TRIPLET ENERGY DELOCALIZATION IN POLYNUCLEOTIDE-ACRIDINE COMPLEXES

Thesis by

William Claude Galley

In Partial Fulfillment of the Requirements

For the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

1967

(Submitted February, 1967)

Dedication

to my wife, HENRIETTE,

and daughter, SUZANNE,

for years of sacrifice

Acknowledgments

I am grateful to Dorothy Hayes who so generously gave her time to type this entire thesis. My wife worked hard in preparing the figures and provided encouragement.

I deeply appreciate the advice that I have received and the interest that has been shown in me by my research advisor, Professor Norman Davidson. His ability to grasp the significance of problems and his genius for rapidly formulating them in a meaningful way are examples not to be forgotten. I have also been educated by him in many specific areas of science. In addition, it has been an encouragement and a pleasure to have been associated with a man who, in a world which sometimes grows too old too fast, continuously generates a youthful enthusiasm toward life in general.

The association with fellow students and postdoctoral fellows at Caltech, particularly in the Davidson group, has contributed significantly to my education in addition to making my stay at Caltech a pleasant one.

The thinking embodied in this thesis has been considerably influenced by the pioneering work of Professor G. W. Robinson and coworkers on triplet energy migration in the solid state.

My education was made possible through support by Caltech in the form of a tuition fellowship and graduate teaching and research assistantships.

I am grateful to Lubert Stryer for the time spent in completing this thesis while under his support.

I

TRIPLET ENERGY DELOCALIZATION IN POLYNUCLEOTIDE-ACRIDINE COMPLEXES

Abstract

Transfer of triplet electronic excitation energy from the purine and/or pyrimidine moities of native DNA and adenine polynucleotides to the acridine dye 9-aminoacridine has been demonstrated at 77°K. The occurrence of such transfers indicates that there is pi electron overlap between the purine and/or pyrimidine bases and the dye bound to the polymer.

The acridine dye has then been used as a trap for the polymer triplet excitation energy. The polymer to dye dependence of the base to dye transfer efficiency indicates that triplet energy is delocalized in native DNA and adenine polynucleotides. Kinetic studies provide evidence that the pathlength for triplet energy transfer in native DNA is determined by trapping within the polymer rather than by diffusion.

Delayed fluorescence from the dye bound to DNA has been observed and its origin in the triplet state of the polymer has been confirmed at high polymer to dye ratios. In addition it has been shown that delayed fluorescence can arise from triplet-triplet annihilation between dyes at low polymer to dye ratios.

TABLE OF CONTENTS

Tables

Figures

INTRODUCTION

The mechanism of binding of the acridines with DNA is of interest in view of the behavior of molecules in this class as mutagens (1), bacteriostatic agents (2) and antimalarial agents (3). Interest lies not only in trying to relate the biological activity of these molecules with the mechanism of binding, but also in determining the forces that are involved in the interaction. In spite of the extensive study that has been devoted to this field, the precise mechanism, or mechanisms of interaction between DNA and the acridine dyes has not yet been elucidated. Current interest in the nature of the dye binding is centered around the "intercalation" model in which the dyes are strongly interacting with the nucleotides in DNA. No compelling experimental evidence, however, has been presented indicating a close range base-dye interaction.

This dissertation is concerned with the detection of such an interaction in DNA in the form of a triplet nucleotide to triplet dye energy transfer. The results will be seen to provide evidence for some degree of pi electron overlap between the electronic systems of the nucleotides and of the acridine dyes.

The occurrence of triplet excitation transfer from the bases in DNA and polynucleotides to dyes means that the acridine dyes are traps for the triplet energy of the polymers. The observed variation of the amount of triplet energy transfer from polymer to dye with the polymer to dye ratio then provides conclusive evidence that triplet energy is delocalized in DNA and in a number of polynucleotides.

The Interaction of Acridine Dyes with DNA

A review of the literature on the binding of acridine dyes to DNA is presented in this section and reveals the current state of our knowledge in this area. It will be evident that considerable uncertainty with regard to the mechanism of binding still exists.

The acridines interact with DNA and polynucleotides under conditions of pH where they are protonated to form cationic dyes. The importance of electrostatic forces in the interaction between the cationic dyes and the negatively charged phosphate groups of the polymer is indicated by several lines of evidence. Acridine orange (4) can interact with polyphosphate, heparin and other negatively charged polymers in the absence of purine and pyrimidine moities. The strength of the binding to DNA is sensitive to the ionic strength of the medium, as is expected if electrostatic forces contribute to the binding energy, (5). In addition, with acridine orange in particular, dye-dye interactions as evidenced by shifts in the absorption spectrum of the dye have been interpreted as arising from stacking of dye bound electrostatically on the outside of the polymer (6) (5). Such evidence has also been seen with acridine yellow (4) and 9-aminoacridine (7) on DNA. The binding of acridine orange (4) and proflavine (8) saturates at a molar ratio of polymer phosphate to dye of one, which is expected on the basis of binding to the phosphates.

On the other hand, there is evidence suggesting that nucleotide-dye interactions are involved. Binding, at high polymer to dye ratios, to DNA and polynucleotides is accompanied by a small red shift

in the absorption spectrum of the dye, while binding to polyphosphate (9) and apurinic acid (8) does not result in a shift. The non-linearity of Scatchard plots of binding data has been interpreted as indicating that molecular heterogeneity of binding sites, arising from the heterogeneous composition of DNA, exists in the case of acriflavine (10). The same conclusion has been reached from variations in the lifetime and quantum efficiency of proflavine fluorescence on binding to DNA (11). Neither of these studies, however, included model compounds such as poly (A + U) to test the hypothesis by removal of the binding site heterogeneity. It has also been suggested that the non-linearity of Scatchard plots arises from dye-dye interactions (8); however, the fact that the binding affinity varies with the type of DNA used indicates that base composition may be important (10). While the binding is in part salt dependent, a component of the binding which is relatively independent of ionic strength has been noted (7). In addition, strong binding to DNA at high ionic strength has been reported for the structurally similar cationic dye ethidium bromide (12), and binding of neutral molecules such as actinomycin (13) and some carcinogenic hydrocarbons is known, although binding in the latter case is much weaker (14).

It has been proposed that the data can be explained on the assumption that two types of complexes exist (8) (15); one type of complex at low polymer to dye ratios where dye-dye interactions occur and a second type of complex at high polymer to dye ratios which might involve dye-nucleotide interactions (8).

The suggestion has been made that at least in the region of

high polymer to dye ratios, the cationic acridine dyes interact with DNA by insertion or "intercalation" between consecutive base pairs in the double helix (16). This requires a local untwisting and extending of the DNA molecule to allow the dye to be intercalated. Several lines of evidence favoring this model have been presented: 1) An increase in the intrinsic viscosity of DNA in the presence of the dye has been interpreted as arising from the expected extention (16). 2) Removal of the planarity of 9-aminoacridine by hydrogenation of one ring results in a decrease in both binding (17) and bacteriostatic activity (18). 3) A decrease in susceptibility of bound dye to attack by nucleophilic reagents has been interpreted as indicating steric hindrance arising as a result of the dye being intercalated within the polymer (19). 4) Polarized fluorescence and absorption of dye bound to flow oriented DNA indicates that the bound dyes are located with the plane of the acridine ring system co-planer with that of the bases (20). The last conclusion has also recently been reached from the anisotropy of the nuclear hyperfine structure of chlorpromazine radical ion bound to flow oriented DNA (21).

The intercalation model holds promise in explaining the biological activity of the acridines (20); however, care must be exercised in interpreting data in favor of such a detailed molecular model. While intercalation of free bases between the pairs of bases of dinucleotides has recently been demonstrated by NMR (22), no such direct evidence for a close range interaction between dye and nucleotides exists in the case of the acridines. It is the purpose of this work to present evidence for such an interaction in the form of an electronic

excitation energy transfer from the bases in DNA and polynucleotides to bound acridine dyes and, in so doing, to simultaneously gain insight into the excited electronic states of the polymers.

Transfer of Electronic Excitation Energy

This section contains a brief review of some of the experimental and theoretical investigations of electronic excitation energy transfer that have been carried out. It indicates that the fundamental mechanism of transfer of triplet excitation is different from the principal mechanism of singlet energy delocalization, the latter involving transfer at a distance while the former occurs only via electron exchange interactions at close range.

The interaction of molecules or atoms in excited electronic states with molecules or atoms in their ground state resulting in electronic excitation energy transfer was initially investigated from two lines of approach.

Electronic excitation in the solid state has been found to give rise to electronically excited states whose energies are characteristic of the aggregates and which were referred to as exciton states (23). Experimental and theoretical investigation of exciton states was extended to crystalline forms by comparison of the energies of allowed electronic transitions in crystals with those of the monomers in the gaseous state (24).

The second approach to the problem was the direct demonstration of singlet energy transfer by sensitized fluorescence experiments. Work on impure crystals has shown, for example, that excitation energy

absorbed only by the host can appear as fluorescence from the guest (25). Experiments carried out much earlier both in the gas phase (26) and in solution (27) have demonstrated singlet-singlet energy transfer between unlike molecules. More recently, concentration depolarization has been used to demonstrate energy transfer between identical molecules (28) in rigid media. Singlet-singlet energy transfer has been shown to involve an actual coupling between molecules and not an emission and reabsorption process by, 1) a decrease in the lifetime of the donor fluorescence, and, 2) a lack of dependence on the geometry of the sample that is expected for radiative transfer (27). The transfer is seen to occur over intermolecular distances of 50 Å or more (29).

In the quantum mechanical treatment of the solid state exciton problem and of singlet-singlet energy transfer in solution, the coupling which gives rise to the exciton splitting and the energy transfer rate arises from a coulombic interaction between the electrons in an atom or molecule in an electronically excited state, with the electrons in neighboring atoms or molecules in the ground state. In the dipole approximation, the interaction Hamiltonian

$$\mathcal{H} = \frac{\mu_1 \mu_2}{R^3} K \qquad (1)$$

where R is the interatomic or intermolecular distance, μ_1, and μ_2 are the transition dipoles of the atoms or molecules, and K is an orientation factor, results in an interaction energy V_{12}, and a transition frequency which is a linear function of V_{12}, that is proportional to R^{-3} (30). Where non-resonance transfer is important, the transition probability varies with $(V_{12})^2$, and, therefore, the transfer rate is proportional to R^{-6} (30). This gradual decrease in

interaction energy with interatomic and intermolecular distances indicates the possibility of energy transfer in a single step over considerable distances. Numerical calculations based on Forster's theory indicate that single step transfer can occur over distances of 50 Å or more (27). The interaction energy in the dipole approximation is proportional to the transition moments of the donor and acceptor, and, therefore, vanishes for forbidden transitions such as in triplet states.

The theoretical possibility of energy transfer by the above mechanism from the triplet state of a donor to the singlet of an acceptor was pointed out by Forster however (27). This triplet-singlet energy transfer is possible in that the forbiddeness of the donor transition is compensated for by the long lifetime of the triplet state. Triplet-singlet energy transfer has recently been observed in thin films by a decrease in the lifetime of the triplet state of the donor, with the simultaneous appearance of delayed fluorescence in the acceptor (31). The appearance of delayed fluorescence in DNA-acridine systems has been interpreted in terms of a triplet polymer to singlet dye transfer (32). This result will be discussed in detail in a later section.

Transfer of electronic excitation energy from the triplet state of a donor molecule to the triplet state of an acceptor, or triplet-triplet energy transfer, was first shown by Terenin and Ermolaev to occur from benzophenone to naphthalene in a rigid glass media (33). It was demonstrated that close approach of the donor and acceptor are required and that the probability of transfer was independent of the oscillator strength of the singlet-triplet transition of the acceptor

(34).

Observation of the fluorescence and phosphorescence of three-component isotopic mixed crystals of benzene (35) and in impure crystals (36) indicates that triplet-triplet transfer occurs in the solid state. In the former case, at low temperature, emission occurred only from the two guests. The fact that the preponderance of the phosphorescence but not the fluorescence arose from one of the two guests, (traps), led to the conclusion that trap to trap triplet energy migration was occurring through the host. In the latter case, transfer was seen to occur from host to guest in impure crystals of naphthalene, benzophenone and triphenylene. The triplet energy transfer was more extensive than singlet energy delocalization.

Transfer of triplet energy occurs readily in solution and the diffusion control of the process again brings out the necessity for close approach of the donor and acceptor molecules (37).

