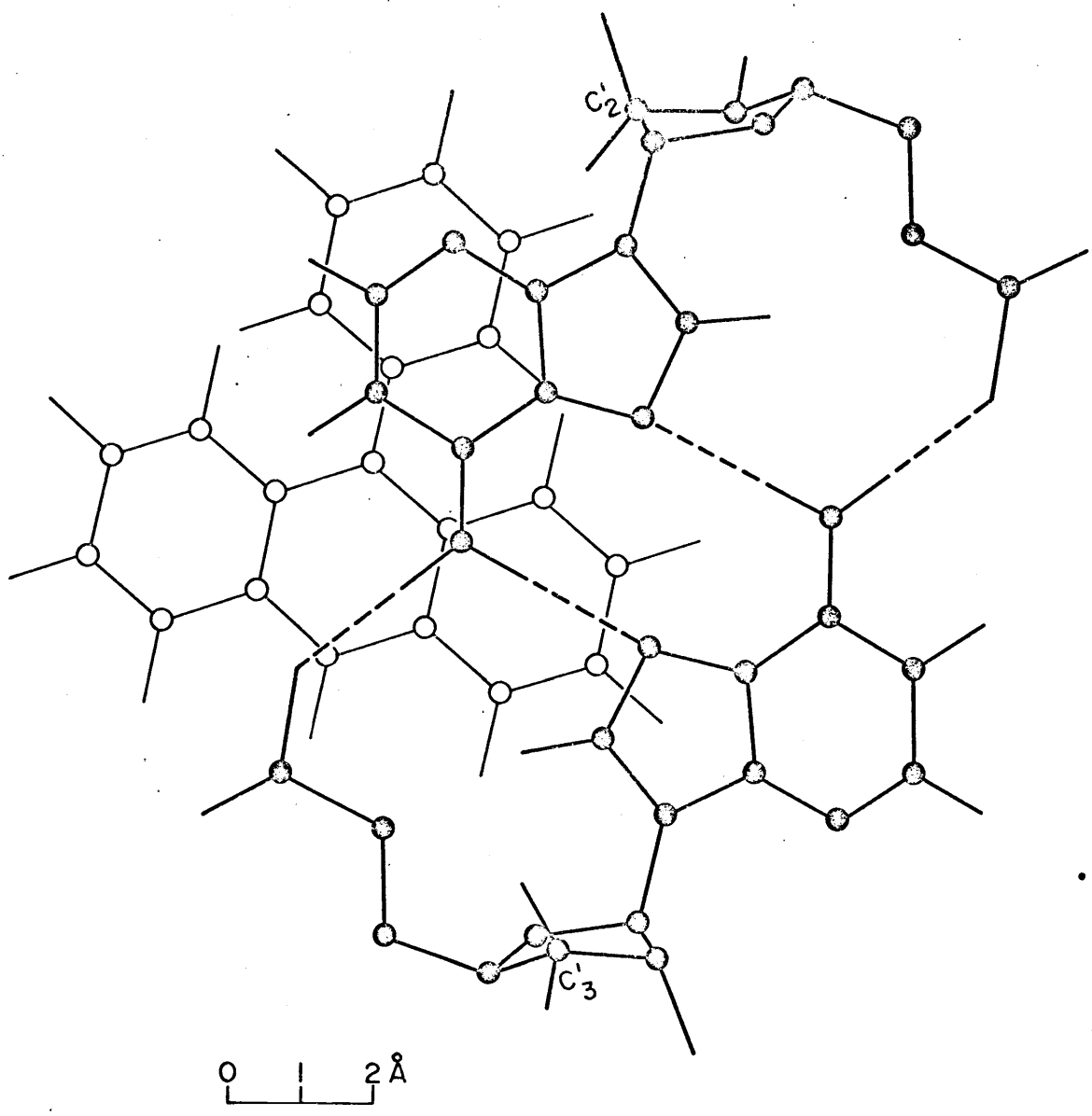

FIGURE 13 : Presumed Model of 9-Phenylanthracene - poly A.

further insertion of the 9-phenyl anthracene in poly A is sterically prevented by the hydrogens on the hydrocarbon and the hydrogens on the phosphodiester backbone. Part of the hydrocarbon juts into the medium, thus, the prediction is for no binding.

Figures 14, 15 and 16 form a third group. This group consists of pyrene and two derivatives. Figure 14 illustrates an intercalation model of pyrene. Like anthracene, pyrene has freedom of orientation under the poly A base pair and is still protected from the aqueous solution. The prediction was that pyrene would bind to poly A.

Figure 15 represents a model of 3,4-benzpyrene intercalation in poly A. 3,4-Benzpyrene may move 3 $\mathring{A}$ between the C_2-C_2 carbons on the base pair and still be protected from the water. 3,4-Benzpyrene was predicted to bind.

Figure 16 illustrates a presumed model of 1,2,3,4-dibenzpyrene intercalated and inserted as far as possible into the helix. 1,2,3,4-Dibenzpyrene protrudes from the poly A. The prediction was it would not bind.

The next two figures show hydrocarbons complexed to DNA. They are presented for comparison with those hydrocarbon intercalated in poly A. One hydrocarbon just fits in the DNA helix; the other is a bit too large. Figure 17 shows a model of 3,4-benzpyrene in DNA. There is only one orientation where 3,4-benzpyrene is shielded from the water. According to the size criterion, 3,4-benzpyrene's position is fixed in DNA. 3,4-Benzpyrene's position in poly A is not as rigidly fixed. However, as in poly A, it was predicted that 3,4-benzpyrene would bind to DNA.

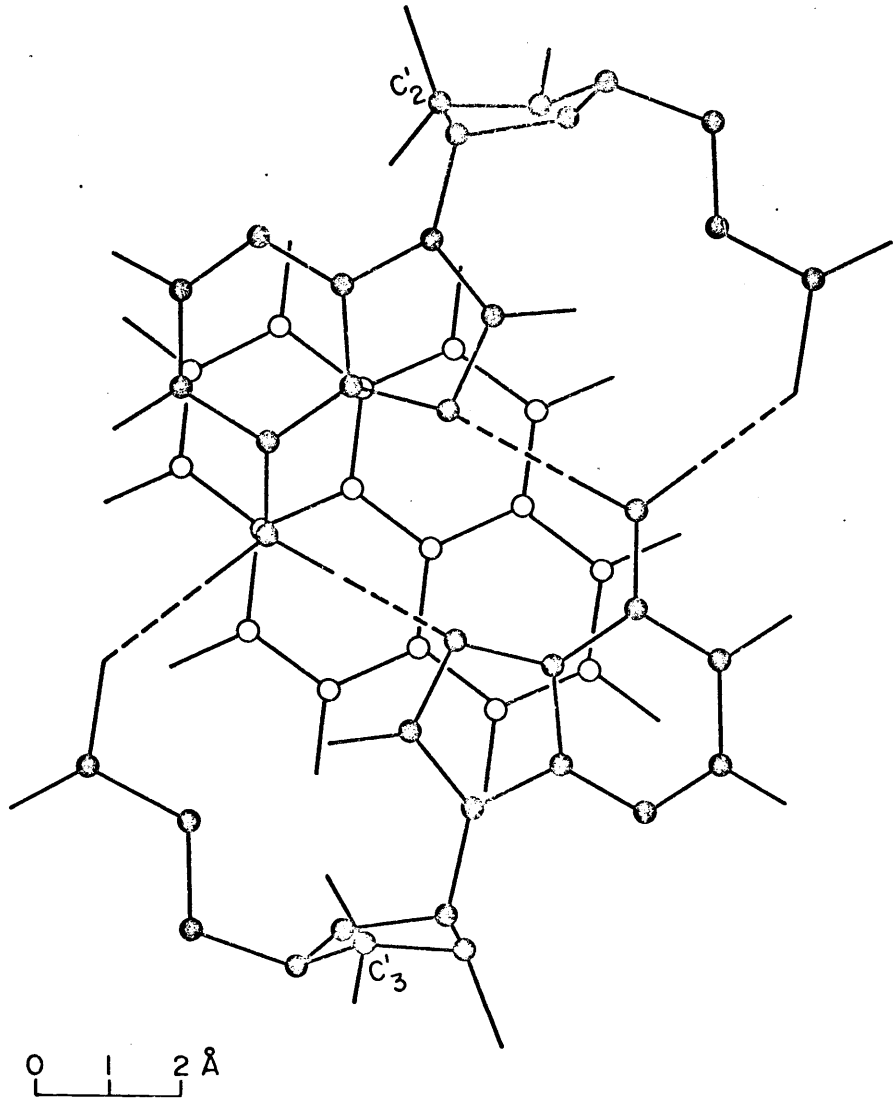

FIGURE 14: Model of Pyrene - poly A in which the hydrocarbon is shielded from contact with the medium.

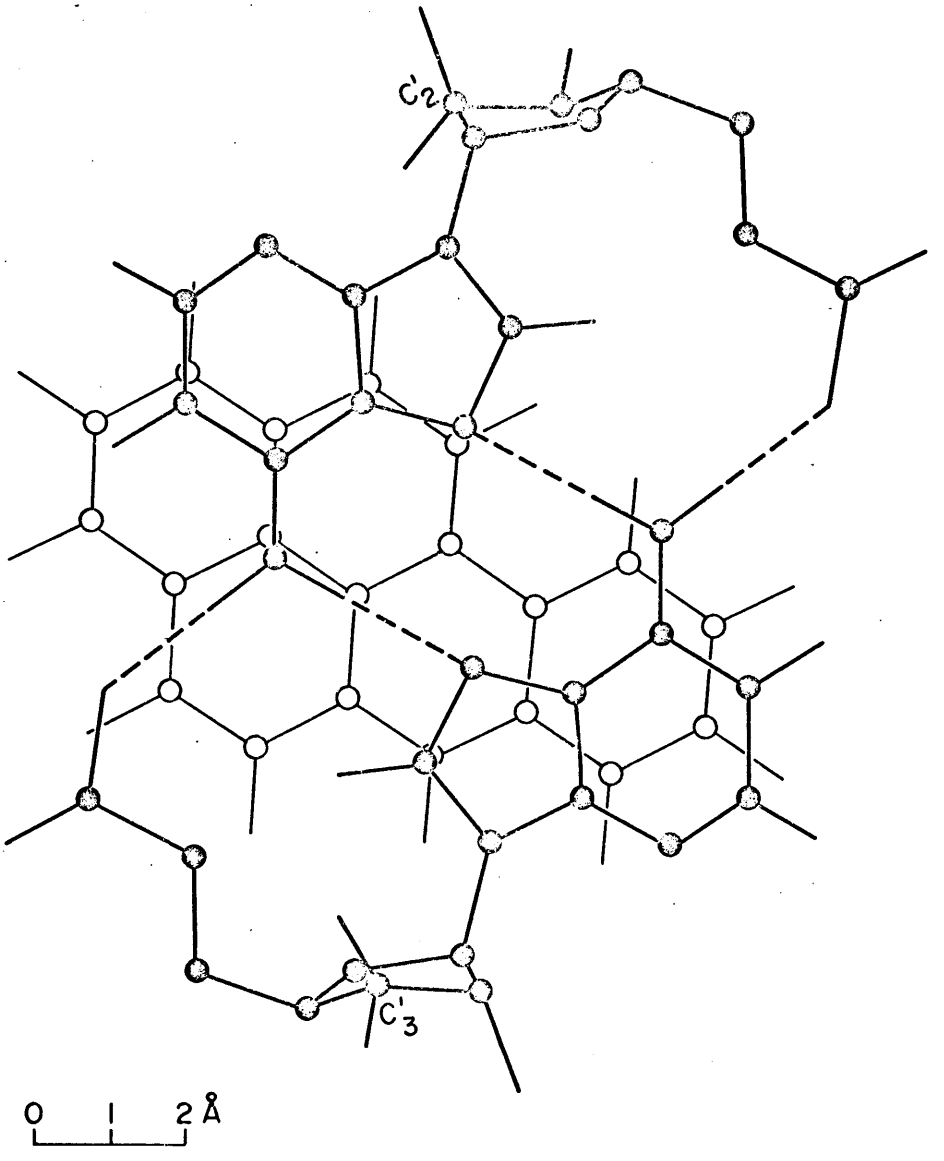

FIGURE 15: Model of 3,4-Benzpyrene - poly A in which the hydrocarbon is maximally shielded from contact with the medium.