Interactions involving forbidden transitions, such as in triplet energy transfer, have been investigated theoretically by Dexter (38). With proper antisymmetrization of the system wavefunctions and use of the complete interaction Hamiltonian, an interaction term arises in the form of an electron exchange integral,

$$\int Q'(r_1)\left(1/r_{12}\right) Q(r_2)\, d\tau_{12} \qquad (2)$$

where $Q'(r_1) = \varphi_s'^{*}(r_1)\,\varphi_a(r_1)$

and $Q(r_2) = \varphi_a^{*}(r_2)\,\varphi_s(r_2)$

φ_a and φ_s represent the molecular wavefunctions for acceptor and donor respectively. The prime superscript denotes the excited triplet states of the molecules and r_1 and r_2 represent the coordinates

of electron 1 and 2. Contribution to the integral only comes from those regions of space in which the electron can be found "simultaneously" on molecule A and D. It is pointed out by Dexter that, since each function φ dies off exponentially with distance from D to A, each product Q will be very small throughout all space, unless D and A have a small separation. Inclusion of spin in the wavefunctions results in the condition that the integral vanishes unless $\chi_s' = \chi_a'$ and $\chi_a = \chi_s$ where the χ's represent the spin functions.

Exchange interaction terms of this type were considered to be negligible even for nearest neighbors, in most molecular and ionic crystals (39). However, Robinson (35) has pointed out that the vanishingly small overlap integral for $2p\pi$ orbitals results from the use of atomic orbitals calculated with an average effective nuclear charge. This underestimates the amplitude of that part of the wavefunction that is important in the overlap. A more realistic value of the exchange interaction and the long lifetime of the triplet state combine to give a triplet energy nearest neighbor transition frequency of 10^{12} sec^{-1} as opposed to 10^{6} sec^{-1} for strongly allowed singlets (40).

Exciton-exciton interactions for triplet states in organic crystals have been demonstrated experimentally and treated theoretically (41). The interaction of triplet excitons results in annihilation of the triplet states with the formation of excited singlet states which can emit. Triplet-triplet annihilation was observed in form of a delayed fluorescence from those states. The intensity of delayed fluorescence under conditions where the annihilation is not efficient

increases with the square of the exciting light intensity. The lifetime of the delayed fluorescence depends on how rapidly the triplet excitons migrate and annihilate. In isotopic mixed crystals of benzene the lifetime was lengthened by long range migration of triplet energy between traps interacting through the host.

The possibility of long range triplet migration through virtual excited states of an intervening host has been demonstrated in both the delayed fluorescence and the phosphorescence studies of isotopic mixed crystals. The one dimensional problem for electron exchange interaction through N intermediate hosts has been solved ([42]) and indicates an exponential decrease in the interaction with the number of intervening hosts:

$$\beta_N = (f_B \beta_o)^2 (f_B' \beta_o')^{N-1} (-\Delta E)^{-N} \qquad (3)$$

where β_N is the trap to trap interaction energy

$f_B \beta_o$ is the nearest neighbor guest-host vibrational and electronic interaction,

$f_B' \beta_o'$ is the corresponding nearest neighbor host-host interaction

ΔE is roughly the difference between the excitation energy of the free guest and that of the free host.

Energy Transfer and the Interaction of Acridine Dyes with DNA

Transfer of electronic excitation energy in the form of a singlet-singlet energy transfer from the bases of DNA to dyes bound to the polymer has recently been demonstrated in several experiments. In experiments on the fluorescence of quinacrine bound to DNA, Lerman (20) observed that the relative uncorrected fluorescence excitation spectrum

in the region of DNA absorption was greater than could be accounted for, on the basis of direct absorption into the dye. An inversion in the fluorescence polarization was also observed in the DNA absorbing region. Weill and Calvin (43) have observed sensitized fluorescence of acridine orange and proflavine bound to DNA. The energy transfer was demonstrated as an increase in the relative quantum yield of bound dye, with excitation in the region of DNA absorption. Their results indicate that a large fraction of the energy absorbed by the DNA is transferred to the dye. Similar observations have recently been made by Le Pecq (44) with the dye ethidium bromide.

Isenberg and coworkers (32) have observed delayed fluorescence from acridine orange, proflavine and quinacrine bound to DNA when the complex was excited in the region of DNA absorption, but not when excited with visible light absorbed by the dye. The excitation spectrum of the delayed fluorescence corresponds with that of DNA phosphorescence. They have interpreted the data as indicating triplet DNA to singlet dye energy transfer of the type postulated by Forster. However, it has been shown that acridine dyes by themselves, in rigid media, show delayed fluorescence only when excited to higher lying excited states and the mechanism is believed to involve photoionization and recombination (45). The delayed fluorescence in this case is of much longer duration than that seen by Isenberg et al; however, the possibility that the delayed fluorescence in their experiments arises from photo-ionization and recombination can not be entirely excluded.

It should be emphasized that both singlet-singlet and triplet-singlet energy can occur over large distances (50 Å or more) between

donor and acceptor with good efficiency by virtue of the dipole-dipole nature of the interaction.* It has been shown that sensitized fluorescence from acridine orange occurs in solutions of adenosine, inosine, thymidine or uridine (46). The presence of such transfers, therefore, should not be taken as evidence favoring "intercalation" or other models in which the dyes are closely associated with the bases.

On the other hand, it has been pointed out that, in the case of triplet-triplet energy transfer, experimental and theoretical evidence demonstrate that there is a requirement for close approach of donor and acceptor moities. In aromatic molecules this corresponds to an overlapping of the pi electron clouds of the participating molecules. This strong dependence on distance then makes triplet energy transfer a sensitive criterion for the association of donor with acceptor. It is for this reason that an investigation of triplet-triplet energy transfer from the bases of DNA and polynucleotides to acridine dyes bound to the polymers was undertaken.

Energy Transfer in DNA, Poly- and Dinucleotides

It was indicated earlier that a quantitative study of triplet polymer to triplet dye energy transfer with polymer to dye ratio is utilized in this work to study the delocalization of triplet excitation

---

* Electron exchange can contribute to the singlet-singlet interaction, but for allowed singlets is negligible in comparison with the dipole-dipole interaction. In the case of the triplet-singlet interaction, however, contrary to the assumptions of Isenberg et al, electron exchange cannot contribute to the interaction by virtue of the fact that an electron exchange interaction does not implicitly involve spin orbit coupling and the total spin must be conserved.

in the polymers. A knowledge of the nature of the excited electronic states of nucleic acids is not only fundamental for an understanding of the photochemistry of these polymers and for the use of optical data in determining secondary and tertiary structures of the polymers, but polynucleotides could also be useful as one-dimensional models for the excited states of molecular aggregates. In addition to their one-dimensional character, they possess possible advantages over crystals, such as the formation of mixed polymers with non-random sequences. A review of current knowledge concerning the nature of the excited states and the question of excitation delocalization in these polymers is therefore presented in this section.

The presence of singlet-singlet interaction between the bases in DNA and polynucleotides has been assumed in the theoretical treatments of the hypochromism (47) seen in these polymers on helix formation. A marked shift in the 260 mμ absorption band, however, which could be attributed to resonance interactions is not seen in going from the denatured to the native state. It has been suggested that the failure to observe a shift in the absorption can be explained on the assumption that DNA forms a weak coupling system according to the criterion of Simpson and Peterson, and, therefore, splitting only occurs in the individual vibronic bands (48). The exciton states to which transitionsare allowed, however, depends on the relative geometry of the transition moments in the aggregate, (49), and it may be that the allowed exciton states in DNA on geometrical consideration are nearly degenerate with the monomer states. Therefore, while singlet-singlet interactions involving higher lying virtual states account

for the hypochromism, there is no positive evidence for resonance interactions in the form of an exciton splitting.

Evidence suggesting that singlet excitation transfer occurs in DNA has recently been presented from a study of the sensitized fluorescence of acridine dyes bound to DNA (43). From the variation of the quantum efficiency and the depolarization of the dye fluorescence with increasing polymer to dye ratio, it was concluded that 20 or more base pairs contribute energy to a dye, and that the energy is transferred from base to base resulting in depolarization. In view of the dipole-dipole nature of the transfer, however, the energy, at least in part, could be transferred directly at a distance. At higher polymer to dye ratios, direct transfer from a number of bases would result in depolarization of the fluorescence.

Singlet energy migration within DNA or efficient energy transfer to dye, in spite of a very low fluorescence efficiency in DNA, would not be surprising in that the lowest excited singlet state could be an exciton state with a long radiative lifetime.

More convincing evidence in the past several years has accumulated, suggesting that delocalization of triplet excitation occurs in DNA, poly- and dinucleotides.

Following from the initial observations of phosphorescence from DNA in glucose and water at 77° K by Steele and Szent-Gyorgyi (50), Bersohn and Isenberg demonstrated phosphorescence from native DNA in glycerol-water solutions at 77°K (51)(52). Based on both the phosphorescence and ESR characteristics of DNA and the constituent nucleotides, Rahn, Shulman and Longworth (53) concluded that the uv-induced state of

native DNA was that of thymine in which a proton had been transferred across the H-bond to adenine.

In the experiments of Bersohn and Isenberg, the binding of Mn++ ion to native DNA resulted in quenching of the phosphorescence but not the fluorescence of DNA, and, from the fact that a small quantity of Mn++ effectively quenched the DNA emission, they concluded that the triplet excitation was delocalized. The experiments have been repeated with a number of paramagnetic ions (54). Rahn, Shulman and Longworth (55) have shown that quenching occurs without a change in the lifetime of the phosphorescence and, therefore, the diffusion model of Bersohn and Isenberg for the excitation migration has been questioned. This point will be discussed in detail in a later section.

The observations of selective quenching of the phosphorescence of polyadenylic acid by small quantities of Mn++ (51) (52) has been confirmed with Mn++, Co++ and Ni++ and studied quantitatively by Eisinger and Shulman (56). They found that approximately 100 residues are quenched by a single bound paramagnetic ion, but that for poly A, which is only seven residues long, one ion per seven residues is required to quench the phosphorescence, thus supporting the idea of a delocalized excitation.

The experiments of Eisinger and Shulman were extended to heterogeneous polymers containing adenine. From a lack of quenching of the adenine phosphorescence in poly A_2U and from the fact that approximately one Co++ ion for every residue is required in order to quench the phosphorescence, it was concluded that triplet energy is not transferred from A to U and that U acts as a barrier to the

migration of the adenine triplet.

The lack of transfer from A to U was also seen in the dinucleotide 3'-5' UpA. No triplet-triplet transfer was seen from A in the dinucleotides ApC and ApG by virtue of the fact that the adenosine phosphorescence lifetime was not shortened. While a decrease in the lifetime of a potential donor is strong evidence that transfer is occurring, failure to note a shortened lifetime in itself does not entirely exclude the possibility of triplet energy transfer. Triplet excitation transfer is known to be very sensitive to distance. If a fraction of the dinucleotides are unstacked, then they could emit with close to their normal lifetime, while the fraction of the dinucleotides which are stacked transfer energy at a sufficiently rapid rate to completely quench the phosphorescence of the donor in those dinucleotides.

Helene, Douzou and Michelson have concluded that triplet energy transfer from A does occur in ApC in ethanol:water and GpA in water: propylene glycol. Helene has shown that in frozen aqueous mixtures of nucleic acid derivatives in which thymine or thymine derivatives are included, the phosphorescence is characteristic of the thymine partner. The possibility of singlet-singlet transfer was not excluded however. The phosphorescence of G_1ACC and A were found, in addition, to be quenched by U. In this case quenching at the singlet state level was excluded by energy considerations. It was shown that neither H-bonding nor proton transfer are a prerequisite for the observation of the thymine triplet state, and the possibility that the uv-induced triplet state of both poly dAT and DNA is that of thymine populated by T-T transfer rather than ionized thymine, (57, 58).

It is apparent from the previous investigations of the excited electronic states of DNA and polynucleotides that there is evidence suggesting delocalization of the excitation. However, the broad absorption bands and the low levels of emission from both the singlet and triplet states makes this evidence not entirely convincing. In the work described in this dissertation some of these difficulties are eliminated with the use of acridine dyes with characteristic emission, as traps for the polymer triplet excitation.

EXPERIMENTAL

THE PRINCIPLE OF THE METHODS

While the matrix elements involved in exciton formation can be measured directly from absorption and emission spectra, such an experimental approach is difficult in the case of triplet excitons in view of the low $T\text{-}S^{o}$ transition probability and the fact that the triplet interaction energy is only expected to be of the order of several wavenumbers ($1\text{-}0$). In the absence then of very sharp emission or absorption lines, interaction between triplet states must be detected by energy transfer experiments.

A number of experimental observations have been used to prove or suggest that triplet-triplet transfer is occurring. The method chosen depends in part on the nature of the system under study. In the DNA-, polynucleotide-dye systems studied in this work, a number of methods are utilized to investigate triplet energy transfer in these complexes.

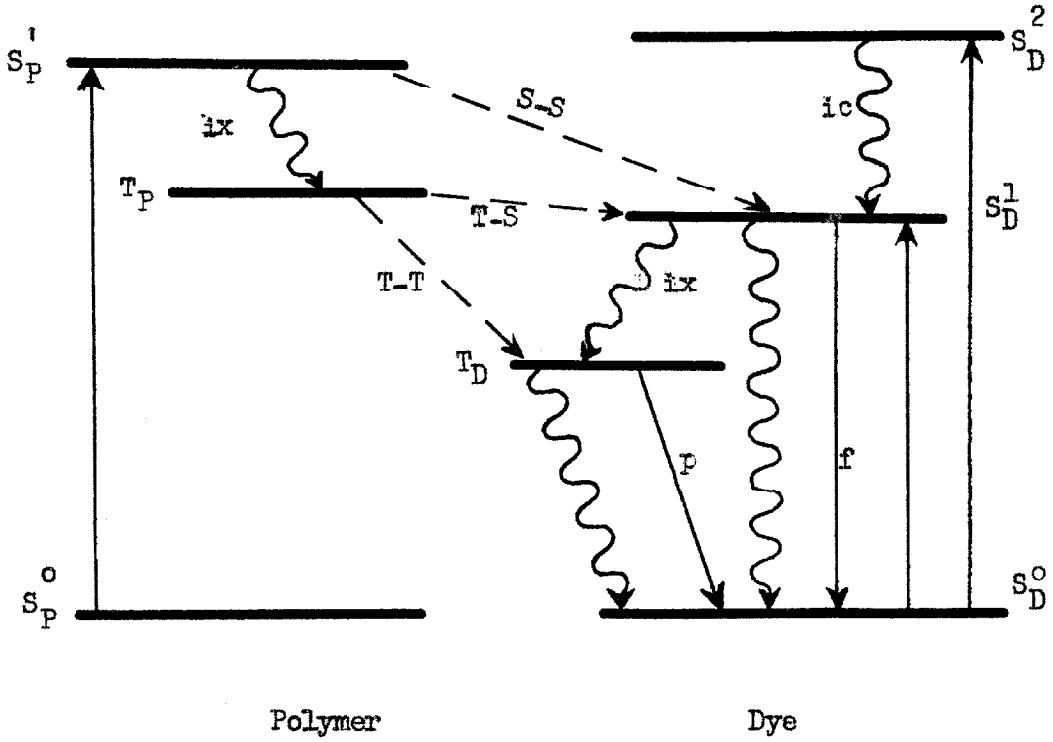
1) Triplet-triplet energy transfer can be demonstrated from

the observation of an enhanced phosphorescence intensity of the acceptor in the presence of a donor. The interpretation of the results, however, can be complicated by the simultaneous occurrence of singlet energy transfer. If singlet energy transfer to the acceptor cannot be ruled out by a failure to conserve energy, then it must be shown that it does not completely account for the sensitized phosphorescence. The problem is simplified if the acceptor has an area of absorption in a part of the spectrum where the acceptor does not absorb. The DNA-, polynucleotide-acridine systems in which the first singlet-singlet absorption band of the dye lies far to the red of the absorption band of the bases of the polynucleotides and DNA, fall in this category.

A criterion for the occurrence of triplet-triplet energy transfer from polymer to dye can then be arrived at, with the aid of the Jablonski diagram in Fig. 1 for the case of a hypothetical polymer-dye complex. Excitation of the complex with visible light results in absorption by the dye, giving rise to the first excited singlet state of the dye ($S_D^o \rightarrow S_D^1$). A dye molecule in the first excited singlet state can then release the excitation energy as fluorescence ($S_D^1 \rightarrow S_D^o$), <u>possibly</u> return to the ground state by a radiationless transition, ($S_D^1 \rightsquigarrow S_D^o$), or undergo intersystem crossing to give rise to a triplet state ($S_D^1 \rightsquigarrow T_D$). From the triplet state the molecule can undergo radiationless transitions to the ground state ($T_D \rightsquigarrow S_D^o$), or emit a quanta of energy as phosphorescence ($T_D \rightarrow S_D^o$). The intensities of fluorescence and phosphorescence are dependent on the amount of light absorption by the dye, that is, on the exciting light intensity and the extinction coefficient at the exciting wavelength; however, the ratio

FIG. 1

Jablonski Diagram For a Hypothetical Polymer - Dye Complex.



ic = Internal conversion

ix = Intersystem crossing

⟶ = Radiative transitions

⇝ = Intramolecular radiationless transitions

⇢ = Intermolecular energy transfer

of intensities of phosphorescence to fluorescence p_{vis}/f_{vis}, where p and f represent phosphorescence and fluorescence intensities respectively and the subscript $_{vis}$ denotes visible excitation, depends only on internal properties of the dye. Excitation of the complex with uv light results in absorption by both the dye ($S_D^o \longrightarrow S_D^2$) and the polymer ($S_P^o \longrightarrow S_P^1$). In the absence of transfer from the polymer to dye, the dye excited to a higher excited singlet state undergoes internal conversion with high efficiency to return to the lowest excited singlet state and, from there, the excitation can then take any of the paths which were available to it on direct excitation to the lowest excited singlet state, with the same probabilities. Therefore, while the absolute *intensities of fluorescence and phosphorescence are different*, the ratio of phosphorescence to fluorescence remains constant with exciting wavelength. Energy transfer from the polymer to the singlet state of the dye by singlet-singlet ($S_P^1 \rightsquigarrow S_D^1$) or triplet-singlet ($T_P \rightsquigarrow S_D^1$) transfer yields the same result. Any process which increases the population of the lowest excited singlet state of the dye does not alter the p/f ratio by virtue of the fact that the triplet state is being populated in proportion to the population of the singlet state. With the occurrence of triplet polymer to triplet dye ($T_P \rightsquigarrow T_D$) energy transfer on the other hand, the triplet state of the dye is populated directly from the polymer, without going through the excited singlet state of the dye and, therefore, this pathway alone selectively enhances the phosphorescence of the dye and increases the p/f ratio over its value with visible light excitation. Therefore, a criterion for triplet-triplet energy transfer can be written in the form:

$\frac{P_{uv}}{f_{uv}} > \frac{P_{vis}}{f_{vis}}$ for T-T transfer

$\frac{P_{uv}}{f_{uv}} = \frac{P_{vis}}{f_{vis}}$ for no detectable transfer

It is evident that the method has the advantage of possessing an internal standard. A comparison is made with all variables except the wavelength of the exciting light remaining fixed. Thus perturbations to the electronic structure of the dye which arise on binding are present under both transfer conditions and control.

While the technique described above provides the principle evidence for triplet-triplet energy transfer in this work, several other approaches are used in support of the first method.

2) Quenching of the phosphorescence of the donor is expected in the presence of triplet energy transfer to an acceptor. The observation of quenching of the donor emission in the presence of a donor does not prove that triplet energy transfer is occurring.

In the polymer-dye case, it must be shown in the form of a decrease in the p/f ratio of the polymer that the quenching is not occurring at the excited singlet state but specifically at the polymer triplet. This observation still does not unequivocally demonstrate triplet-triplet energy transfer, in that the p/f ratio of the polymer must be determined in the presence and absence of the dye, and perturbations by the dye to the electronic structure of the polymer could lower the p/f ratio by enhancing $T-S^{o}$ radiationless transitions or decreasing intersystem crossing.

3) If either an enhanced dye phosphorescence from triplet-

triplet energy transfer from the polymer or a specific quenching of the polymer phosphorescence occurs, then determination of the magnitude of the enhancement or the quenching as a function of the number of polymer residues per dye (ratio of hosts to traps) indicates the number of residues which can interact indirectly with a dye (trap), and, therefore, provides an indirect measurement of energy transfer in the polymer (host).

4) A decrease in the lifetime of the triplet state of the donor in the presence of the acceptor directly indicates an interaction at the triplet level. The possibility, however, that the interaction shortens the triplet lifetime by enhancing, for example, spin orbit dependent T-S transitions in the polymer, rather than by triplet-triplet transfer, must be considered.

5) With pulsed excitation of the donor, the intensity of the phosphorescence of the acceptor following the flash initially rises and then decays normally. The transient rise comes from triplet excitation being transferred from donor to acceptor after the pulse. This transient rise in the acceptor phosphorescence is only observable when the transfer time is long compared with the flash duration. If this is not the case, all transfer occurs during the flash period and the emission decays normally following the pulse. This technique has recently been used in the study of triplet-triplet transfer between deutero-phenanthrene and naphthalene in a viscous medium (59).

6) Triplet-triplet transfer can be demonstrated by the observation of delayed fluorescence from the singlet states formed on annihilation of triplets. It should be pointed out that it must be shown from

the properties of the delayed fluorescence, such as a square dependence on the exciting light intensity, that it arises from triplet-triplet annihilation and not from other processes.

APPARATUS AND EXPERIMENTAL PROCEDURES

Steady State Emission

Emission intensities with constant illumination were measured with a spectrofluorimeter,- phosphorimeter built in this laboratory. The source of illumination was an Osram HBO-200 high pressure Hg arc, which was operated on dc current supplied by 120 v batteries. The wavelength of the exciting light was selected by a Bausch and Lomb 500 mm focal length, 3.3 millimicrons per millimeter linear dispersion grating monochromator, fitted with quartz optics and blazed in the uv. The exciting light was focussed onto the sample with a 50 mm focal length quartz lens and emission was observed at right angles to excitation. The wavelength of the emitted light was selected by a second Bausch and Lomb monochromator of the same type only blazed for 500 mμ. The variable slits on the monochromators were normally 2 mm. The output of the emission monochromator was focussed onto the cathode of an RCA IP-21 photomultiplier tube which was routinely cooled with dry ice to reduce the photomultiplier dark current. The photocurrent was measured as a voltage across a variable resistance (10^3 - $10^{12}\Omega$) by a Keithley electrometer and the unity voltage gain output was used to drive the Y axis of a Moseley X-Y recorder. The signal gain was variable over a further range of 10^4 with the Y-axis amplifier of the recorder. The X-axis of the recorder was connected to a potentiometer

circuit in which the variable resistance was geared to the wavelength drum of the emission monochromator. A variable voltage (0 - 4.5 volts) was supplied to the X-axis with changes in wavelength. An emission spectrum was then obtained by manual rotation of the wavelength drum of the emission monochromator, while recording the emission signal along the Y-axis.

The sample compartment which was connected to the emission monochromator at the entrance slit was constructed around an Aminco Keirs phosphoroscope. The rotating cup of the phosphoroscope which alternately permits excitation and observation of delayed emission was easily removed with its motor from the bottom of the sample compartment, without altering the position of the sample, to allow fluorescence measurements to be made. The speed of the rotating cup was variable with a Variac transformer, but was normally operated under conditions with an approximately 2 msec delay between excitation and observation of emission.

The sample was held in a 1mm inside diameter quartz micro sample tube and the sample tube was held in the center of a small quartz dewar flask which was placed in the sample compartment from above. Dry nitrogen was constantly circulated through the sample compartment to prevent condensation of water vapor on the wall of the liquid nitrogen filled dewar and, in addition, was circulated over the outer face of a quartz window on the photomultiplier housing for the same reason. The inability to accurately center the sample tubes in the quartz dewar resulted in large errors in the measured emission intensities. The sample tubes were centered as accurately as possible by eye and the dewar was rotated

in its holder until the maximum intensity was attained. This still results in considerable errors (~20%) in determining absolute intensities, and the most reliable results are those which depend only on ratios of emission intensities. Measurements of the dye emission in triplet energy transfer experiments were then performed by determining the phosphorescence intensity with excitation in the visible and the uv. The rotating cup was removed and the fluorescence was measured with the same two exciting wavelengths. In this way the four required components of the emission could be measured on a single sample, without altering the position of the sample holder.

Kinetic Experiments - Lifetimes

Lifetimes of delayed emission were determined with the phosphoroscope rotating. The emission monochromator was set at a fixed wavelength and the X - axis of the X - Y recorder was operated in the time mode of operation. With the recorder scanning as a function of time, the exciting beam was closed with a camera shutter and the decay of the emission was recorded. The system was only useful for lifetimes greater than 200 msec.