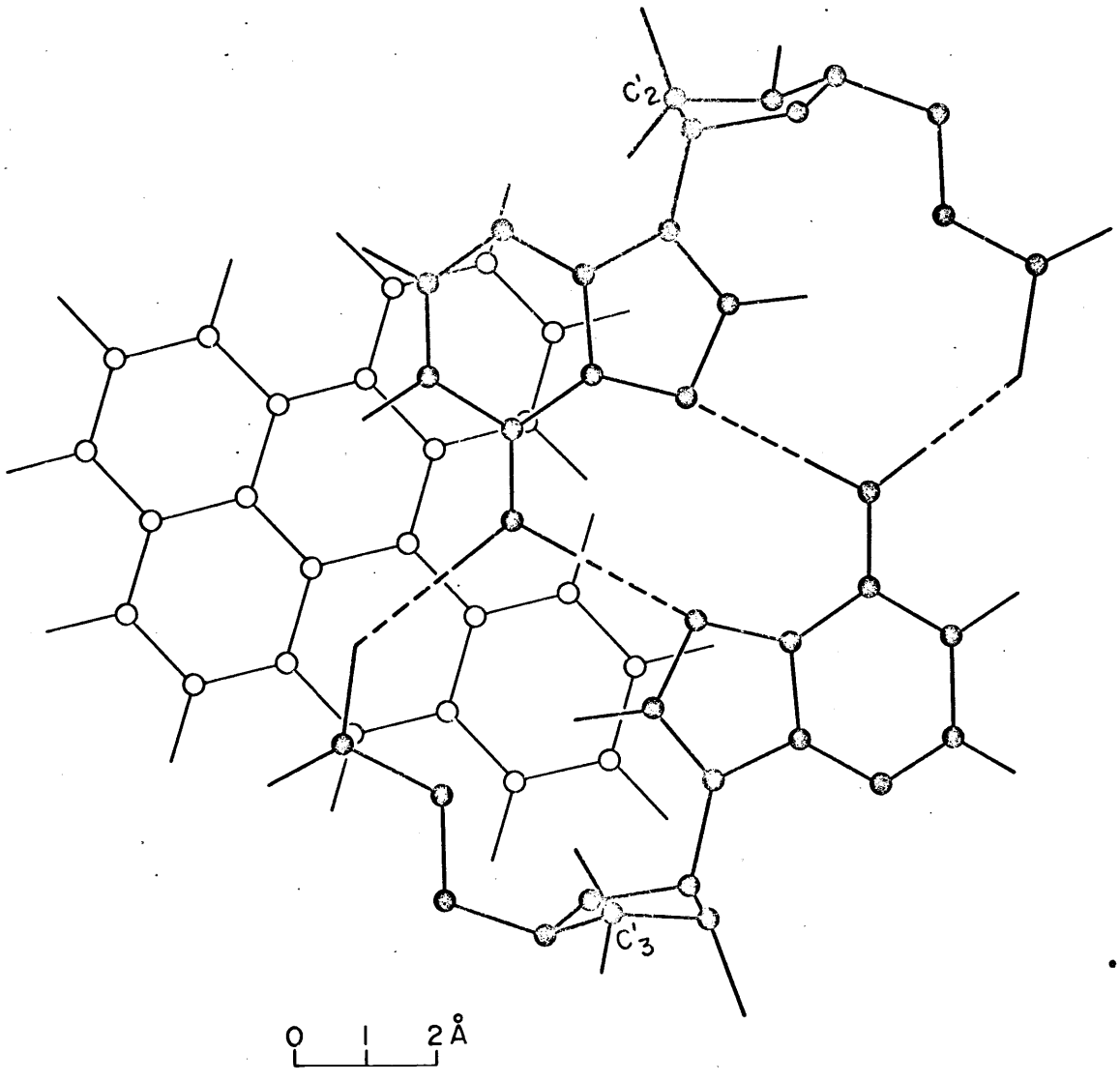

FIGURE 16: Presumed Model 1,2,3,4-Dibenzpyrene - poly A.

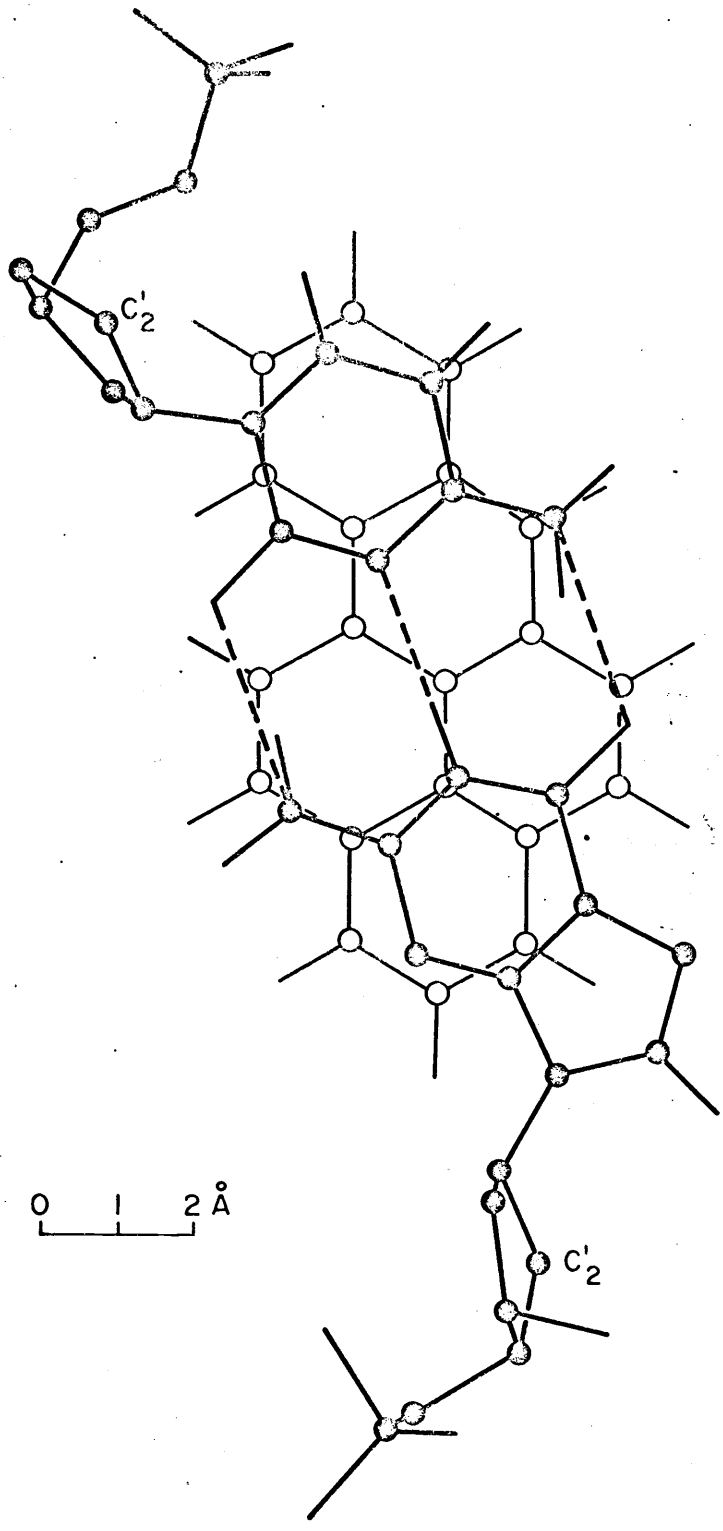

FIGURE 17: Model of 3,4-Benzpyrene - DNA showing hydrocarbon maximally shielded from contact with the medium.

In Figure 18, tetracene cannot be shielded by DNA. The steric hindrance preventing such shielding is between the hydrocarbon hydrogens and the C_2' hydrogens of the sugars. In poly A, tetracene was protected from contact with the solvent, and the prediction was that it would bind. However, in DNA it is not protected, and the prediction is that tetracene will not bind.

Testing of the Size Criterion For Poly A

Space filling models were built of the hydrocarbons and a short segment of poly A. By examining these models in the context of the size criterion, the binding and orientation of each hydrocarbon was predicted. To reiterate, a statement of the size criterion is that hydrocarbons that are not protected from water by the base pairs of poly A do not bind.

Table II summarizes the results for binding of hydrocarbons to poly A. All the predictions were verified experimentally. For convenience in Table II, the hydrocarbons are divided into Class 1 or Class 2. Class 1 hydrocarbons bind. Class 2 do not. The less-than sign in front of Class 2 hydrocarbons means no binding was found, and the number is the estimated upper limit of solubilization.

Some of the hydrocarbons that complex to poly A did not complex to DNA. In TableII, note that both tetracene and 1,2,5,6-dibenzanthracene complex to poly A. Neither of these hydrocarbons complexed to DNA. By inspecting Figures 8, 9 and 18, it can be seen that poly A protects these hydrocarbons from the aqueous medium, whereas, DNA does not. On the other hand, there is an example of a hydrocarbon

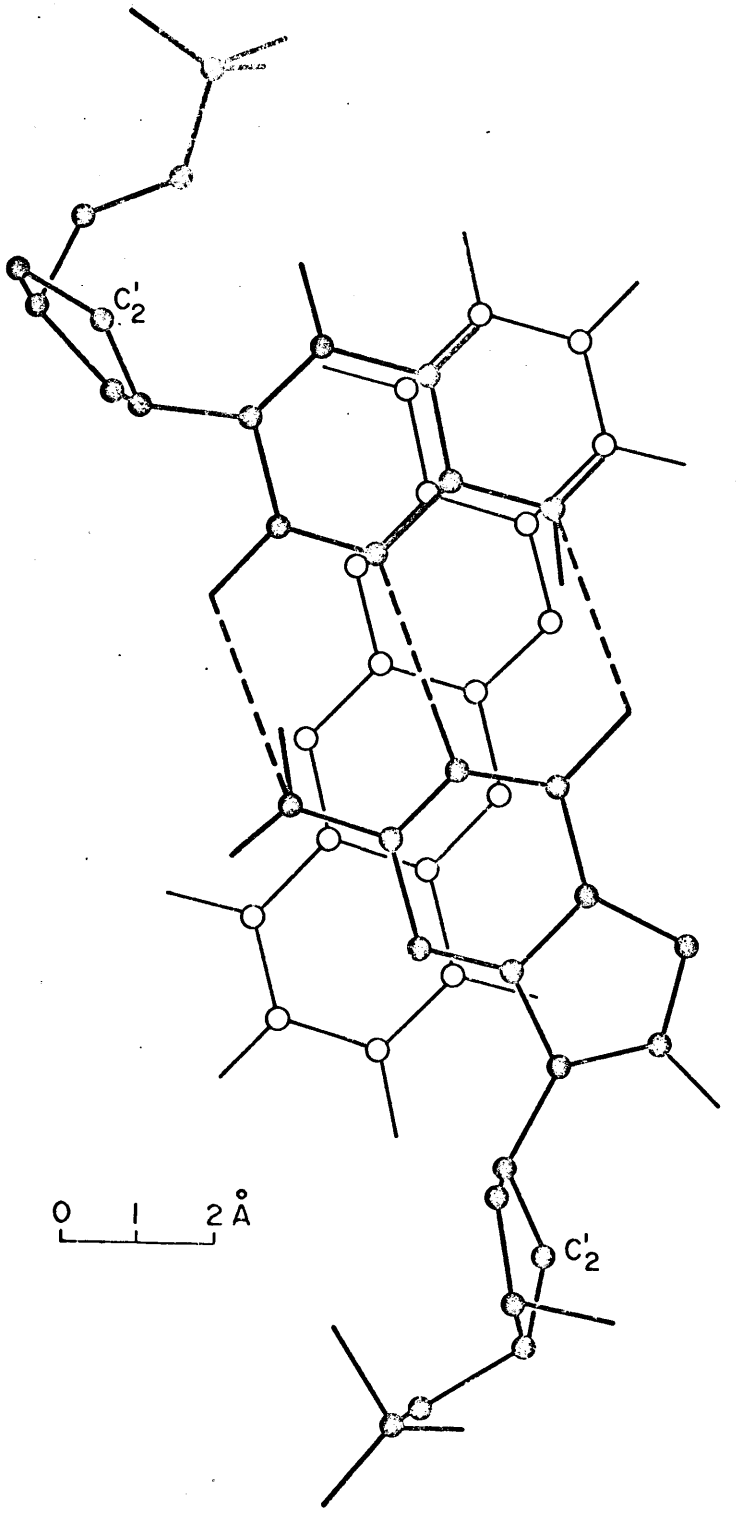

FIGURE 18: Presumed model of Tetracene - DNA.

TABLE II: Saturation Binding Values for Hydrocarbon-poly A Complexes. All values are averages of two or more determinations. A less than sign means no complexed hydrocarbon was found. The value after the less than sign is an estimated upper limit of experimental error. N. F. means flow dichroism measurements were not feasible. Group A contains benzyl derivatives of anthracene of various sizes. Group B contains anthracene derivatives with substitutions in the 9-position. Group C consists of three pyrene derivatives. Classes 1 and 2 are hydrocarbons that do and do not bind respectively.

	Flow Dichroism at 21,250 sec^{-1} average	Moles Hydrocarbon Per Mole Poly A Phosphate Measured by	
		Direct Absorption	Extraction by cyclohexane
Group A			
Class 1			
Anthracene	N.F.	1.4×10^{-4}	2.0×10^{-4}
1,2-Benzanthracene	$\frac{\Delta A}{A} = .07$	5.9×10^{-4}	3.9×10^{-4}
1,2,5,6-Dibenzanthracene	N.F.	0.24×10^{-4}	0.39×10^{-4}
Tetracene	N.F.	0.32×10^{-4}	0.57×10^{-4}
Class 2			
1,2,3,4-Dibenzanthracene	N.F.	$<7.4 \times 10^{-6}$	$<7.4 \times 10^{-6}$
Pentacene	N.F.	$<0.1 \times 10^{-6}$	$<0.1 \times 10^{-6}$

TABLE II (Cont'd.)