Flash Experiments

The steady state apparatus was modified to perform millisecond flash experiments. A 15 msec. flash was achieved with a multileaf camera shutter in the exciting beam. The electrometer and recorder were eliminated because of their slow response time and the photocurrent was measured directly across the $10^6\Omega$ input resistance of a Tektronic

535 oscilloscope. A low leakage semi-conductor diode in parallel with the input resistance prevented the signal during the flash period from becoming greater than 0.4 v. This clipping increased the recovery rate of the oscilloscope electronics following the large fluorescence signal in the flash period. A 400 channel computer of average transients (CAT) was connected in parallel with the main vertical amplifier of the oscilloscope at a point, one stage of amplification lower than the vertical deflection plates. The CAT was triggered at the start of each sweep with the +20 volt gate of the oscilloscope. The CAT then sampled the vertical signal at the 400 channels as a function of time. In this way, the time history of the signal following each flash was displayed on the oscilloscope and accumulated in the memory of the CAT. The accumulated signal was displayed on the CAT oscilloscope screen, and plotted on an X - Y recorder through an analog output in the CAT. Accumulation of flashes in this manner results in averaging of the noise, and, therefore, enhancement of the signal to noise ratio.

Polarization Measurements

Polarization of the emission was determined with Glan-Thompson prisms in the excitation and emission beams of the steady state apparatus.

The degree of polarization p is given by the general expression:

$$p = \frac{Vv - Hv}{V_v + H_v} \qquad (4)$$

where V and H represent the fluorescence intensities in the vertical and horizontal components respectively and the subscript denotes the polarization of the excitation; it was used in its corrected form:

$$p = \frac{V'_v - t_2 H'_v}{V'_v + t_2 H'_v} \qquad (5)$$

where the prime ' superscript indicates that the components are the measured quantities and t_2 is a factor which corrects for polarization effects in the analysing monochromator.

The ratio of intensities of the vertical to horizontal components of light transmitted by the excitation and by the emission monochromators, referred to as t_1 and t_2 respectively, were determined essentially by the methods of Weill and Calvin (43) and Azumi and McGlynn (60).*

The t_1 factor was determined at room temperature, using the fluorescence from a dilute solution of 9-aminoacridine as a source of depolarized light; the ratio of fluorescence intensity with the exciting light polarized vertically to that, with the exciting light polarized horizontally was taken equal to t_1. The second method is based on the fact that for unpolarized light

$$H_v = H_h$$

while with polarization effects present:

$$H_v = t_1 H_h$$

Therefore t_1 can be determined under conditions of the experiment at 77°K by measuring the horizontal component of emission with the excitation polarized vertically and horizontally.

The t_2 factor was similarly determined. At room temperature, the fluorescence from dilute solutions of acridine orange and rhodamine B were also used to obtain t_2 at the wavelengths of 9-aminoacridine

---

* The notation of Weill and Calvin is used here.

phosphorescence. In this case the ratio of intensities of vertically and horizontally emitted light was determined with unpolarized light or with light with a fixed polarization. At low temperature, t_2 was determined from the ratio

$$t_2 = \frac{V_h}{H_h}$$

While only the t_2 factor is used in obtaining polarization values, values for both t_1 and t_2 are required for corrections due to polarization by the monochromators.

Corrections to Emission Intensities from Anisotropy of Emission and Polarization by the Monochromators

Emission intensities observed at right angles to excitation with unpolarized light must be corrected in general for the anisotropy of the emitted light. A correction factor for this anisotropy is derived in Appendix I and is given by:*

$$C_A = \frac{2\,(3 - p)}{3\,(2 - p)} \qquad (6)$$

where p is the degree of polarization defined above. Multiplication of the emission intensities observed at right angles to excitation by C_A corrects all intensities to zero polarization.

---

* The anisotropy correction factor $\frac{3 + p}{3}$ derived by Weber is used incorrectly by Weill and Calvin, in that the p in this expression signifies polarization measured with unpolarized exciting light. Weill and Calvin measured the degree of polarization with polarized exciting light and, therefore, the correct expression for the correction factor in this case is the one given in the text. That the two expressions give the same result when used in conjunction with the proper p value can be readily seen by substituting the maximum and minimum values of p in the two cases, 0.5, - 0.33 respectively for polarized exciting light, and 0.33, - 0.143 for unpolarized light, into the corresponding expressions.

In addition to the above correction for unpolarized light an additional correction must be made for partial polarization of the exciting and emitted light which occurs on transmission through the monochromators. This factor is given by:

$$C_m = \frac{(4 - 2p)}{(1 - p)\quad 1 + t_1 + t_2 + t_1 t_2 \frac{(1 + p)}{(1 - p)}} \qquad (7)$$

where t_1, t_2 and p are given above. While this expression differs from that derived by Weill and Calvin, an obvious error occurs in their final expression and, therefore, the derivation will not be repeated here. The above correction reduces to unity in the absence of polarization effects by the monochromators ($t_1 = t_2 = 1$).

With the determination of t_1 and t_2 at the exciting and emission wavelengths respectively and of the degree of polarization p, emission intensities can be corrected by multiplication by C_A and C_m.

Materials

9-Aminoacridine was obtained as the hydrochloride from Dr. R. L. Peterson in this laboratory. It was synthesized by the method of Albert and Ritchie (80). The hydrochloride was obtained by passing dry HCl into an ethanol solution of the base. The precipitated hydrochloride was recrystalized three times from 75% aqueous ETOH.

Acridine Orange was a product of National Aniline Division of Allied Chemical and Dye Corp. It was further purified in this laboratory by T. Burke by recrystallization as the free base from aqueous ethanol solution (9:1) and chromographed on alumina with elution by $CHCl_3$.

DNA. Calf thymus DNA was obtained from Worthington Biochemicals as the K^+ salt. It was dissolved by dropwise addition of buffer, and in most cases was used without further purification. In one set of experiments the DNA was extracted three times with buffer saturated phenol, and the phenol removed with ether extraction and dialysis against the final buffer.

Native DNA concentrations were measured by absorbance at 257 mμ using a molar extinction coefficient of $\epsilon_{257} = 6.56 \times 10^3$ l mole^{-1} cm^{-1} where the DNA concentration is expressed in term of molar concentration of phosphate.

The CT-DNA was denatured in 2×10^{-3}F salt by heating to 80°C for 10 min. and quenching in ice water.

Polyadenylic acid (poly A), oligioadenylic acid $(Ap)_7$, and polyuridylic acid (poly U), were obtained from the Miles Chemical Co.

as the ammonium salts. They were dissolved in buffer and used without further purification. The concentrations were determined by absorbance using ϵ_{257} (poly A) = 1.04×10^4 1 mole^{-1}cm^{-1}, ϵ_{260} (poly U) = 0.96×10^4 1 mole^{-1}cm^{-1}.

Solutions.

Complexes between the acridine dyes and the DNA and polynucleotides were formed, in general, in 2×10^{-3}F cacodylate, buffer pH-7.0, and 2×10^{-3}F NaCl. The low salt was used to assure complex formation between the polymer and dye. Polymer concentrations varied from 2×10^{-4} M, in phosphate, to 2×10^{-3} M. Dye concentrations ranged from 3.0×10^{-6} - 1.2×10^{-4} M. The final solutions were formed by the addition of an equal volume of ethylene glycol or glycerol. The 1:1 glycerol-H_2O solutions were allowed to stand for at least $\frac{1}{2}$ hr. at 0°C prior to cooling to liquid nitrogen temperature. In the case of the copolymers the solutions were allowed to stand for 48-72 hrs. at 4°C to assure formation of the polynucleotide complexes prior to cooling to 77°K. Dry nitrogen was bubbled through the solutions for 20 minutes.

The solutions were cooled to 77°K in microsample tubes into rigid glasses. Cracked glasses were obtained in nearly all cases.

RESULTS AND DISCUSSION

Emission Characteristics

In order to provide background information for the later sections, the emission characteristics of the DNA- and polynucleotide - acridine dye complexes investigated in this work are briefly described and illustrated in the following section.

Excitation of 9-aminoacridine in a 1:1 glycerol-H_2O or 1:1 ethylene glycol-H_2O glass at 77°K and pH 7.0 with either visible or ultraviolet light results in a characteristic fluorescence spectrum which is a "mirror image" of the lowest solution singlet-singlet absorption band. The positions of the peaks of the low temperature spectrum in the glycerol-H_2O glass in fig. 2(a) are very close to those in the room temperature spectrum in fig. 2(b); however the individual bands are sharper at the low temperature. The wavelength maxima and the shape of the fluorescence spectrum is somewhat dependent on concentration; the most distinctive feature being a decrease in the intensity of the high energy band with concentrations above 10^{-4}M. Radiative transfer cannot account for the magnitude of the decrease and it must arise from dye-dye interactions involving the 0-0 band. In addition to the structured fluorescence spectrum at low temperatures, a much weaker phosphorescence is seen in fig. 3 with wavelength maxima at 556 mμ (18,000 cm^{-1}). It is attributed to a $T \rightarrow S^{\circ}$ transition by virtue of its position and its 2.1 second lifetime. Phosphorescence is only observed with the phosphoroscope and at high gain, and is much too weak to be seen in the fluorescence spectrum. A second broad band with a wavelength maximum around 450 mμ appearing in the delayed emission spectrum

FIG. 2 Fluorescence Spectra of 9-aminoacridine

Dye concentration = 4.8×10^{-5} M, spectrum #1 - λ_{ex} = 405 mμ,

spectrum #2 - λ_{ex} = 280 mμ.

(a) free dye, 77°K, relative gain of spectrum 2 to spectrum 1 = x 2.

(b) free dye, 298°K, relative gain of 2 = x 2.

(c) bound dye, P/D = 19, 77°K, relative gain of 2 = x 20

(d) bound dye, P/D = 19, 298°K, relative gain of 2 = x 10

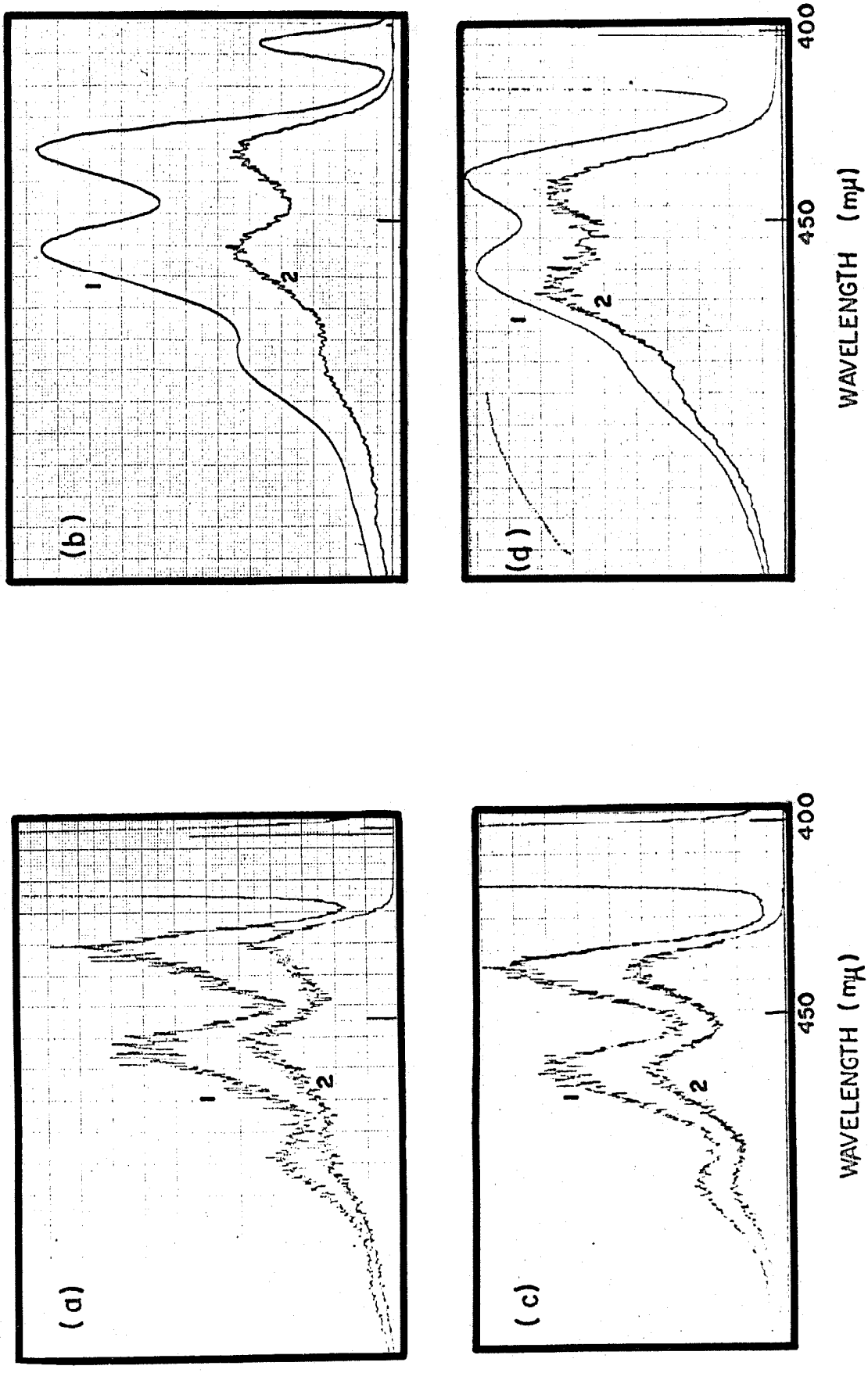
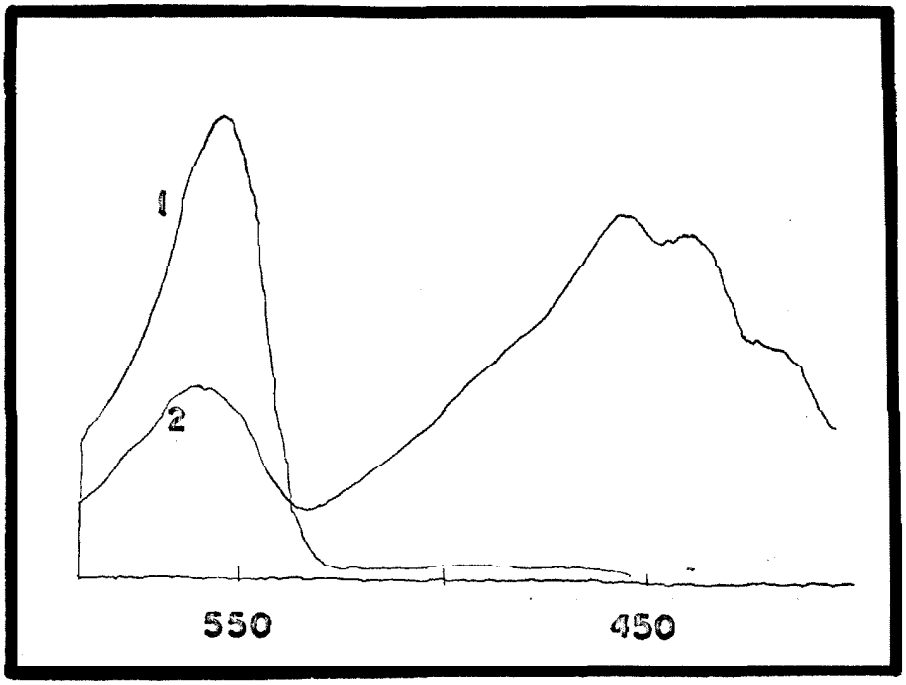

(a)
(b)
(c)
(d)
1
2
400
450
WAVELENGTH (mμ)

FIG. 3



Delayed Emission Spectra of 9-aminoacridine.

Dye concentration = 1.5×10^{-4} M.

1 - λ_{ex} = 405 mμ.

2 - λ_{ex} = 280 mμ rel. gain = x 10.

Table 1

Fluorescence behavior of 9-aminoacridine on binding to native CT-DNA (9-aminoacridine conc. = 4.8×10^{-5}M). Data from one experiment.

Sample	Temp. (°K)	f_{vis} (arb.units)	f_{uv}/f_{vis}
free dye	77	1.00 1.32	0.027 0.025
free dye	room temp.	1.08	0.020
bound dye (P/D = 19)	77	.94 .79	0.087 0.085
bound dye (P/D = 19)	room temp.	.006	0.075

---

f_{vis} and f_{uv} represent the fluorescence intensity with visible (405 mμ) and uv (280 mμ) excitation. The fluorescence intensity was routinely determined at the height of the center band to minimize interference from scattered radiation with 405 mμ excitation. Small errors introduced due to changes in the height to area ratio are cancelled in taking ratios of intensities at two exciting wavelengths.

with uv excitation is attributed to emission from the glass. Emission in this region was somewhat lower in glycerol than ethylene-glycol. In the glycerol-H_2O glass peaks appear in the uv-induced spectrum in this region at positions corresponding to the normal fluorescence of the dye. Delayed fluorescence of the type observed by Lim et al. in acridine (45) systems and attributed to photo-ionization and recombination could account for these peaks. Their contribution, however, is too small to be seriously considered.

The emission properties of the dye on binding to DNA and polynucleotides at high polymer to dye ratio are not markedly perturbed from those of the free dye. As shown in Fig. 2(c), the shape of the spectrum remains the same but is shifted to the red by 6 - 8 mμ. This red shift in the fluorescence spectrum of 9-aminoacridine on binding to DNA is observed both in aqueous solution* and 1:1 glycerol-H_2O mixtures at room temperature [Fig. 2(d)]. The fluorescence intensity of the dye induced with visible light remains essentially constant on formation of the high polymer to dye complex, as shown in table 1. When the solutions are warmed to room temperature, however, the intensity of the free dye remains constant while the fluorescence from the bound dye is quenched to about 7% of the low temperature intensity. Quenching of 9-aminoacridine fluorescence on binding to DNA in aqueous solution at room temperature has been previously observed (5). The fact that quenching occurs in the bound dye at room temperature suggests that perhaps nuclear

---

* A small red shift in the absorption spectra of the acridines on binding to DNA is frequently observed and occurs with 9-aminoacridine and DNA in a 1:1 glycerol-H_2O solution at room temperature.

motions within the DNA enhance intersystem crossing in the dye. This suggestion, however, is purely speculative. A study of the fluorescence behavior of bound 9-aminoacridine at intermediate temperatures would indicate the critical temperature range for the quenching and might prove useful in determining the mechanism.

The ratio f_{uv}/f_{vis} of the dye is enhanced by a factor of three on going from the free to the bound form, both at room temperature and at 77°K. While, as stated earlier, this cannot be quantitatively interpreted in the absence of absorption data, in view of the relatively small perturbations to the optical properties of the dye on binding the magnitude of the increase can only be explained by singlet energy transfer from the DNA. Crude calculations based on the assumption that the absorption probabilities are not changed indicates that 3 - 4 bases can transfer singlet energy completely to a dye. These data confirm the earlier observations of singlet energy transfer in these systems.

The position of the wavelength maximum of the bound dye phosphorescence shown in Fig. 4 is blue shifted by about 5 mμ from the free dye and the lifetime is still 2.1 sec. The intensity with visible excitation remains within about 10% of that of the free dye at the same concentration. No careful quantitative measures of the differences in emission intensities between free and bound dye were made in view of the expected shifts in the absorption spectrum of the dye on binding. A small amount of delayed emission not present in the free dye spectrum appears at shorter wavelengths (450-550mμ) than the dye phosphorescence. Its presence was seen to a variable extent. Its probable origin will be considered later and it was ignored in calculating the phosphorescence

FIG. 4. Delayed Emission from 9-aminoacridine - Native DNA complex.

(a) 1 - λ_{ex} = 405 mμ,

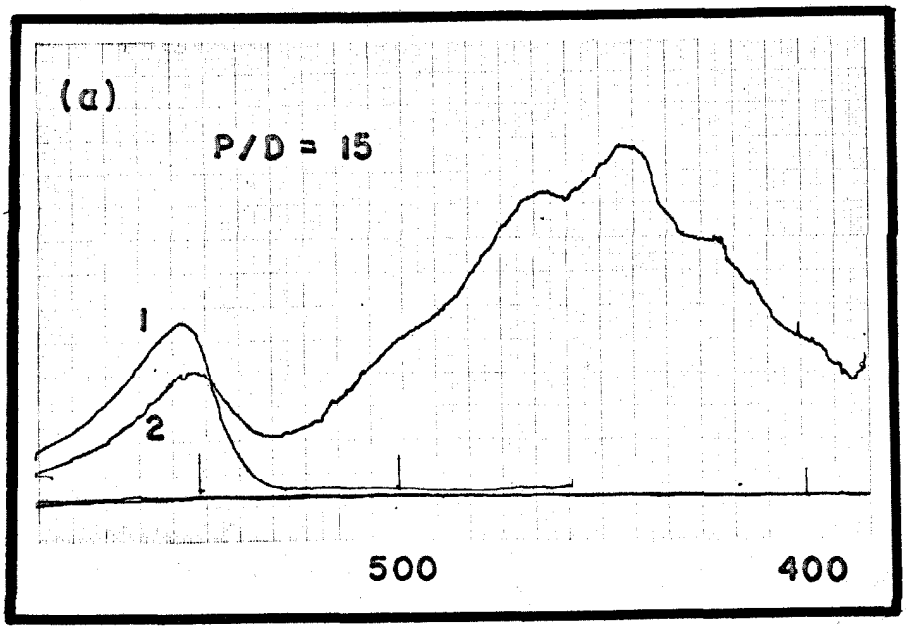
2 - λ_{ex} = 280 mμ, rel. gain = x 1.

DNA concentration = 9.0×10^{-4}M.

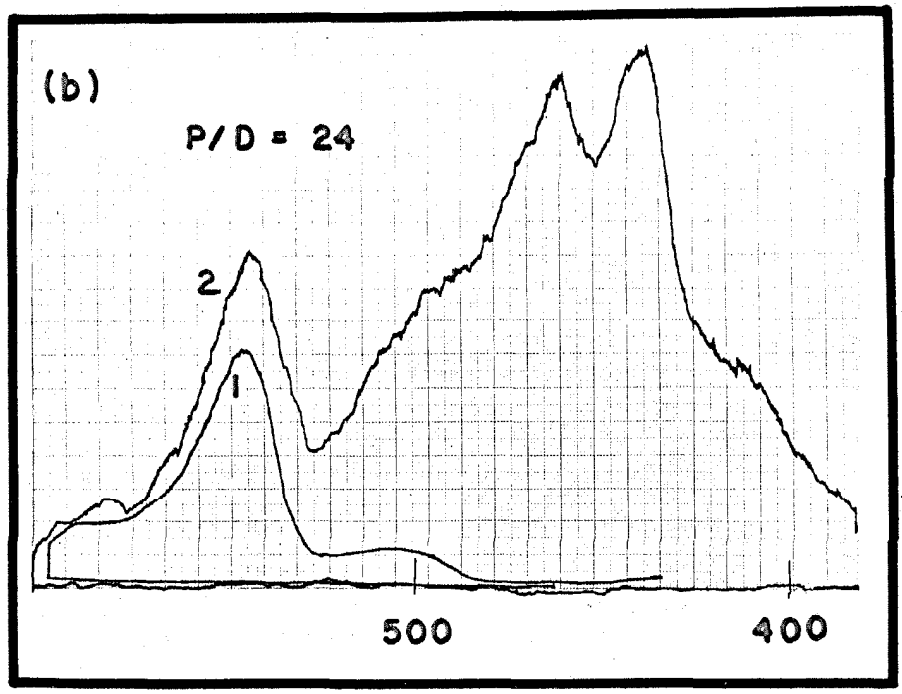
(b) 1 - λ_{ex} = 405 mμ,

2 - λ_{ex} = 247 mμ, rel. gain = x 5

DNA concentration = 2.3×10^{-3}M.



WAVELENGTH (mμ)



WAVELENGTH (mμ)

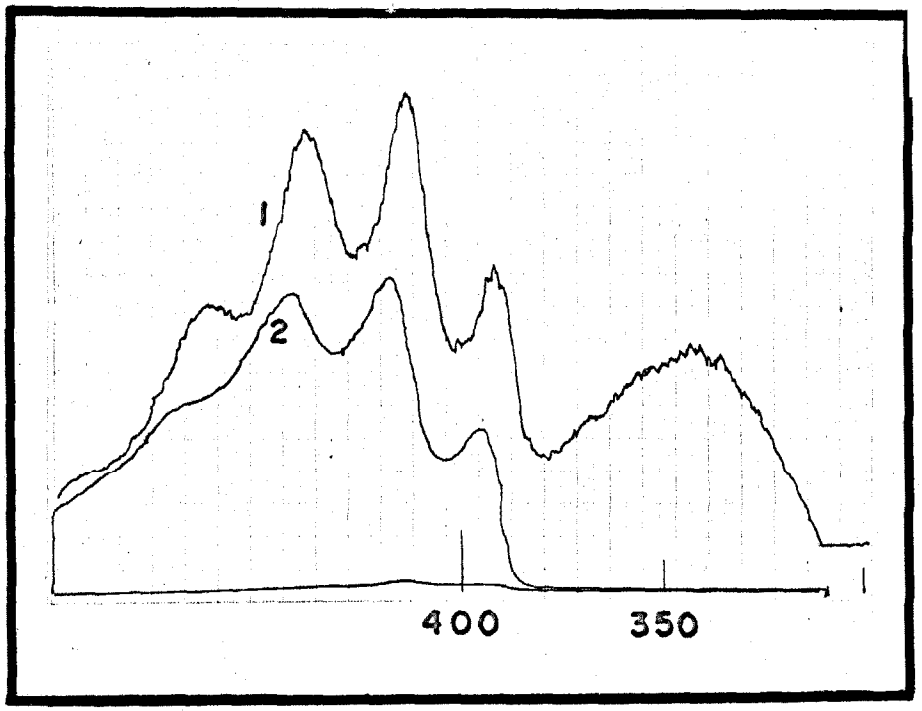
intensities.