	Flow Dichrosim$_1$ at 21,150 sec^{-1} average	Direct Absorption	Extraction by cyclohexane
Group B			
Class 1			
Anthracene	N.F.	1.4×10^{-4}	2.0×10^{-4}
9-Methylanthracene	$\frac{\Delta A}{A} = .07$	10.7×10^{-4}	9.9×10^{-4}
Class 2			
9-Phenylanthracene	N.F.	$<5.8 \times 10^{-9}$	$<5.8 \times 10^{-9}$
Group C			
Class 1			
Pyrene	$\frac{\Delta A}{A} = .07$	7.5×10^{-4}	8.4×10^{-4}
3,4-Benzpyrene	$\frac{\Delta A}{A} = .07$	4.5×10^{-4}	6.8×10^{-4}
Class 2			
1,2,3,4-Dibenzpyrene	N.F.	$<5.4 \times 10^{-9}$	$<5.4 \times 10^{-9}$

that binds to DNA but not to poly A. 1,2,3,4-Dibenzanthracene does not bind to the acid form of poly A, but binds to DNA. 1,2,3,4-Dibenzanthracene is not protected from the aqueous medium by poly A, but is protected by DNA.

The error, of the results presented in Table II, is within a factor of two. Both absorption and extraction techniques agreed to within that factor. By using two techniques for determining the amount of hydrocarbon bound, the chance of error was decreased. This "double check" is needed since hydrocarbons bind in such small amounts.

Table III is a qualitative summary of the work on the size criterion hypothesis for both poly A and DNA (13). The prediction can be compared with all the hydrocarbons tested for both polynucleotides. As previously stated, all predictions for both DNA and poly A have been verified.

Analogous to DNA complexes, the spectra of the hydrocarbon when complexed to poly A were red shifted 12 nm. The fluorescence of the hydrocarbons was quenched to various degrees (Figures 19-25). These figures are redrawn from original data.

Figure 19 shows the excitation and emission spectra of 3,4-benzpyrene in DNA, poly A and cyclohexane. The excitation spectra of the three solutions are normalized to give equal absorptions. Thus, the fluorescence is relative. The fluorescence of 3,4-benzpyrene, when complexed to either poly A or DNA, is quenched about one third. Moreover, both the excitation and emission spectra are red shifted 10 nm. This result is in variance with Green and McCarter (20) who stated the emission spectra were only red shifted 3 nm.

TABLE III: Comparison of Binding Predicted by the Size Criterion for DNA and Double Stranded Poly A.

	DNA	Poly A
Group A		
Anthracene	Yes	Yes
1,2-Benzanthracene	Yes	Yes
1,2,3,4-Dibenzanthracene	Yes	No
1,2,5,6-Dibenzanthracene	No	Yes
Tetracene	No	Yes
Pentacene	No	No
Group B		
Anthracene	Yes	Yes
9-Methyl Anthracene	Yes	Yes
9-Phenyl Anthracene	No	No
Group C		
Pyrene	Yes	Yes
3,4-Benzpyrene	Yes	Yes
1,2,3,4-Dibenzpyrene	No	No
1,2,5,6-Dibenzpyrene	No	No

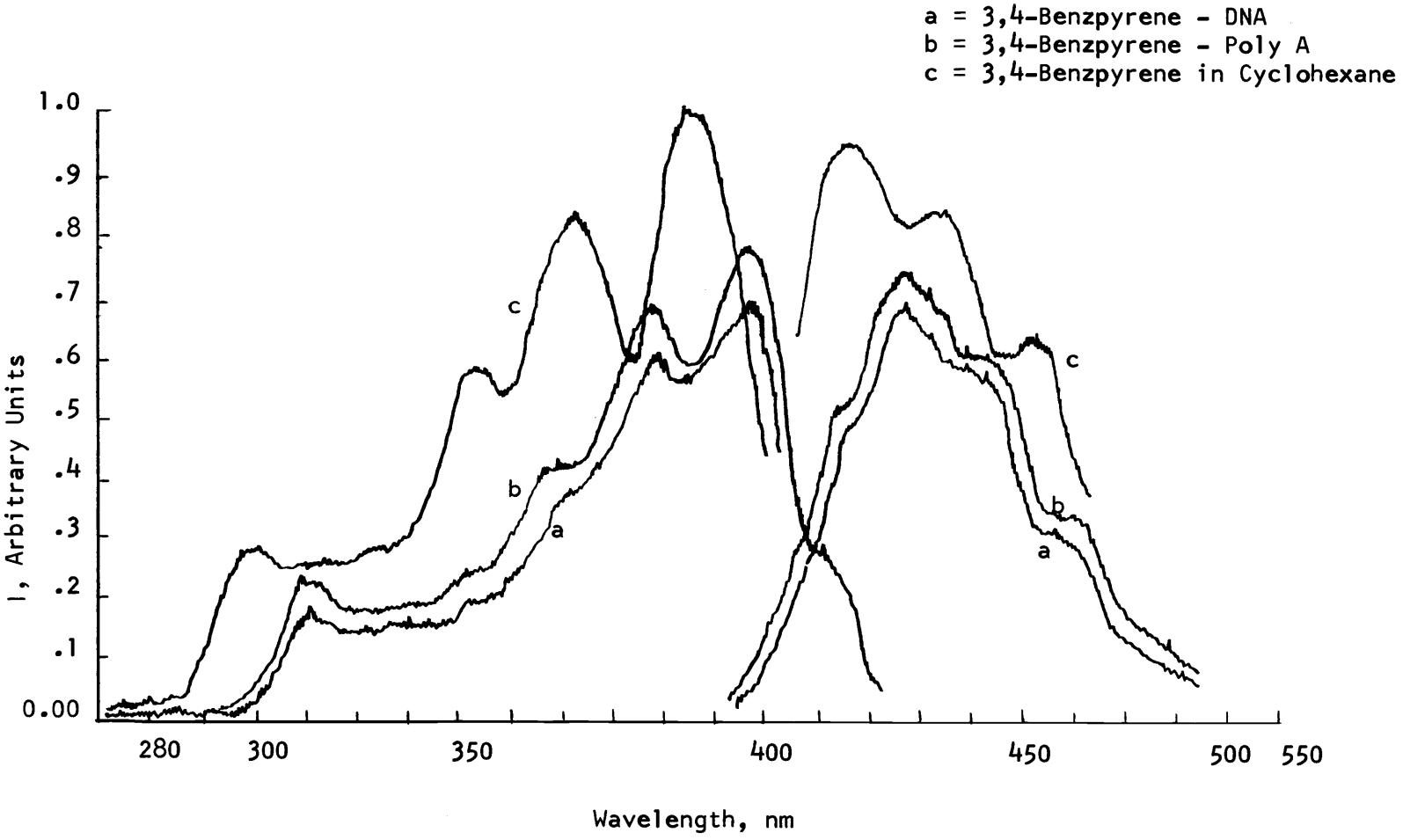

FIGURE 19: Excitation and Emission Spectra of 3,4-Benzyprene in DNA, Poly A and Cyclohexane. Note the red shift of the hydrocarbon spectra in DNA and poly A.

The vibrational structure of the excitation spectra was resolved. Figure 20 shows that the anisotropy of emission of 3,4-benzpyrene in DNA and poly A is constant over the vibrational frequencies. The anisotropy of emission from the complexed hydrocarbons in 0.26 $\pm$ .02. The anisotropy of the hydrocarbon in cyclohexane averages 0.00 $\pm$ .01. When in cyclohexane, the hydrocarbon's emission is completely depolarized by Brownian rotation. The hydrocarbon, when complexed to a polynucleotide, has the much slower rotational diffusion of the polynucleotide. Thus, the emission from the hydrocarbon is polarized.

Figure 21 shows the fluorescence of anthracene in DNA, poly A and cyclohexane. Since not much hydrocarbon binds, the fluorescence of anthracene is weak; moreover, it is further quenched by a factor of one-half. Again the excitation and emission spectra of anthracene in DNA and poly A are red shifted 10 nm. The vibrational structure of the complexes is resolved, and it is the same as that for anthracene in cyclohexane. The anisotropy for the DNA and poly A complexes averages about 0.26. The anisotropy for anthracene in cyclohexane is again around 0.00. Most hydrocarbons are so insoluble in water that no fluorescence can be detected. However, the fluorescence of anthracene could be measured. Note that the spectrum of anthracene in water is not red shifted (Figure 23). Moreover, no quenching could be observed.

Figure 24 and 25 contain the emission and excitation spectra of 1,2,5,6-dibenzanthracene and tetracene in poly A and cyclohexane. Again the spectrum of hydrocarbon-poly A complex is red shifted 10 nm. Neither tetracene nor 1,2,5,6-dibenzanthracene bound to DNA (Table III).

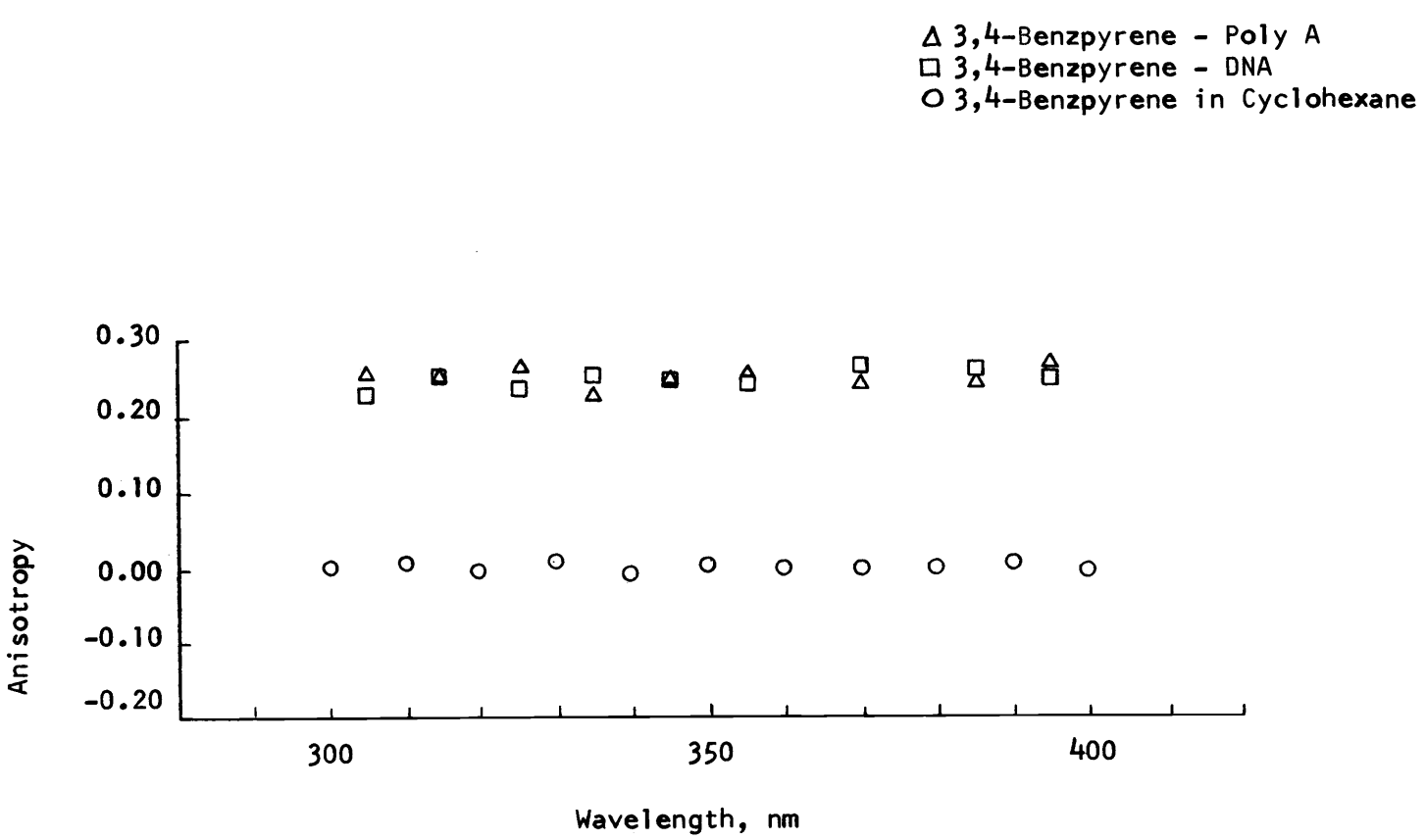

FIGURE 20: Anisotropy of 3,4-Benzpyrene in DNA, Poly A and Cyclohexane.

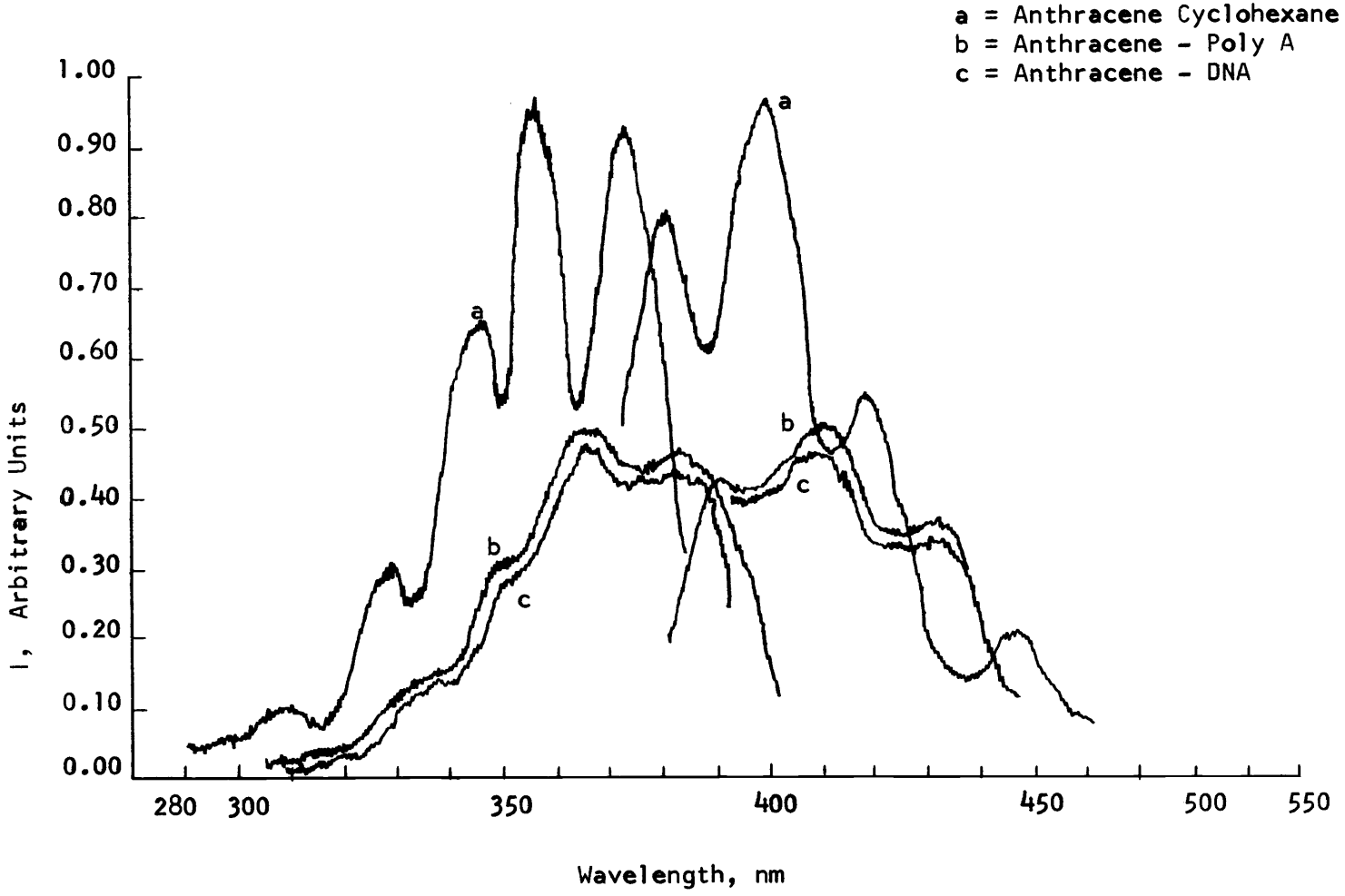

FIGURE 21: Excitation and Emission Spectra of Anthracene in DNA, Poly A and Cyclohexane. Note the red shift of the hydrocarbon spectra in DNA and poly A.

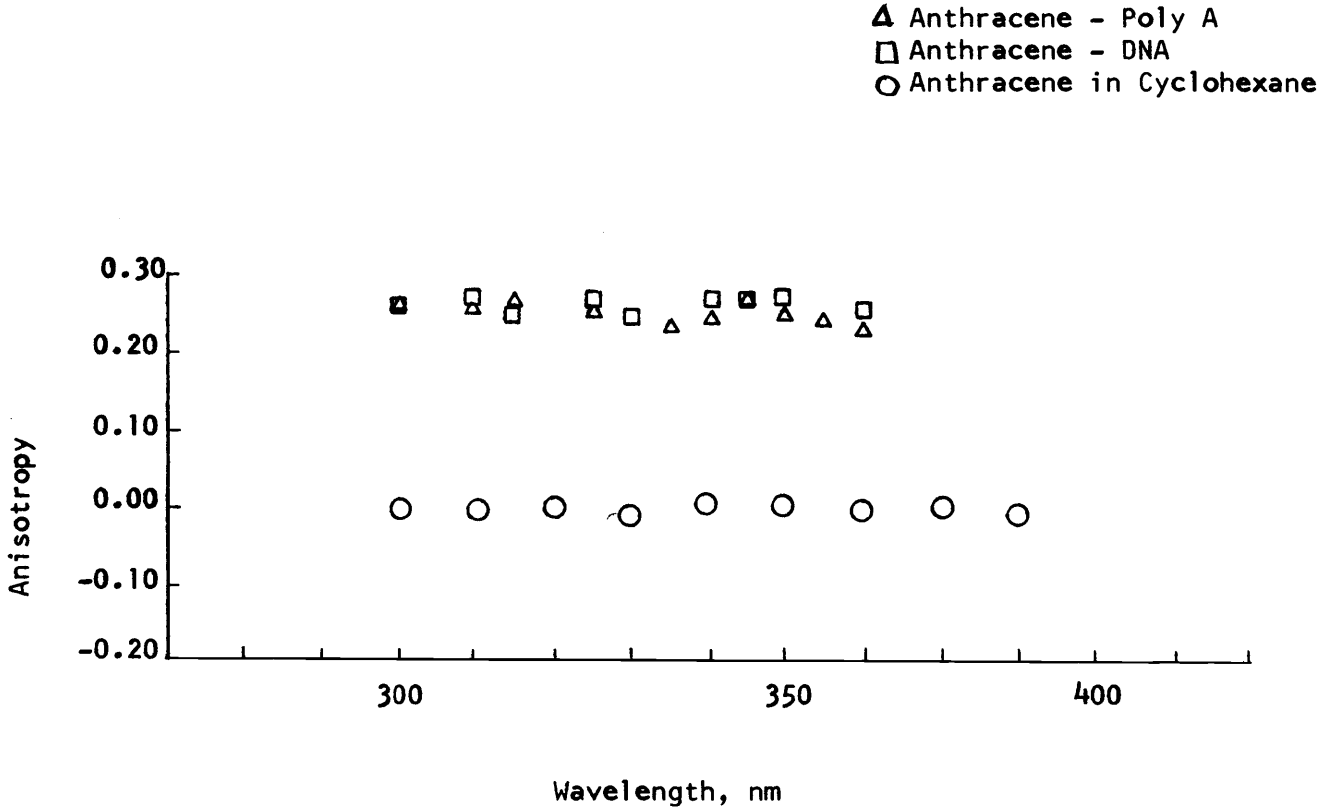

FIGURE 22: Anisotropy of Anthracene in DNA, Poly A and Cyclohexane.

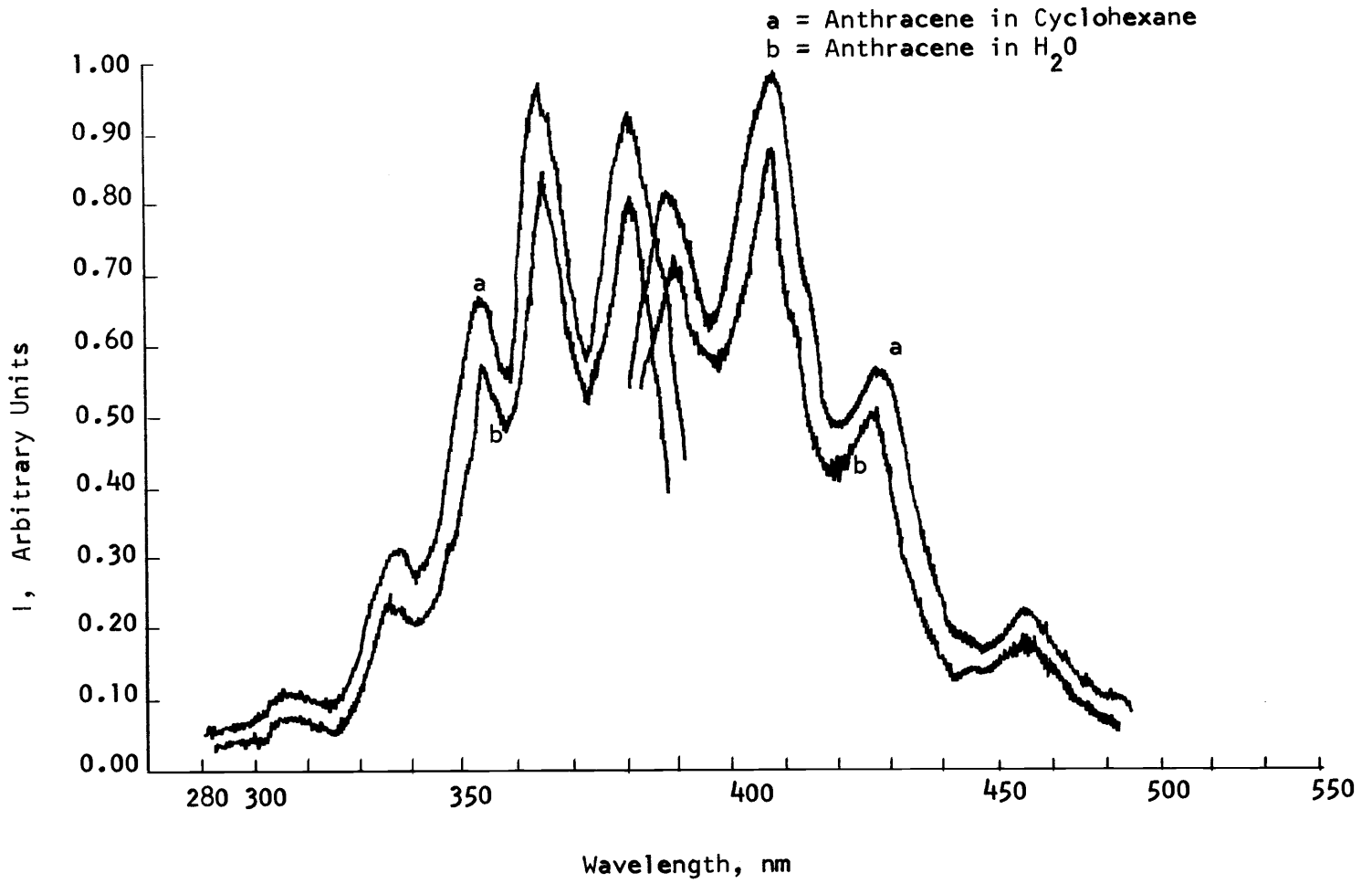

FIGURE 23: Excitatior and Emission Spectra of Anthracene in Water and Cyclohexane. No red shift in the hydrocarbon spectra.