In the uv-induced delayed emission spectrum of the high P/D complex of 9-aminoacridine with DNA seen in fig. 4, a broad emission occurs between 400 and 550 mμ. Emission from both the glycerol and the DNA occur in this region and contribute to the intensity, and, in addition, there is delayed fluorescence from the dye, with the characteristic fluorescence peaks of 9-aminoacridine in evidence. The observation of delayed fluorescence with uv but not with visible light is consistent with the observations of Isenberg et al in complexes of DNA and acridine orange, proflavine and quinacrine (32). The DNA phosphorescence is partially quenched at high P/D, but the degree of quenching is difficult to estimate.

The intensity and nature of the emission in this region of polymer emission depends on the polymer present in the complex. The emission is most clearly defined in complexes with polyadenylic acid. The phosphorescence and fluorescence from poly A at neutral pH are at least an order of magnitude greater than in DNA and the phosphorescence has a characteristic structured appearance. Under these conditions the emission from glycerol can be ignored and the polymer emission reliably measured. Emission from poly A at neutral pH in the absence of dye is shown in Fig. 5.

In the presence of poly U the fluorescence maxima of 9-aminoacridine shown in Fig. 6(a) appears to be red shifted by only 1 or 2 mμ at high P/D. This is consistent with the lack of a red shift observed for the binding of dye to apurinic acid (8). No emission from the polymer is observed and the characteristic peaks in the spectrum cor-

FIG. 5



Emission Spectra of Poly A (neutral pH)

1 - Total emission spectrum.

2 - Emission with the phosphoroscope, rel. gain = x 10.

FIG. 6. Emission From the 9-aminoacridine - Poly U.

Poly U concentration = 6.7×10^{-4}M.

(a) Fluorescence (total emission)

1 - λ_{ex} = 405 mμ,

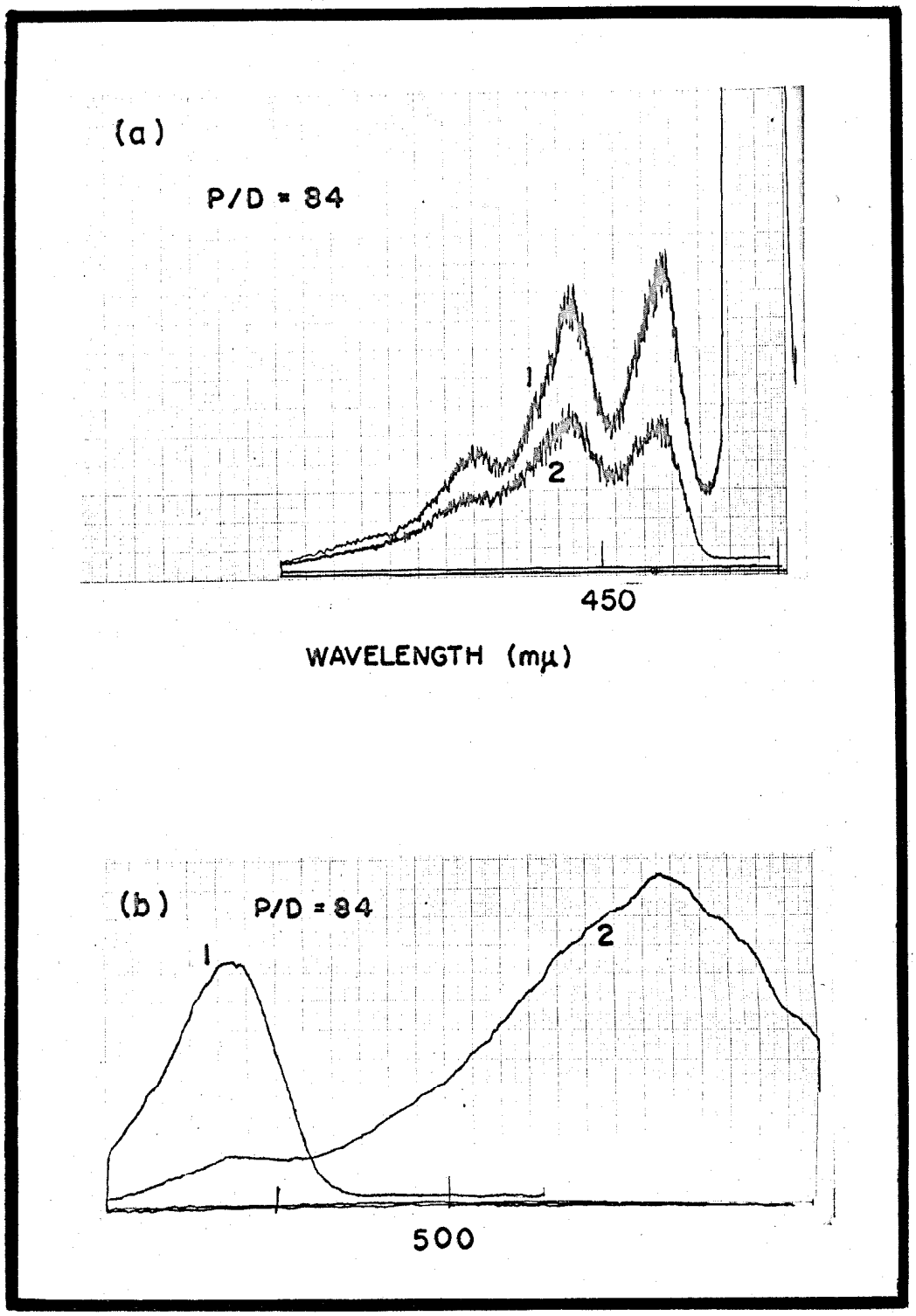
2 - λ_{ex} = 280 mμ, rel. gain = x 10.

(b) Delayed Emission

1 - λ_{ex} = 405 mμ

2 - λ_{ex} = 280 mμ, rel. gain = x 1.*

* gain of (b1) relative to (a1) = 10^4


(a)
P/D = 84
1
2
450
WAVELENGTH (mμ)
(b)
P/D = 84
1
2
500
WAVELENGTH (mμ)

responding to uv-induced delayed fluorescence of the dye are absent; the emission in the delayed spectrum in the 450 mμ range being essentially that of glycerol [fig. 6(b)].

The normal and delayed emission spectra for acridine orange bound to native DNA at high P/D are shown in Fig. 7(a) and Fig. 7(b). The emission in general appears at higher wavelengths than 9-aminoacridine. The fluorescence maximum appears at 520 mμ and the phosphorescence at 610 mμ. In the delayed emission spectrum, in addition to the phosphorescence, delayed emission is observed with the same shape, and appearing at the same wavelength as the normal fluorescence. This emission only appears on uv excitation, again confirming the observations of Isenberg et al (32).

The low P/D spectrum of acridine orange shown in Fig. 8 is not markedly altered from the high P/D emission, except that,in this case, delayed fluorescence also appears with visible excitation. The probable source of this emission which does not involve excitation of the DNA will be considered in a later section.

As can be seen from Fig. 8 the fluorescence spectrum of 9-aminoacridine at low P/D ratios is complicated. The fact that the spectra are no longer identical with visible and uv excitation indicates that more than one type of complex is present and that one or the other is preferentially excited at the visible or uv exciting wavelengths. The formation of a new complex at low P/D ratios could arise from occupation of a second type of binding site on the polymer, from interactions between dyes on nearby binding sites, or a combination of both. In the low P/D complex the emission in the 400-500 mμ region appears to be due

FIG. 7. Emission from the Acridine Orange - Native DNA complex.

DNA concentration = 9.0×10^{-4}

(a) Total Emission

1 - λ_{ex} = 436 mμ

2 - λ_{ex} = 280 mμ, rel. gain x 10

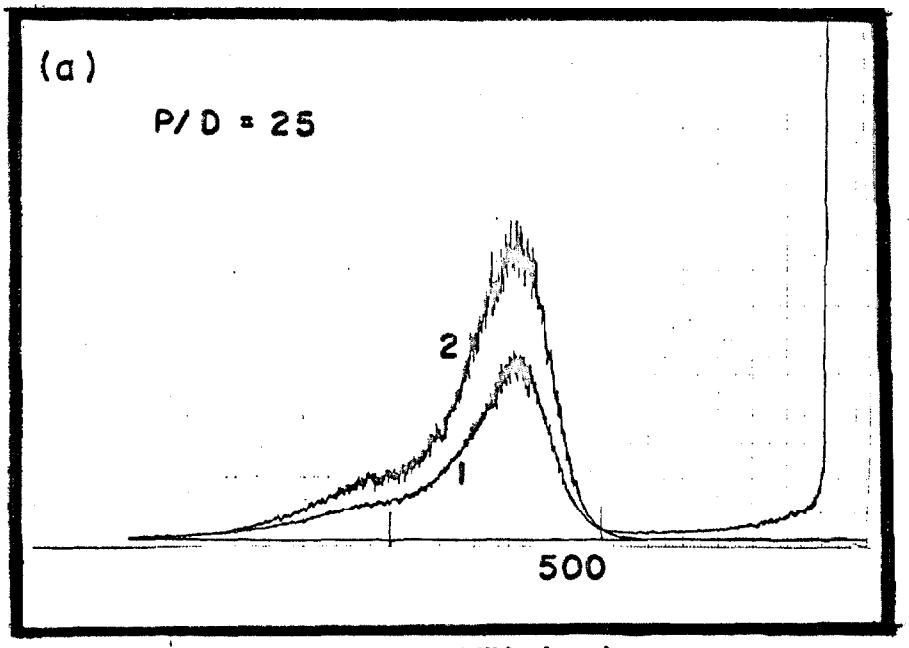
(b) Delayed Emission

1 - λ_{ex} = 436 mμ

2 - λ_{ex} = 280 mμ, rel. gain - x 1

The small peak in the delayed emission spectrum at the exciting wavelength (436 mμ) arises from exciting light which is scattered outside of the phosphoroscope cup.

Rel. gain of (a) - 1 to (b) - 1 = 10^4


(a)
P/D = 25
2
1
500
WAVELENGTH (mμ)

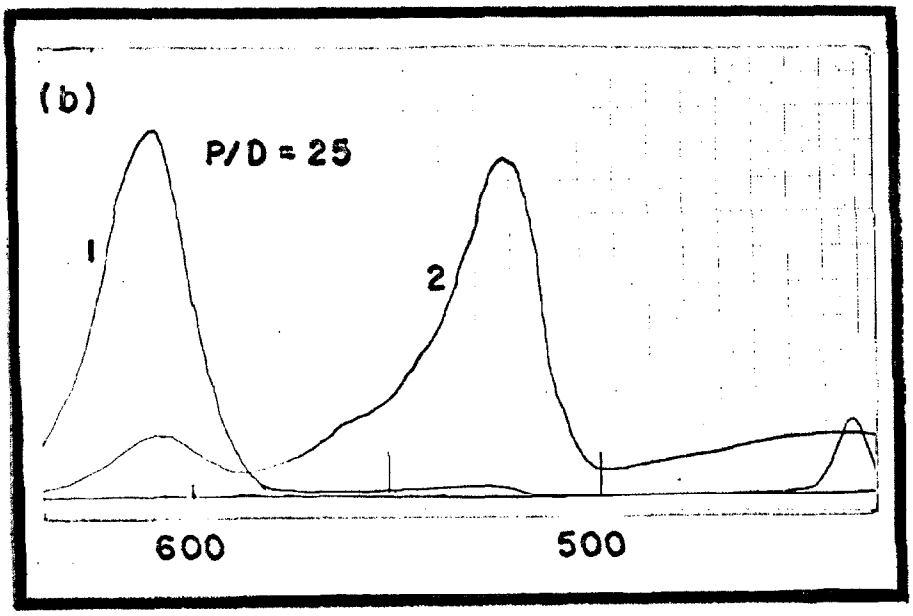

(b)
P/D = 25
1
2
600
500
WAVELENGTH (mμ)

FIG. 8. Emission Spectra at Low P/D

(a) Fluorescence from Acridine Orange

DNA concentration = 9.0×10^{-4} M.

1 - λ_{ex} = 436 mμ,

2 - λ_{ex} = 280 mμ, rel. gain = x 10

(b) Delayed Emission from Acridine Orange - DNA

1 - λ_{ex} = 436 mμ,

2 - λ_{ex} = 280 mμ, rel. gain = x 10

(c) 9-aminoacridine Fluorescence

DNA concentration = 4.5×10^{-4} M.