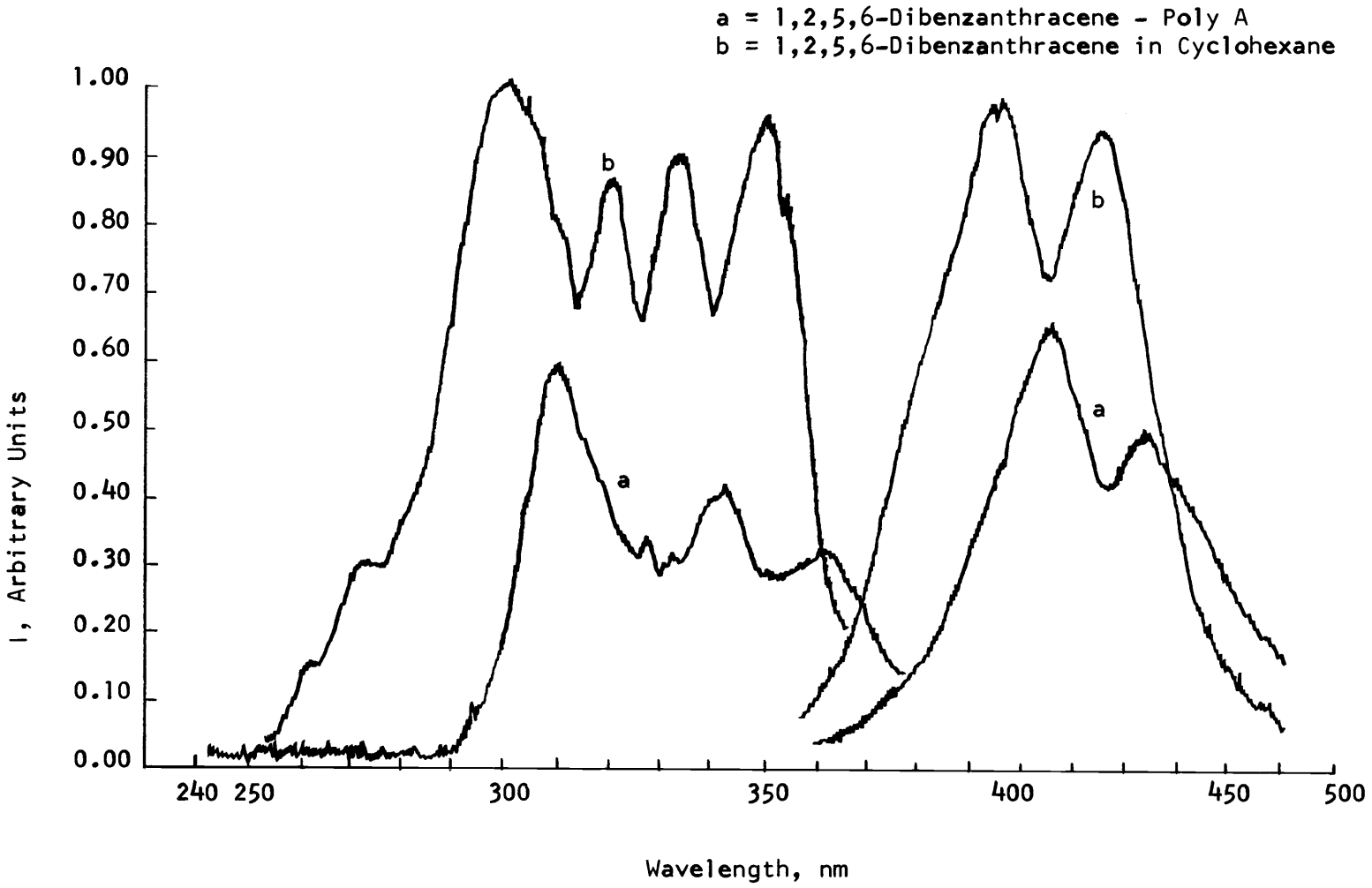

FIGURE 24: Excitation and Emission Spectra of 1,2,5,6-Dibenzanthracene in Poly A and Cyclohexane. Note the red shift of the hydrocarbon spectra in poly A.

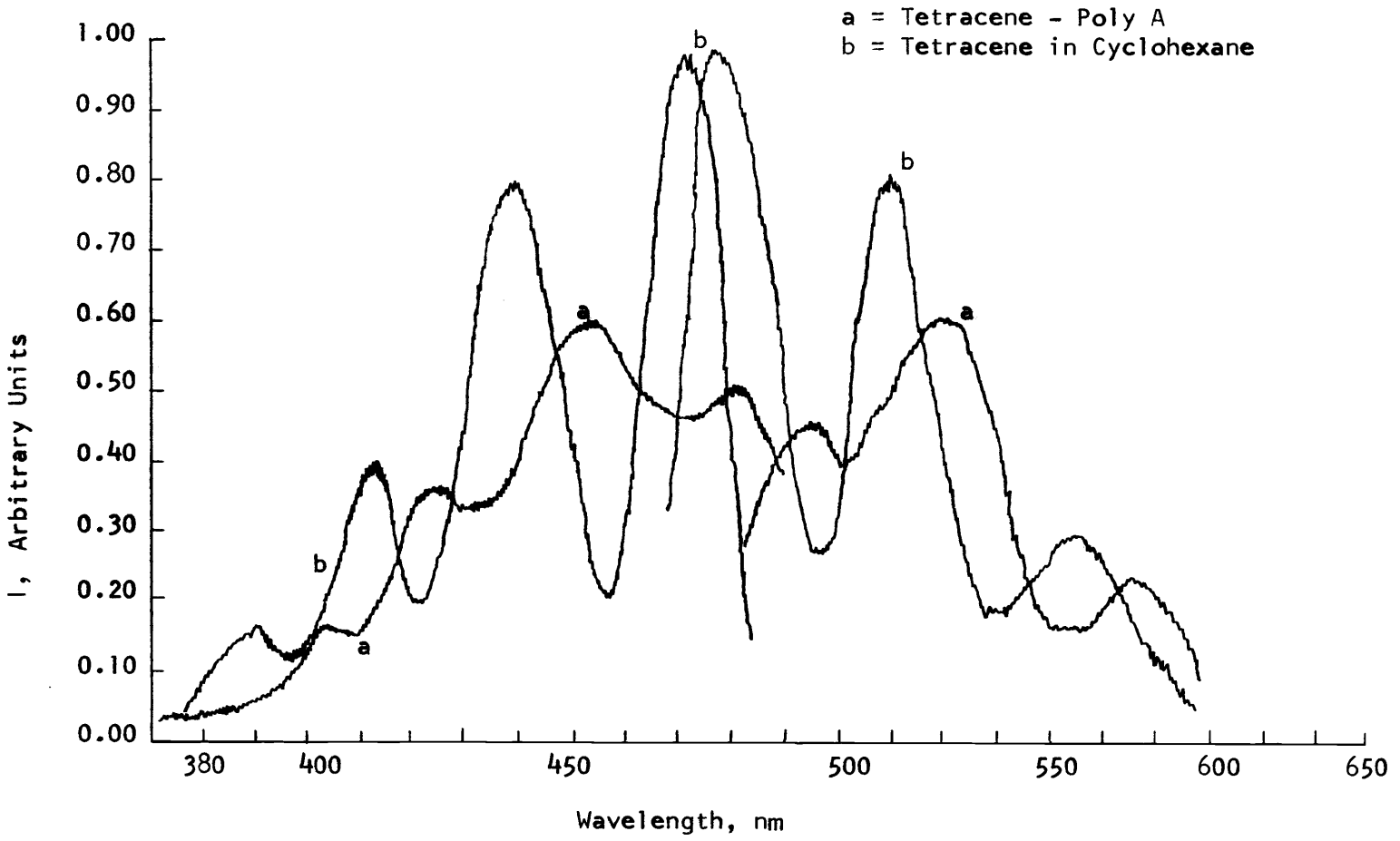

FIGURE 25: Excitation and Emission Spectra of Tetracene in Poly A and Cyclohexane. Note the red shift of the hydrocarbon spectra in poly A.

Since both tetracene and dibenzanthracene complex to such a small extent, it was difficult to obtain accurate values for quenching. The values reported in the figures are probably only good to a factor of four. Figure 26 presents the anisotropy of these two hydrocarbons in poly A complex and cyclohexane. Again the anisotropy, when hydrocarbons are complexed, is relatively high, and the anisotropy of hydrocarbons in cyclohexane is near zero.

Figures 27 and 28 present a different problem. Figure 27 contains the emission and excitation spectra of 9-methyl anthracene bound to DNA, poly A and dissolved in cyclohexane. Figure 28 presents the spectra of pyrene in the same solvents. In both figures the fluorescence is severely quenched. Moreover, the spectra of the complexed hydrocarbons are not red shifted. This indicates that the emission is from the monomer hydrocarbon in aqueous solution and no emission is observed from the complexed hydrocarbon. Moreover, to support this conclusion, the anisotropy, for the DNA, poly A and cyclohexane solutions of 9-methyl anthracene and pyrene, averages 0.00 (Figure 29).

All the results of fluorescence anisotropy are summarized in Table IV. The anisotropy values in Table IV are representative of the average values over the excitation wavelengths. In general, the anisotropy of complexed hydrocarbons is relatively large, and the anisotropy of hydrocarbons in cyclohexane is nearly zero.

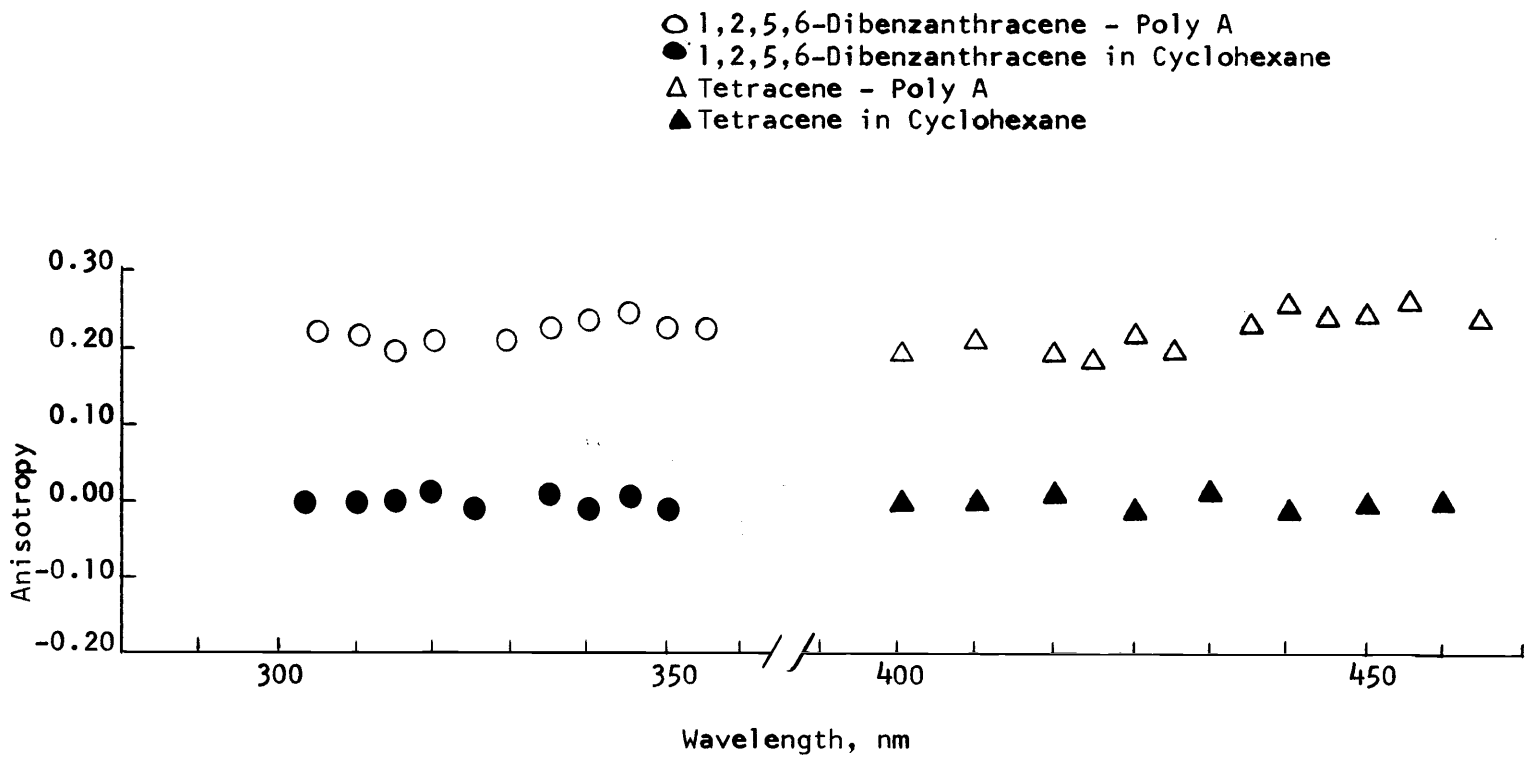

FIGURE 26: Anisotropy of 1,2,5,6-Dibenzathracene and Tetracene in Poly A and Cyclohexane.