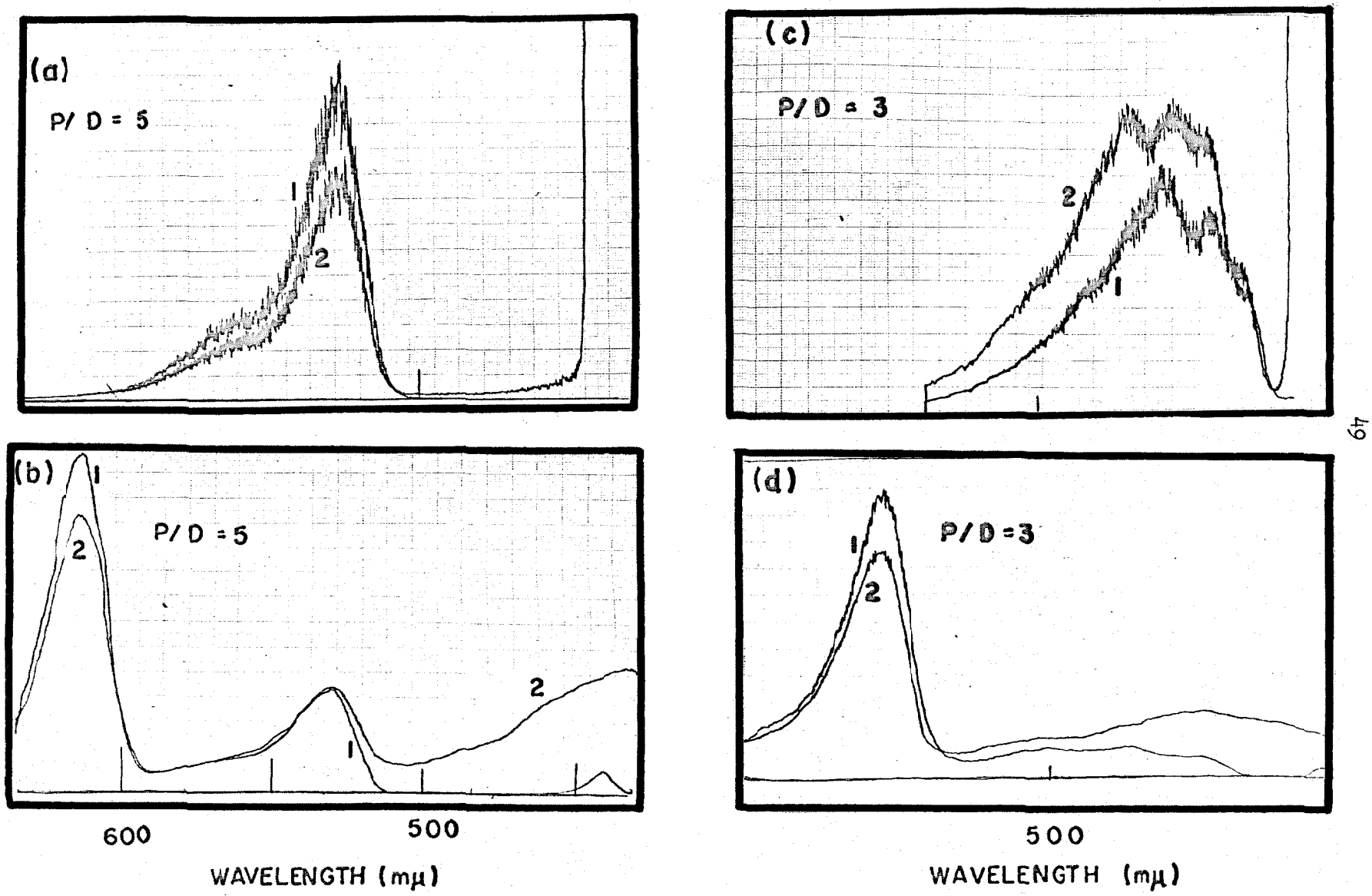
1 - λ_{ex} = 405 mμ,

2 - λ_{ex} = 280 mμ, rel. gain = 2.5

(d) Delayed Emission from 9-aminoacridine

1 - λ_{ex} = 405 mμ,

2 - λ_{ex} = 280 mμ, rel. gain = x 10


(a)
P/ D = 5
1
2
(c)
P/ D = 3
2
1
(b)
1
2
P/ D = 5
2
1
(d)
1
2
P/D = 3
600
500
WAVELENGTH (mμ)
500
WAVELENGTH (mμ)

essentially to glycerol, the DNA phosphorescence being entirely quenched. In view of the complex nature of the fluorescence spectrum at low P/D, the high P/D case will be primarily considered here. The low P/D dye spectrum will be considered again in discussing dye-dye interactions.

The observation of delayed fluorescence from the dyes bound to DNA will be dealt with and discussed in relation to the original observation of delayed fluorescence in these systems by Isenberg et al (32) in a later section. The important observation with regard to the investigation of triplet energy transfer is that emission from both the singlet and triplet state of the dye can be detected and measured, and, therefore, the data can be analyzed in terms of triplet polymer to triplet dye energy transfer.

TRIPLET-TRIPLET ENERGY TRANSFER IN THE 9-AMINOACRIDINE-NATIVE DNA COMPLEX

Phosphorescence to Fluorescence Ratios of Bound and Free 9-Aminoacridine

The phosphorescence and fluorescence intensities of 9-aminoacridine in a high P/D complex with native DNA and in the free form, were determined from the phosphorescence and the fluorescence spectra. The ratio of dye phosphorescence to fluorescence intensities with uv excitation was normalized by the corresponding ratio with visible excitation, and the results from a number of experiments are given in table II. The values given in table II represent the maximum value obtained at high P/D. The R values shown in table II for 9-aminoacridine in the native DNA complex indicate that the total uv-induced phosphorescence in the dye is 7 - 11 times greater than that component of the phosphorescence which arises via intersystem crossing from the lowest singlet

Table II

Relative Enhancement in 9-Aminoacridine Phosphorescence to Fluorescence Ratio with UV Excitation.

Dye conc. (M)	P/D	p_{VIS}/f^{*}_{VIS}	R $\frac{p_{uv}/f_{uv}}{p_{vis}/f_{vis}}$
9.4×10^{-5}	24	5.6×10^{-5}	11
3.6×10^{-5}	25	3.3×10^{-5}	7.5
5.0×10^{-6}	33	3.4×10^{-5}	8.2
4.8×10^{-5}	0	6.0×10^{-5}	1.46
4.8×10^{-5}	0	5.4×10^{-5}	1.74
2.8×10^{-5}	0	4.6×10^{-5}	1.55
3.0×10^{-4}	0	2.0×10^{-4}	1.46

$\bar{R}$ (DNA) = 8.1 ± 1.4 (mean of 10 samples)

$\bar{R}$ (Free Dye) = 1.48 ± .12 (mean of 7 samples)

* The values given for the p/f ratios are approximately 60 X smaller than the real values due to averaging of the phosphorescence intensity by the rotating cup of the phosphoroscope and the decrease in photomultiplier sensitivity at the wavelengths of phosphorescence relative to fluorescence.

state of the dye. On the other hand, the R values for free 9-aminoacridine are close to one. These uncorrected R values for free dye, however, are significantly different from unity.

The reason for the rather wide variations (± 1.4) in the R values for 9-aminoacridine - native DNA complexes from experiment to experiment is unknown. Considerable error is undoubtedly made in determining the intensities of dye phosphorescence with uv excitation in view of the necessity of correcting for the emission from DNA and glycerol. The correction is made with use of the ratio of emission intensity in the absence of dye at the wavelength of the dye phosphorescence, to a reference intensity at a wavelength outside of the dye phosphorescence band. In spite of changes in the relative amount of DNA and glycerol emission on complex formation with the dye, under the conditions of the experiments given in table II, this error could not amount to more than 15%. Reproducibility in a given experiment is within ± 5%. These errors, therefore, cannot totally account for the lack of reproducibility from experiment to experiment. While phenol extraction of the DNA increased the maximum R value over non-extracted DNA in one experiment, higher values were seen in other experiments with non-extracted DNA, suggesting that the variation is not caused by the presence of protein impurities in the DNA.

The absolute values of R given in table II are in error due to the anisotropy of each of the emission components which constitute a single R value, and corrections for this anisotropy must be made to obtain the correct R values. Polarization measurements were carried out in the experiments described in the following section. The values of R

determined here will be seen to essentially be unaltered by these corrections.

Emission Corrections

Monochromator Correction Factors

The t_1 and t_2 factors for the excitation and emission monochromators respectively, measured at the wavelengths of excitation and emission, are given in table III. The t_1 and t_2 values at given wavelengths are seen to depend on the method of determination. This variation has been observed and discussed by Azumi and McGlynn (60). In measuring the degree of polarization the value of t_2 determined under the same conditions as for the polarization measurements, from the relation $t_2 = V_h/H_h$, should be used; however, this value undoubtedly does not represent the true t_2 value for the emission monochromator in view of the known errors resulting from the use of a Glan-Thompson prism close to the monochromator. The t_2 value obtained with the use of a polaroid between the sample and emission monochromator was used in the expression for the correction due to monochromator polarization effects. It is more difficult to ascertain which value of t_1 should be used. Measurements at low temperature account for the effects of the cylindrical geometry of the dewar and sample holder and the refractive index of the liquid nitrogen; however, the displacement of the beam on rotation of the Glan-Thompson prism undoubtedly introduces more error under these conditions than when the large square cell sample holder was used in the room temperature measurements.

Table III

Correction Factors for Preferential Transmission through Monochromators of One Polarized Component

Emission (mμ)	Method	t_2
455.	G-T, prism, 77°K	0.72 ± .04
455.	G-T, prism, 298°K	0.74 ± .04
455.	polaroid, 298°K	1.00 ± .01
550.	G-T, prism, 77°K	0.77
	G-T, prism, 298°K	0.75
	polaroid, 298°K	0.95 ± .01

Excitation (mμ)	Method	t_1
403	G-T prism, 77°K	0.95 ± .04
403	G-T prism, 298°K	1.10 ± .01
403	polaroid, 298°K	1.05 ± .04
247	G-T prism, 77°K	0.85 ± .04
247	G-T prism, 298°K	1.03 ± .03

Polarization of Emission of Free and Bound 9-Aminoacridine

Measurements of the degree of polarization of the fluorescence and phosphorescence of 9-aminoacridine excited with visible and uv light were carried out on the free dye and the dye in the DNA complex and the results are given in table IV for the wavelengths at which the emission was routinely determined. The values for the degree of polarization given in table IV were used along with the t_1 and t_2 values in calculating the emission correction factors C_A and C_m (cf. p. 28).

Polarization measurements were carried out in only a few experiments. In addition to the technical difficulties involved in using Glan-Thompson prisms, the phosphorescence intensity excited with uv light was too low to be reliably measured with polarizers. More reliable determinations could be made by using Polaroid type polarizers instead of the Glan-Thompson prisms. This would eliminate the errors which arise from displacement of the beam with rotation of the Glan-Thompson prism and, in addition, the absorption type polarizers have a much larger acceptance angle and would allow measurements of the degree of polarization of lower emission intensities.

The polarization data on the free 9-aminoacridine molecule is of interest for its own sake. The degree of polarization of the dye fluorescence with visible excitation is considerably lower than the theoretical value (+0.5) for parallel absorption and emission transition moments. This probably arises, at least in part, from the fact that excitation was not in the 0-0 band of the dye. In addition, as indicated in the footnote to table IV, the highest absolute magnitudes of the degree of polarization were observed from emission in the 0-0 band. The absolute

Table IV

Polarization of 9-Aminoacridine Emission in the Free and Bound Form

Excitation (mμ)	Emission (mμ)	P
	Free dye (1.5×10^{-4}M)	
403	fluor. 455	+0.18
247	fluor. 455	-0.02
403	phosp. 553	-0.03
247	phosp. 553	-0.26
	Bound dye (9.4×10^{-5}M, P/D = 24)	
403	fluor. 464	+0.09
247	fluor. 464	-0.01
403	phosp. 550	0.00
247	phosp. 550	-0.08

The values reported here for the degree of polarization are for emission at the wavelengths where it was routinely determined. Higher absolute magnitudesfor the degree of polarization p were obtained with emission from the shortest wavelength (0-0) emission band.

magnitude of p for fluorescence decreased toward the long wavelength end of the spectra. The degree of polarization of the phosphorescence of the free dye is seen to be negative with respect to both 405 and 247 mμ excitation. On the assumption that all singlet-singlet transition moments are in the plane of the molecule, the fact that the phosphorescence is negatively polarized with excitation both at 405 mμ and 247 mμ while the fluorescence excited with 247 mμ is also negatively polarized, suggests that the T-S^{o} transition moment is polarized out of the plane of the molecule. However, substituting the values of p obtained from emission in the 0-0 band into the expression for the degree of polarization

$$p = \frac{(3\cos^2\alpha - 1)}{(\cos^2\alpha + 3)} \qquad (8)$$

where α is the angle between absorption and emission transition dipole moments, does not unambiguously lead to an out of plane T-S^{o} transition moment. An experiment carried out with excitation into the 0-0 band of the dye would probably lead to a firm assignment of the T-S^{o} transition moment direction.