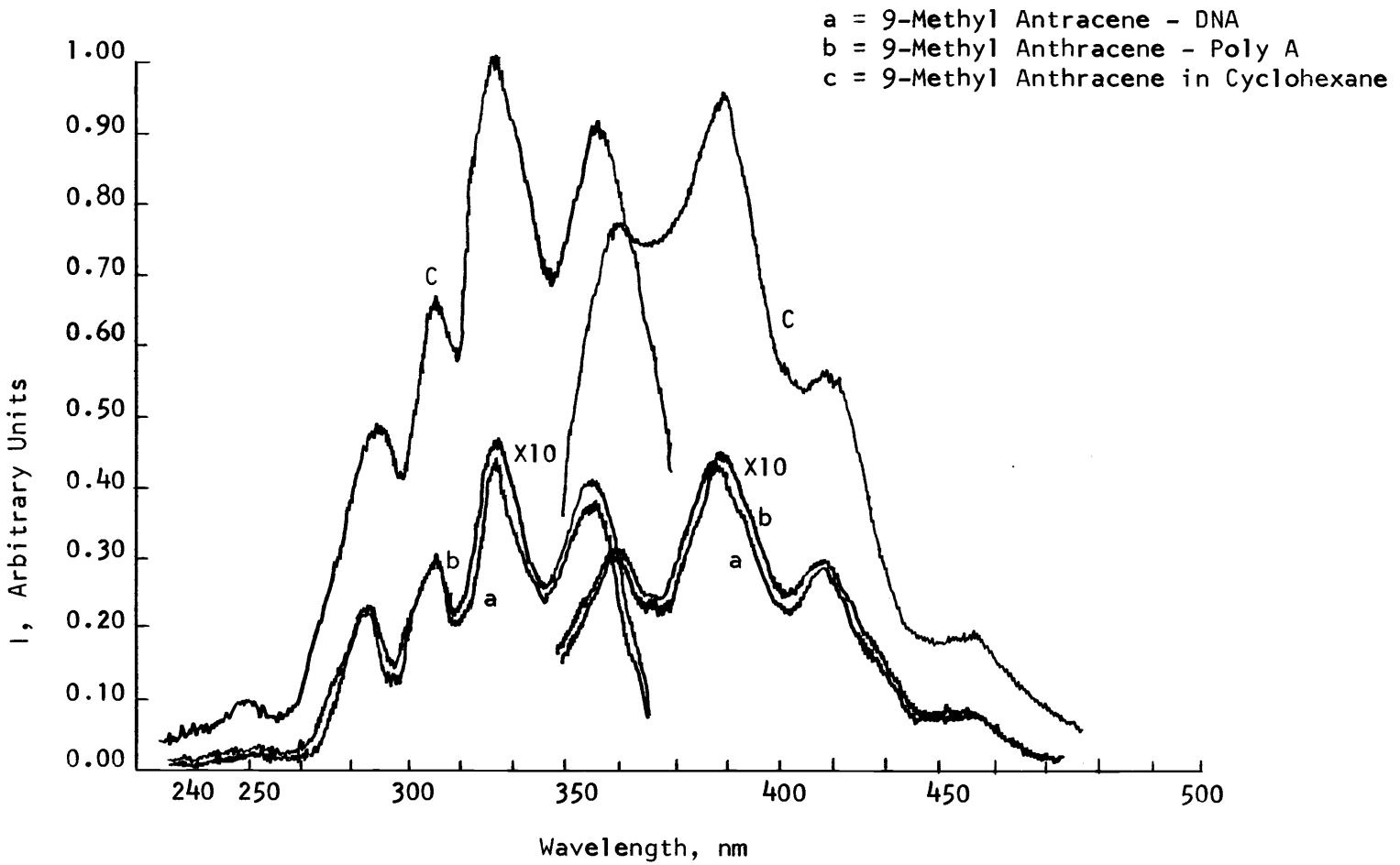

FIGURE 27: Excitation and Emission Spectra of 9-Methyl Anthracene in DNA, Poly A and Cyclohexane. Note no red shift.

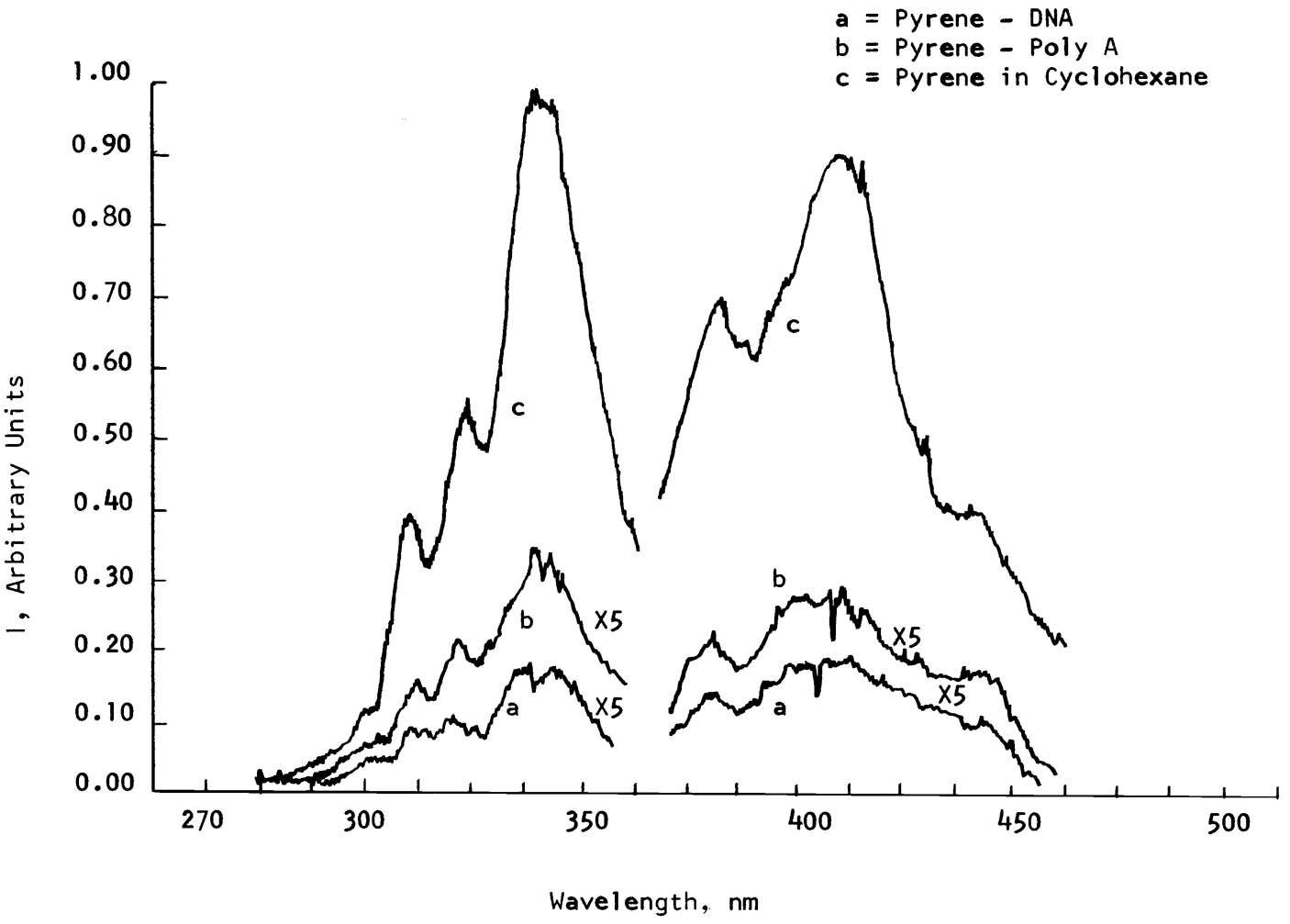

FIGURE 28: Excitation and Emission Spectra of Pyrene in DNA and Poly A. Note no red shift.

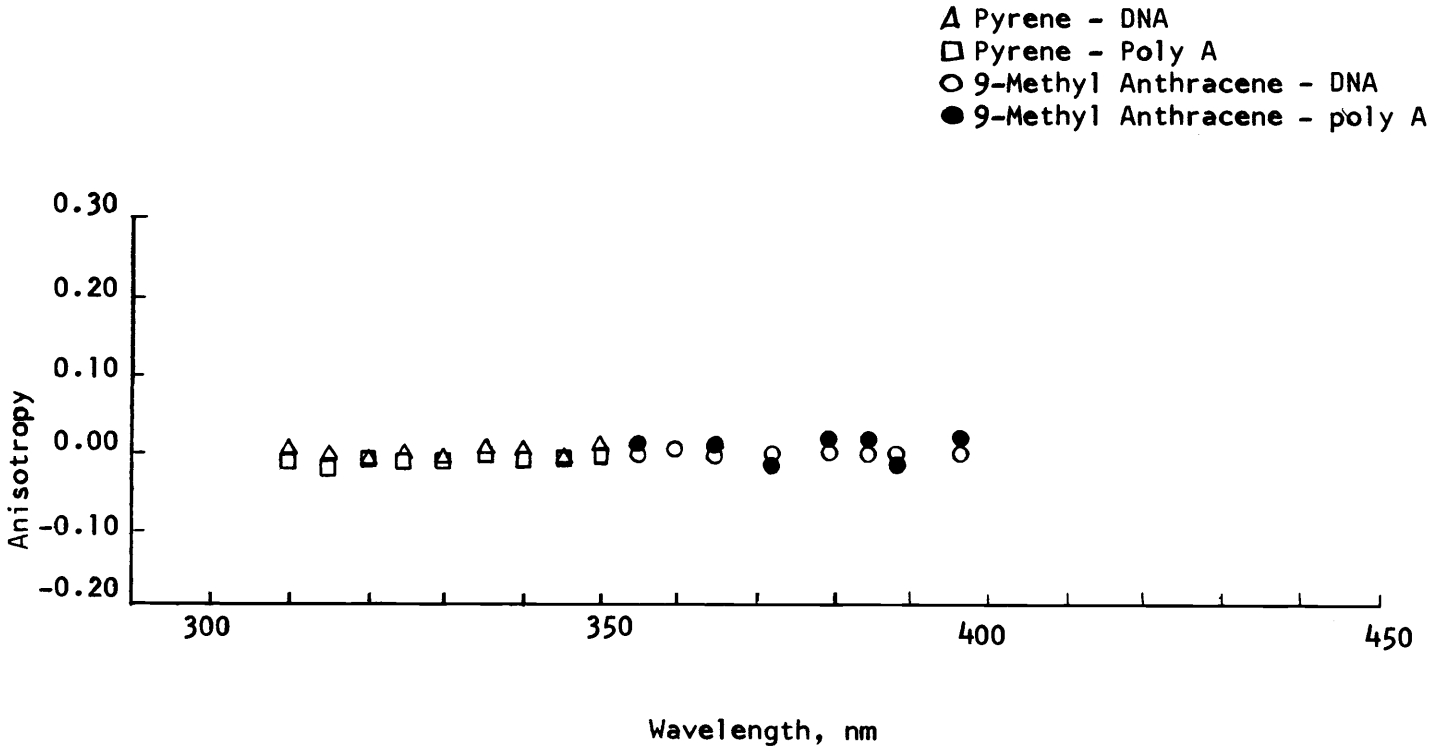

FIGURE 29: Anisotropy of Pyrene and 9-Methyl Anthracene in DNA and Poly A.

TABLE IV: Fluorescence Anisotropy of Polycyclic Aromatic Hydrocarbon Emissions

$$\mu = \frac{3}{2}\frac{E - B}{E + 2B}$$

Hydrocarbon	Excitation λ nm	DNA μ	Poly A μ	Excitation λ nm	Cyclohexane μ
3,4-Benzpyrene	376	.26 ± .01	.25 ± .01	365	.00 ± .01
Anthracene	360	.28 ± .02	.26 ± .02	348	.00 ± .02
1,2,5,6-Dibenzanthracene	340	no signal*	.26 ± .01	330	.01 ± .01
Tetracene	450	no signal*	.25 ± .02	440	.01 ± .02
Pentacene	---	no signal*	no signal*	---	---------
9-Phenyl Anthracene	392	---------	no signal*	---	---------
9-Methyl Anthracene	365**	.01± .01	.01 ± .01	365	.00 ± .01
Pyrene	335**	.00 ± .01	.01 ± .01	335	.01 ± .01

* "No signal" means there was no detectable emission. This is a trival observation since we found that these hydrocarbons did not complex. See Table II.

** No characteristic red shift in either excitation or emission spectra. See text.

Flow Dichroism

As stated previously, the change in absorbance ΔA is due to the intrinsic anisotropic of the base and the asymmetry of the molecule. As the helical polynucleotides line up in the velocity gradient, the absorbance of $\pi \longrightarrow \pi^*$ transitions will increase over random orientation. This transition is perpendicular to the DNA helical axis. The flow dichroism, $\Delta A/A$, is a function of the molecular weight and also the sheer gradient. For instance, for a given molecular weight, the higher the average sheer gradient, $\bar{G}$, the higher the flow dichroism, $\Delta A/A$. However, if the molecular weight of the sample is increased, the dichroism, $\Delta A/A$, will be higher than the previous values at each $\bar{G}$.

The purpose of the flow dichroism was to show the orientation of the hydrocarbon with respect to the poly A. Thus the orientation of the poly A had to be determined. This was determined in a series of steps. Since the flow dichroism cell has an absorbance path length of 10 cm,the solution must be dilute. The stock solution of salmon sperm DNA was diluted from 1.12×10^{-2}M (0.001M NaCl, 0.003M cacodylate buffer pH 6.8) to 1.35×10^{-5}M. This concentration corresponds to an absorbance $A_{260}^{1cm} = 0.094$. The flow dichroism, $\Delta A/A$, versus $\bar{G}$ was then obtained. The curve was similar to those obtained by Callis and Davidson and the same as that in Figure 30. The dichroism at a sheer gradient of 21,250 sec^{-1} at 255 nm was $\Delta A/A = 0.097$. Proflavin, 1.2×10^{-5}M, was then complexed to 12 ml of the stock solution of DNA, 1.12×10^{-2}M. Figure 30 presents a plot of $\Delta A/A$ versus $\bar{G}$ for this solution. The absorbance was taken at 444 nm; $\Delta A/A = 0.096$ at a

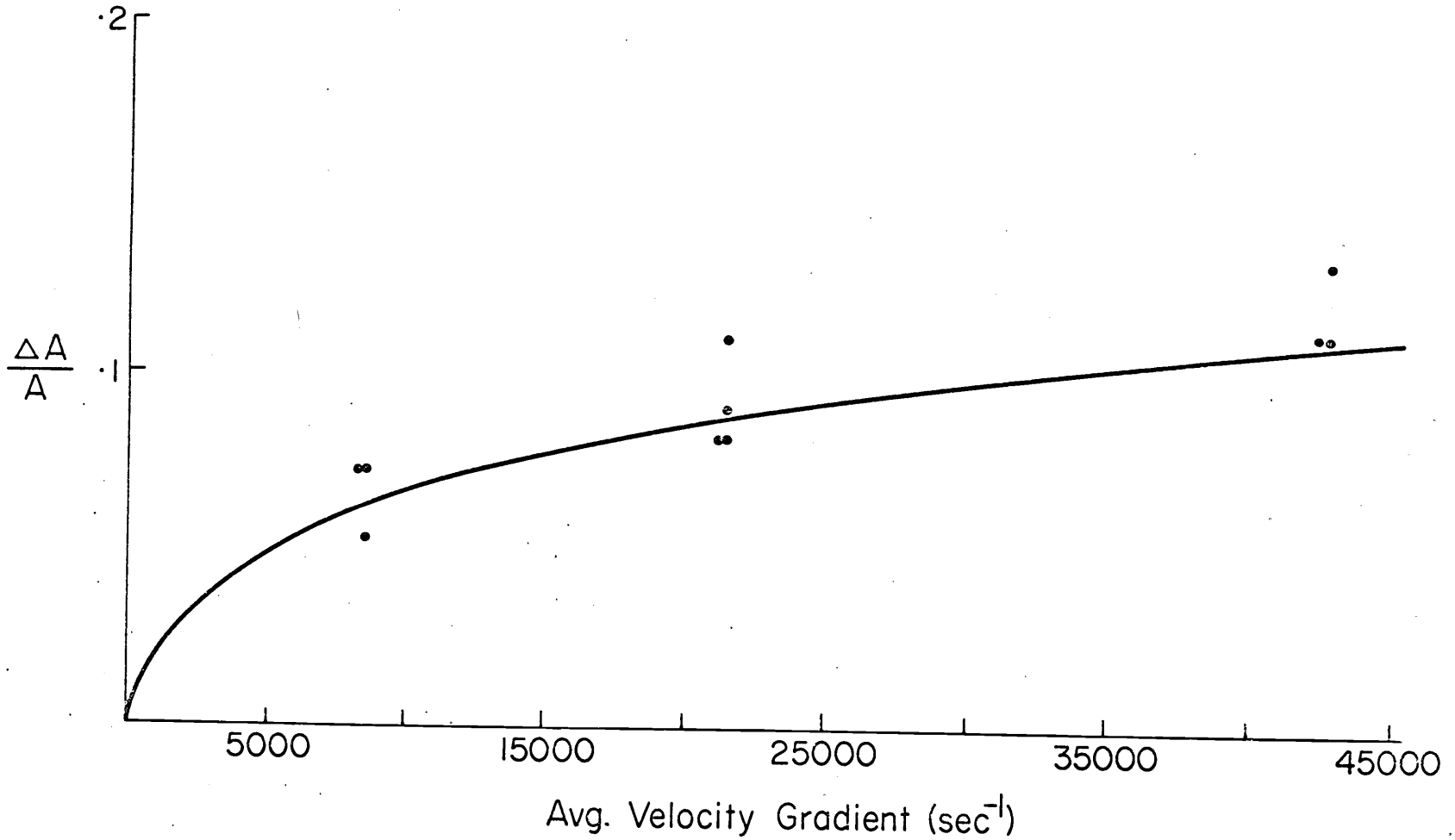

FIGURE 30: Flow Dichroism of Proflavin - DNA Complex, λ = 444nm.

sheer gradient of 21,250 sec^{-1}. The dichroism at both the lower concentration, 10^{-5}M, and higher concentration, 10^{-2}M, are the same. This shows that the DNA at a higher concentration and in a more viscous solution will still align in the flow.

The next step, in determining the orientation of poly A in a flow gradient, was to obtain the dichroism of a dilute solution of poly A, 1.45×10^{-5}M. The dichroism at λ = 255 nm versus $\bar{G}$ appears in Figure 31 (a) for a gradient of 21,250 sec^{-1} $\Delta A/A = 0.07$. In Figure 31 (b) the concentration of the poly A was increased to 5×10^{-5}M, and the dichroism was measured at λ = 275 nm. However, the concentration of the poly A in the hydrocarbon complexes is 1.45×10^{-2}M. Since the absorbance at this concentration is too great to obtain a dichroism directly, proflavin was complexed to poly A. The $\Delta A/A$ versus $\bar{G}$ appears in Figure 32. For a gradient of 21,250 sec^{-1}, $\Delta A/A = 0.08$. At this concentration air bubbles and dust particles caused significant noise problems, thus, our error was $\pm$ 0.02 absorbance units. However, it is evident, for this poly A solution at a sheer gradient of 21,250 sec^{-1}, molecules that absorb in a plane perpendicular to the helical axis, such as the bases or dyes, have a $\Delta A/A$ around 0.07 to 0.08.

Flow dichroism measurement were made on double standard poly A-hydrocarbons complexes. Absorbances were measured at 351 nm for 1,2-benzanthracene, 395 nm for 9-methyl anthracene, 364 nm for 3,4-benzpyrene and 345 nm for pyrene. Only these four hydrocarbons had an absorbance large enough to see a $\Delta A/A$ of 0.07. $\Delta A/A$ is observed at $\bar{G}$ = 21,250 sec^{-1} in these four cases (Table II). The

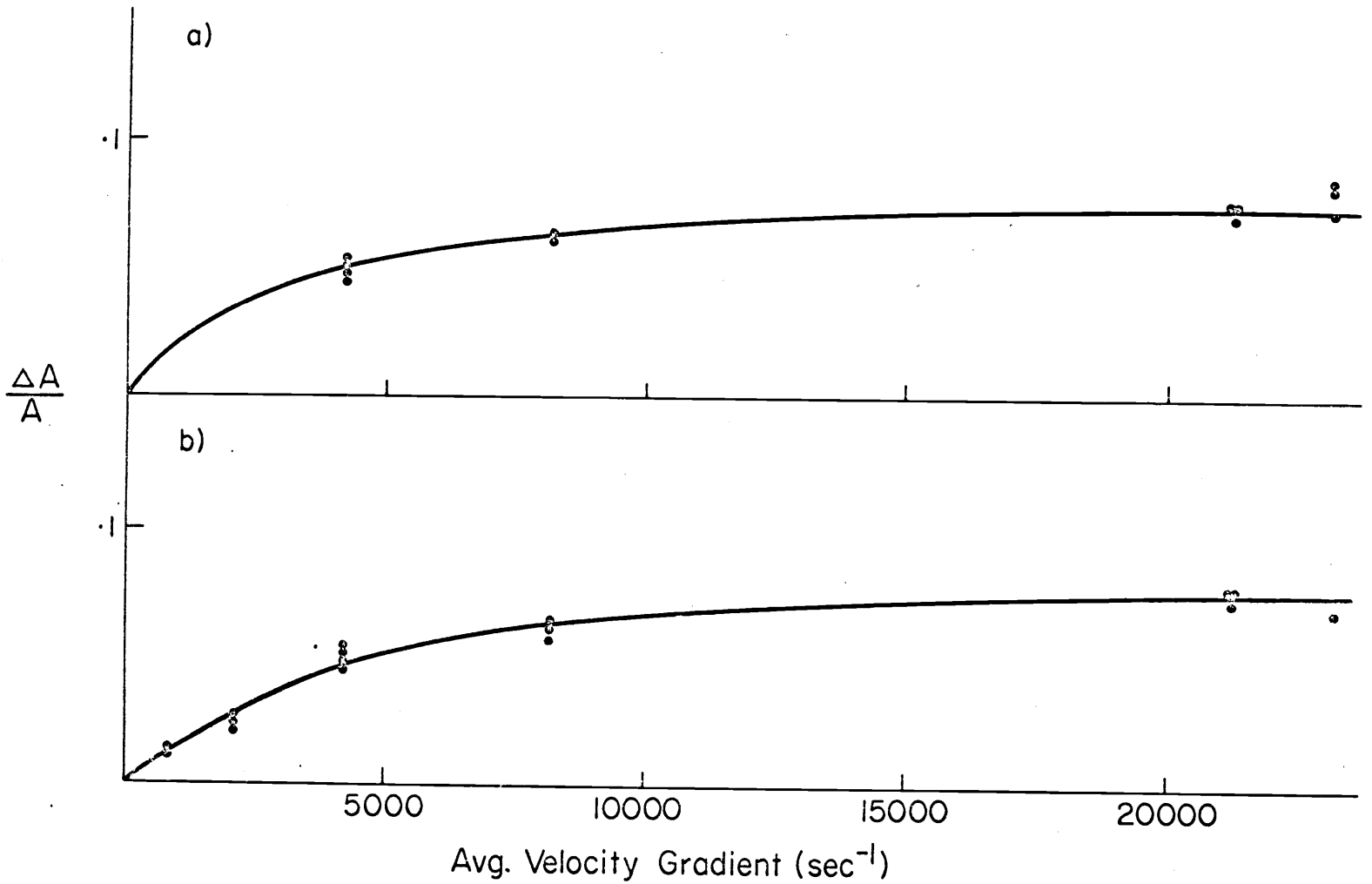

FIGURE 31: Flow Dichroism of Poly A: a) concentration 1.45×10^{-5}M at λ = 255nm,
b) concentration 5×10^{-4}M at λ = 275nm.

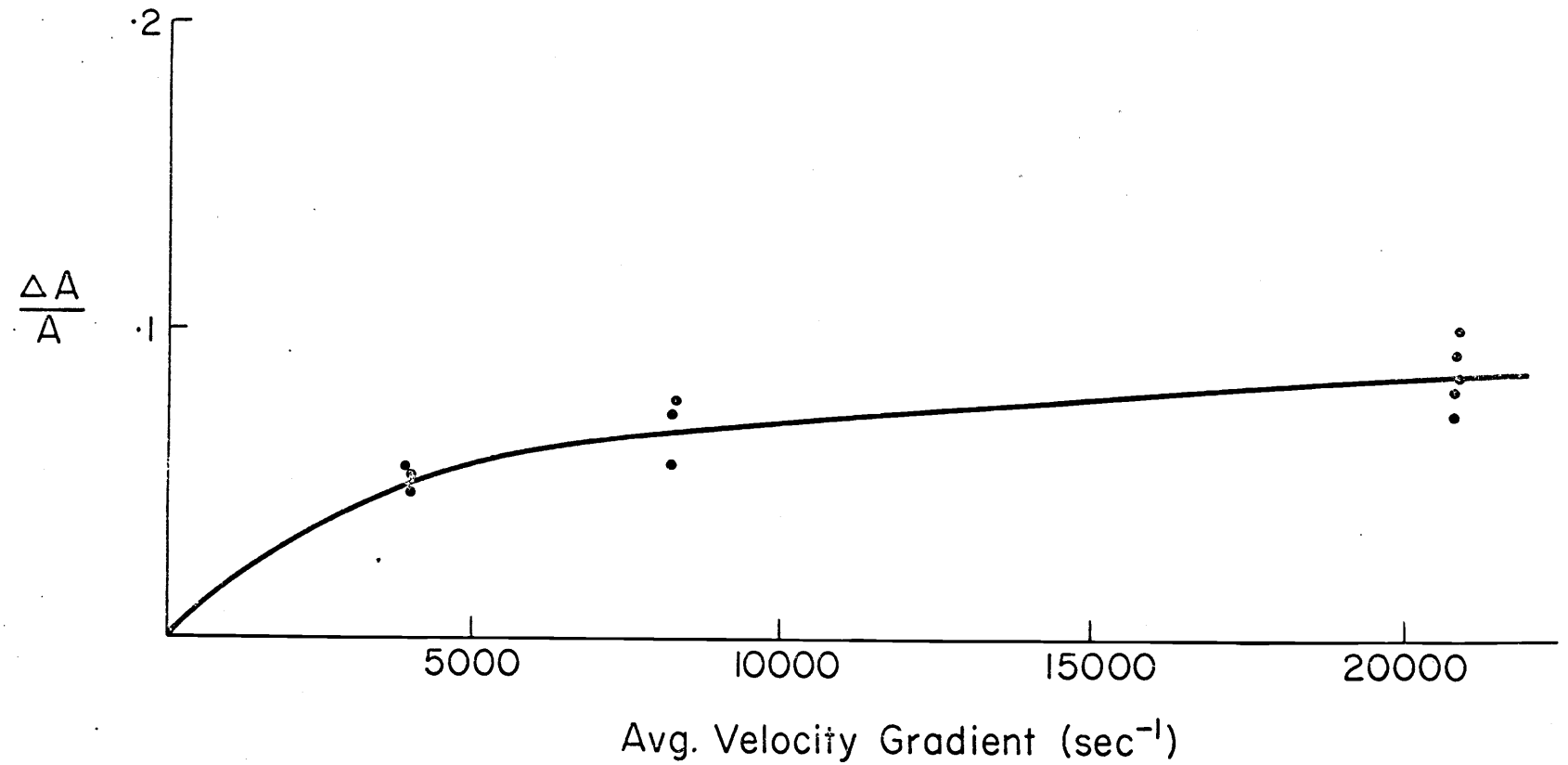

FIGURE 32: Flow Dichrosim of Poly A-Proflavin Complex. Poly A at concentration 1.45×10^{-2}M; Wavelength for Dichroism λ = 444nm.

other three hydrocarbons that complexed had a change in absorbance, ΔA, that was less than the noise, 0.02 absorbance units. In the flow dichroism assembly, a positive value of $\Delta A/A$ implies an orientation in which a $\pi \longrightarrow \pi^*$ transition moment is perpendicular to the helical axis. In Table II note that all complexed hydrocarbon had a dichroism of 0.07. The values of 0.07 obtained both for complexed hydrocarbons and for poly A are consistent with an intercalation model.

Vapor Pressure

The equilibrium that is measured in the poly A-hydrocarbon complexes is between the solid hydrocarbon and the poly A-hydrocarbon complex. In a statement of the size criterion, it is assumed that the dominant factor in binding may be described by saying that a hydrocarbon that binds will not protrude in the polar medium. If it does, it will not bind. However, equally valid, by a priori reasoning, is the argument that the lattice energy of the larger hydrocarbons is so great that they do not bind because they cannot dissociate from the crystal. To help resolve this question, the free energy of the crystalline states of several hydrocarbons was obtained (Table V). Through the Clausius--Clapeyron equation, the vapor pressure of the hydrocarbon can be related to the free energy of the crystal:

$$\log p = A - B/T \quad (38)$$

Where the heat of sublimation is $\Delta H = 2.303 \cdot R \cdot B$ in Kcal/mole (38), and the entropy of sublimation is $\Delta S^{\circ} = 2.303 \cdot R(A-2.881)$ in cal/mole deg. (38).

TABLE V: Comparison of ΔG of Hydrocarbons From Vapor Data.

HYDROCARBONS	ΔG Kcal
Anthracene (26)(32)	10.6
1,2-Benzanthracene (32)(38)	15.7
Tetracene (33)(38)	17.0
1,2,5,6-Dibenzanthracene (38)	19.3
9-Phenyl Anthracene	14.6 14.2
Coronene (38)	20.0
Pentacene (38)	23.3

Figure 33 illustrates a log p in mm versus 1/T plot for 9-phenyl-anthracene. The slope is B and the intercept is A. As explained before, there were two cells. One with larger orifices; one with smaller. For 9-phenyl anthracene, A = 13.99 and B = 6.32 for the first cell, and A = 13.66 and B = 6.167 for the second. These values correspond to ΔG of 14.2 Kcal/mole and 14.6 Kcal/mole.

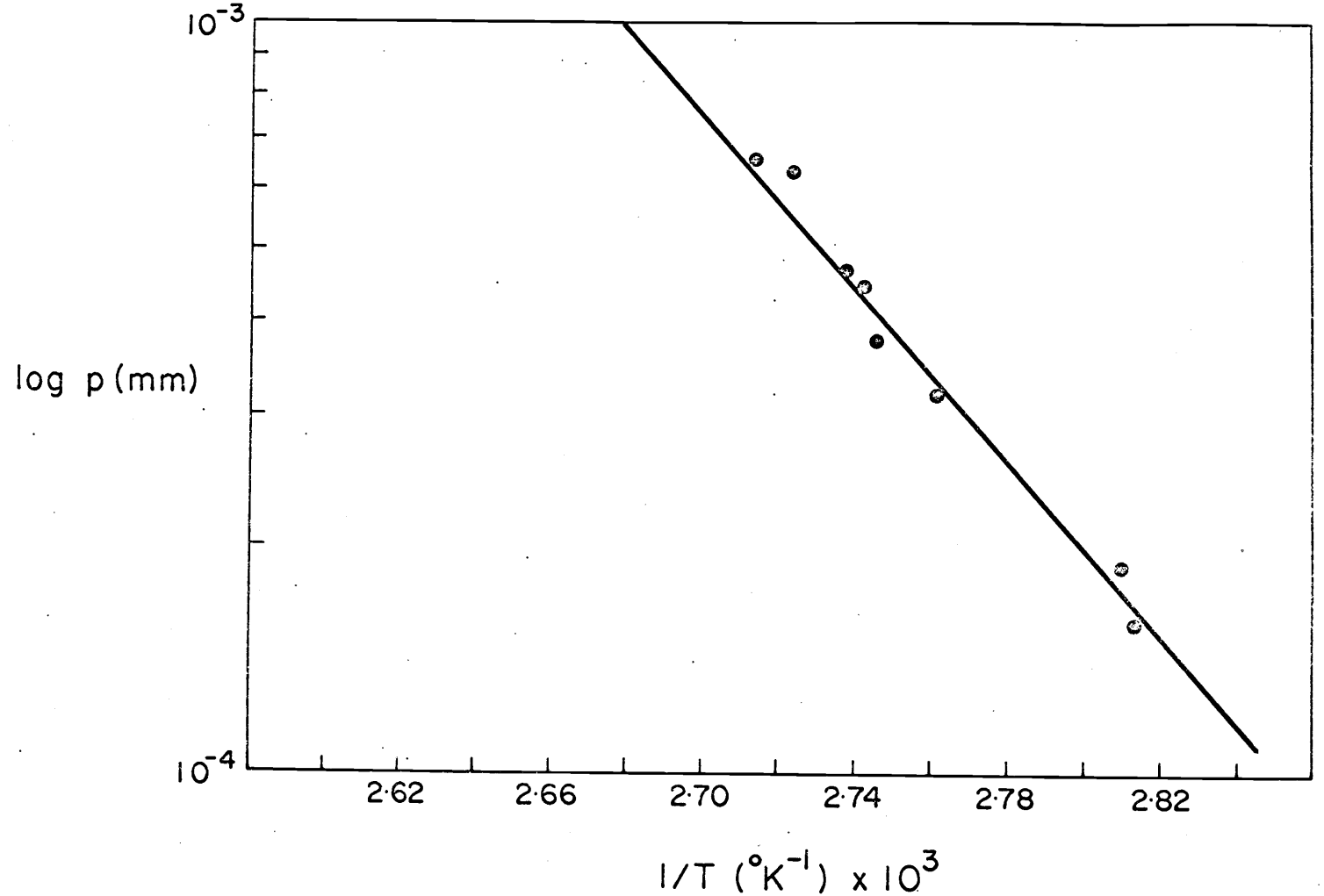

FIGURE 33: Vapor Pressure of 9-Phenyl Anthracene.

DISCUSSION

The goal of this dissertation was to present demonstrated evidence that polycyclic, aromatic hydrocarbons intercalate in double helical polynucleotides, that is, DNA and acid form of poly A. To this end, the validity of the size criterion has been demonstrated. The size criterion is based on the hypothesis that hydrophobic interactions dominate binding. If hydrophobic interactions did not dominate binding, small protrusions of hydrocarbon from the base pair would not inhibit binding. Therefore, the size criterion could not be demonstrated.

All the predictions of the size criterion that have been tested to date have been fulfilled. Only hydrocarbons complex to poly A that are small enough to intercalate and be protected from contact with the aqueous medium.

The set of hydrocarbons that bind to poly A is different than that which binds to DNA. Tetracene and 1,2,5,6-dibenzanthracene bind to poly A, but do not bind to DNA. Model building experiments predicted that these hydrocarbons would bind to poly A but not to DNA. 1,2,3,4-Dibenzanthracene did not bind to poly A, but bound to DNA. This, too, was predicted by the size criterion.

At the time when Craig and Isenberg (13) tested the size criterion for DNA, one serious failure existed in their demonstration of the validity of the size criterion. They had not shown that the differences in binding between Class 1 hydrocarbons and Class 2 hydrocarbons was not due to different crystal energies of the two groups of hydrocarbons.

In general, the lattice interactions do increase with an increase in the number of aromatic rings (Table V). In Table V, there is a progression of ΔG from anthracene to pentacene. However, the exception to this progression is 9-phenyl anthracene. 9-Phenyl-anthracene does not bind to poly A. If lattice energy determined whether a hydrocarbon would bind, then 9-phenyl anthracene would have a high ΔG value. But in fact, this hydrocarbon has a low ΔG value. Moreover, anthracene has a crystal free energy of 10.6 Kcal/mole, whereas, 1,2-benzanthracene has a free energy of 15.7 Kcal/mole. If binding was dominated by the crystalline energy, the binding of anthracene to poly A should be about 100,000 times greater than that of 1,2-benzanthracene to poly A. In fact, twice as much benzanthracene as anthracene binds to poly A. Moreover, tetracene and 1,2,5,6-dibenzanthracene binds to poly A in nearly equal amounts. The free energy change of tetracene from solid to vapor is 17.0 Kcal (33,38). The free energy change of 1,2,5,6-dibenzanthracene is 19.3 Kcal (38). If the crystal energies dominated the binding of hydrocarbons to polynucleotides, then tetracene should bind two-hundred times as strongly as 1,2,5,6-dibenzanthracene.

All these facts indicate that crystal lattice interactions do not determine whether or not hydrocarbons will build to polynucleotides. Moreover, they show that the size criterion is not a solubility artifact. The size criterion implies that hydrophobic interactions are the dominant factor in the binding of hydrocarbon to polynucleotide. This, in turn, indicates water around the helix behaves as liquid water, at least, with respect to hydrophobic interactions.

It is of interest to see how much of the hydorcarbon need project into the medium to prevent complex formation. Only a small piece of 1,2,3,4-dibenzanthracene sticks out from poly A; yet, this is enough to prevent complex formation (Figure 10). A tetracene-DNA model may be built in which only a small fraction of tetracene protrudes (Figure 18). In addition models of 9-phenyl anthracene and pentacene-poly A also show small pieces of hydrocarbon extending from the helix. None of these complexes form. At first it was hoped that the flow dichroism studies could demonstrate in a quantative manner that hydrocarbons intercalate; this hope was not realized. In this thesis, the flow dichroism studies have a more supplementary role. The result of the dichroism will be combined are consistent with and confirm the intercalation model.

One of the pitfalls of flow dichroism is that poly A is a heterogeneous material. This distribution of molecular weights in a velocity gradient creates a distribution of orientation of the poly A strands. This distribution unfortunately varies with time; a sensitive measurement of dichroism would vary with time. The dichroism, $\Delta A/A$, results from a distribution of particles aligned in a flow. This is the problem that Nagata et al., Green and McCarter and this study have encountered. Since there is distribution of particles, no more than qualitative results can be obtained. Even considering these limitations, the dichroism of the complexed hydrocarbons was determined. These two values for the flow dichroism were the same, and a qualitative con clusion was reached.

From the size criterion, it is known that the hydrocarbon will avoid the aqueous medium. Moreover, from flow dichroism studies, it is known that, in general, the planes of the bases and hydrocarbons are parallel. Both of these stipulations are satisfied by an intercalation model where the hydrocarbon can avoid the water. This conclusion in conjunction with the size criterion, lends strong evidence for the intercalation of hydrocarbons in polynucleotides.

Two interesting observations were noted while building models. In poly A-hydrocarbon complexes, the helix simply extended to accommodate the hydrocarbon. However, in DNA complexes, the angle between the neighboring base pairs decreased. In the case of anthracene, the angle went from 36°, without the hydrocarbon, to 25° with the hydrocarbon. For 1,2-benzanthracene the angle decreased from 36° to 20°, for 1,2,3,4-dibenzanthracene from 36° to 20°, and for pyrene from 36° to 26°. However, for 3,4-benzpyrene the helix angle decreased from 36° to 15° after intercalation. This was the largest rotation.

Possibly the most significant observation is that the orientation of at least two hydrocarbons in polynucleotide complexes is now known. Nearly all structural information about the coordinates of biological molecules comes from X-ray data. However, model building studies, and the size criterion, almost completely determine the orientation of 1,2,5,6-dibenzanthracene in poly A and 3,4-benzpyrene in DNA.

1,2,5,6-Dibenzanthracene may assume one orientation intercalated in poly A within the limits of the error of the models. If other positions of intercalation were assumed, the dibenzanthracene would not be protected from the aqueous medium and would project from the

poly A helix. The same conditions exist for 3,4-benzpyrene intercalated in DNA. According to the size criterion, there is only one orientation for 3,4-benzpyrene intercalated in DNA. Both these conclusions rest on the assumption that the size criterion is valid and that a hydrocarbon cannot jut from the polynucleotide helix. With the validation of the size criterion, the orientation of these two hydrocarbons in polynucleotides is known.

In summary, the size criterion is found to be valid for poly A and DNA. It does not reflect differences in hydrocarbon crystal energy. There can be little remaining doubt that intercalation is the mode of binding.

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