Calculation of Corrected R Functions.

The phosphorescence and fluorescence intensities in the R functions were corrected by multiplication by C_A and C_M (cf. p.28). The total correction for the R function of the 9-aminoacridine - DNA complex was calculated from the polarization data for each of the four emission intensities, and was found to be 1.01. This lack of correction arises not only as a result of cancellation among the individual correction factors, but also as a result of the low degree of polarization.

The R values given in table II for the 9-aminoacridine-native DNA complex are then essentially unaltered, and corrections were not made on further measurements of R. The total correction factor for the R function for free 9-aminoacridine was calculated using the two most extreme values for t_1. This gives a range for the total R correction factor of 0.71 to 0.84 and a corrected R value for free 9-aminoacridine of 1.03-1.30. It should be pointed out that there was no way of predicting a priori that the corrections to the experimental values of R would be small. Having made these corrections it is possible to consider the interpretation of these data.

Anomalous p/f Ratios with UV Excitation.

According to the scheme outlined briefly in the experimental section for the possible paths of excitation in the complex, R values greater than unity indicate that triplet polymer to triplet dye energy transfer is occurring. This can be placed on a quantitative basis with a kinetic analysis which is presented in detail in Appendix II. The analysis results in the following expression for the R function determined experimentally above:

$$R = \frac{(p/f)uv}{(p/f)vis} = 1 + \frac{k_{t-t}\,(T_p)}{\Phi_i \left[I_D^{\circ} + k_{s-s}\,(S_p^1) + k_{t-s}(T_p) \right]} \qquad (II.11)$$

where k_{t-t} is the rate constant for triplet polymer to triplet dye transfer, (T_p) represents the steady state polymer triplet population*, and

* A kinetic analysis gives a poor representation of the system in characterizing the transfer with a single rate constant and in describing the population of excited states of polymer. This will become clearer later in dealing with the excited states of the polymers. The kinetic analysis has the advantage that it connects the theory with experimentally measurable quantities in a simple way.

Φ_i the intersystem crossing efficiency in the dye, I_D^1 the rate of uv absorption by the dye, k_{s-s} the rate constant for singlet polymer - singlet dye energy transfer, (S_p^1) the population of excited singlet state of the polymer, and k_{t-s} the rate constant for triplet polymer - singlet dye energy transfer. The analysis again illustrates the point that R values greater than unity indicate triplet-triplet energy transfer. The magnitude of the additional term is given by the rate of populating the dye triplet state by triplet-triplet energy transfer $k_{t-t}(T_p)$ relative to the rate of populating the dye triplet state by all other pathways, i.e., the denomination is given by a sum of terms $I_D^1 + k_{s-s}(S_p^1) + k_{t-s}(T_p)$ which represents the rate of populating the lowest excited singlet state of the dye multiplied by the intersystem crossing efficiency in the dye Φ_i. On the basis of this analysis an average value of (R-1) = 7 in the 9-aminoacridine - native DNA complex indicates that the rate of populating the triplet state of the dye is 7 X greater by triplet-triplet energy transfer than by other pathways which go through the lowest excited singlet state in the dye.

The fact that the corrected R value for free 9-aminoacridine still appears to be greater than one, indicates that with uv excitation a small component of the phosphorescence does not arise by way of the lowest excited singlet state of the dye. According to the analysis above, in the absence of triplet-triplet transfer there is no other pathway for selectively populating the triplet state. The analysis, however, is based on the assumption that internal conversion from the higher excited singlet state to the lowest excited singlet state occurs with unit efficiency. This assumption is based largely on the observa-

tion that fluorescence spectra and quantum yields, in general, are found to be independent of the wavelength of excitation (61). Emission in azulene, however, occurs from the second excited singlet state (62) and anomalous p/f ratios with excitation to higher excited singlet states have been reported (63), indicating that rapid internal conversion does not always occur, nor is it required theoretically (64). The alternatives to internal conversion among excited singlet states are emission from the higher excited singlet states or intersystem crossing from the higher excited singlet state to the triplet manifold of states. The latter process is obviously operating here and our results indicate that $\sim 15\%$ of the excitation reaching the lowest triplet state of 9-aminoacridine arrives by way of the triplet manifold following intersystem crossing from a higher excited singlet state.

Having acknowledged the possibility of intersystem crossing among higher excited states, the proposition can be made that the large R values in the 9-aminoacridine - DNA complex arise by an enhancement in the efficiency of this pathway on binding of the dye to DNA rather than by triplet polymer to triplet dye energy transfer. While the possibility of such an event cannot be completely ruled out on the basis of the data presented up until this point, the results in a later section do exclude the enhanced intersystem crossing hypothesis.

DNA-ACRIDINE ORANGE COMPLEX

Having demonstrated triplet energy transfer from the bases of native DNA to 9-aminoacridine, it was decided to investigate triplet energy transfer in a second acridine dye - DNA complex.

The phosphorescence and fluorescence intensities from acridine orange bound to native DNA were determined. The intensity ratios were calculated in the same manner as for 9-aminoacridine, and the results are given in table V.

Table V

Triplet Energy Transfer in Native DNA-Acridine Orange

P/D	p_{vis}/f_{vis}	R
9.1	2.0×10^{-4}	1.06
19.0	2.2×10^{-4}	0.94
57	2.28×10^{-4}	0.98

DNA conc.= 9.0×10^{-4}M

It can be seen that there is no enhancement in the p/f ratio on uv excitation, indicating that there is no detectable triplet DNA to triplet acridine orange energy transfer.

The failure to detect triplet-triplet transfer in the acridine orange complex is not altogether unexpected. It is evident from equation (II.11) that the magnitude of R-1 is inversely proportional to the intersystem crossing efficiency in the dye. The p_{vis}/f_{vis} ratio in acridine orange is seen from table V to be almost a factor of 10 higher than in 9-aminoacridine. On the assumption that the 10-fold difference in the p_{vis}/f_{vis} ratio reflects a difference in intersystem crossing the R-1 value would be expected to be < 0.1 in the acridine orange complex if the triplet transfer efficiency from DNA to dye were the same as in 9-aminoacridine. The initial choice of 9-aminoacridine as

the dye to be studied was based on its high fluorescence efficiency and, therefore, low intersystem crossing efficiency.

Comparison of experimental data on triplet energy transfer for a number of dyes by this technique, therefore, is not meaningful without knowledge of their relative intersystem crossing efficiencies. Given this information, it must be borne in mind that the efficiency of triplet transfer depends not only on the extent of overlap of the electron clouds of the donor and acceptor, but also on their Franck-Condon factors. An empirical measure of these factors can theoretically be obtained from the degree of overlapping of the polymer phosphorescence spectrum and the $S^{o} \rightarrow T$ absorption spectrum of the dye, but the latter in general is difficult to obtain.

NUCLEOTIDE-NUCLEOTIDE TRIPLET ENERGY TRANSFER IN NATIVE DNA

The data in the preceding section indicate that 9-aminoacridine bound to native DNA is a trap for the nucleotide triplet excitation energy. The amount of triplet energy being transferred to the dye was, therefore, investigated as a function of the nucleotide to dye ratio to determine the number of bases that can transfer triplet excitation to a 9-aminoacridine molecule.

The Dependence of the Selective Enhancement of UV-Induced Phosphorescence on the P/D Ratio in the 9-Aminoacridine - Native DNA Complex.

The R values given in table II were the maximum limiting values at high P/D. The magnitude of R, however, depends on the P/D ratio.

The R values for a number of P/D ratios for the 9-aminoacridine - native DNA complex are shown in table VI:

Table VI

Values of R for the Native DNA-9-Aminoacridine Complex

P/D	R
5.0	1.86
9.0	3.90
15	4.54
18.7	7.20
25	7.50

DNA conc. = 9.0×10^{-4} M

Data are from one experiment.

The selective enhancement in the uv-induced dye phosphorescence is seen to increase with increasing P/D.

According to the kinetic analysis presented above, increases in the R function given by equation (II.11) can arise from: a) an increased rate of triplet-triplet energy transfer $k_{t-t}(T_p)$, b) a decreased intersystem crossing in the dye Φ_i, or c) a decreased rate of populating the excited dye singlet, either by direct absorption I_D^1 or by energy transfer to the excited dye singlet state $k_{s-s}(S_p^1) + k_{T-S}(T_p)$.

Some of the ambiguity in interpretation of the results is eliminated with the use of an alternate criterion for triplet-triplet energy transfer. The data are calculated in terms of a difference between the ratio of dye phosphorescence intensities with uv to visible

excitation and the corresponding ratio for the dye fluorescence. The kinetic analysis in Appendix II gives the following expression for this difference:

$$\frac{P_{uv}}{P_{vis}} - \frac{f_{uv}}{f_{vis}} \equiv \Delta = \frac{k_{t-t}\,(T_p)}{\Phi_i\ I_D} \qquad \text{(II.12)}$$

This expression is again seen to be a measure of triplet polymer to triplet dye energy transfer; $\Delta > 0$ indicating the presence of triplet-triplet energy transfer. The data calculated in this way no longer have significance of a relative enhancement in the uv-induced phosphorescence due to triplet-triplet energy transfer. The absolute values of Δ are of an arbitrary nature in that a comparison is now made between the rate of triplet-triplet transfer $k_{t-t}\,(T_p)$ and the rate of intersystem crossing in the dye with visible excitation ($\Phi_i\ I_D$), and therefore depends on the relative intensities of the exciting light at the uv and visible wavelengths. On the other hand, the Δ function has the advantage that <u>with the internal dye parameters Φ_i and the extinction coefficient at the wavelength of visible excitation remaining constant, an increase in Δ with P/D can only be accounted for by an increase in the rate of triplet excitation transfer per dye</u>. With the use of Δ transfer of energy to the singlet state of the dye does not appear explicitly in the results, as is evident from the absence of the terms $k_{s-s}(S_p^1)$, $k_{t-s}(T_p)$ which appear in the denominator of the R-1 function. This does not mean that singlet-singlet and triplet polymer to singlet dye energy transfer do not affect the magnitude of Δ. They do by affecting the population of the triplet state of the polymer. Obviously if singlet energy transfer is highly efficient, there can be no polymer triplets formed and,

therefore, (T_p) and $\triangle$ will go to zero.

A kinetic analysis gives a poor representation of the system in that the term (T_p) does not in general represent the total pool of triplet excitation in the polymer, but only that fraction which contributes to triplet-triplet energy transfer. Direct transfer of triplet energy can almost certainly only occur between bases adjacent to a dye molecule, and the dye molecule, but not from bases which are non-nearest neighbors to a dye. In view of this, the term (T_p) can be regarded as the population of triplet excitation occurring on purines or pyrimidines adjacent to a dye without specifying where this excitation initially arose. With this interpretation, k_{t-t} is the rate constant for transfer to a dye from a nearest neighbor base.

The ratio of dye phosphorescence intensities at the two exciting wavelengths, and the corresponding ratio of fluorescence intensities were calculated and the individual ratios are plotted as a function of P/D in Fig. 9 for one experiment, and the difference $\triangle$ is plotted as a function of P/D for a number of experiments in Fig. 10.

The ratio p_{uv}/p_{vis} increases with increasing P/D to a limiting value, while the ratio f_{uv}/f_{vis} remains essentially constant with P/D*,

* While in the absence of absorption data, the constant f_{uv}/f_{vis} ratio for P/D > 4 cannot be reliably interpreted as a contribution of only 4 or 5 bases in the DNA to singlet energy transfer to a dye, this conclusion is consistent with the rough calculations made earlier. This suggests that the contribution of singlet excitation from a larger number of bases in DNA to acridine orange fluorescence may be due in part to direct interactions between dye and distant bases as a result of the higher oscillator strength in acridine orange than in 9-aminoacridine, and not entirely as a result of singlet energy delocalization within the polymer, as this should result in contribution from an equal number of bases in both cases. A comparison of the change in the actual quantum efficiency of 9-aminoacridine fluorescence as a function of P/D with the data obtained for acridine orange by Weill and Calvin (43) would be of interest.

Fig. 9 Variation of the ratio of emission with uv and visible excitation with P/D.

Data are from one experiment.

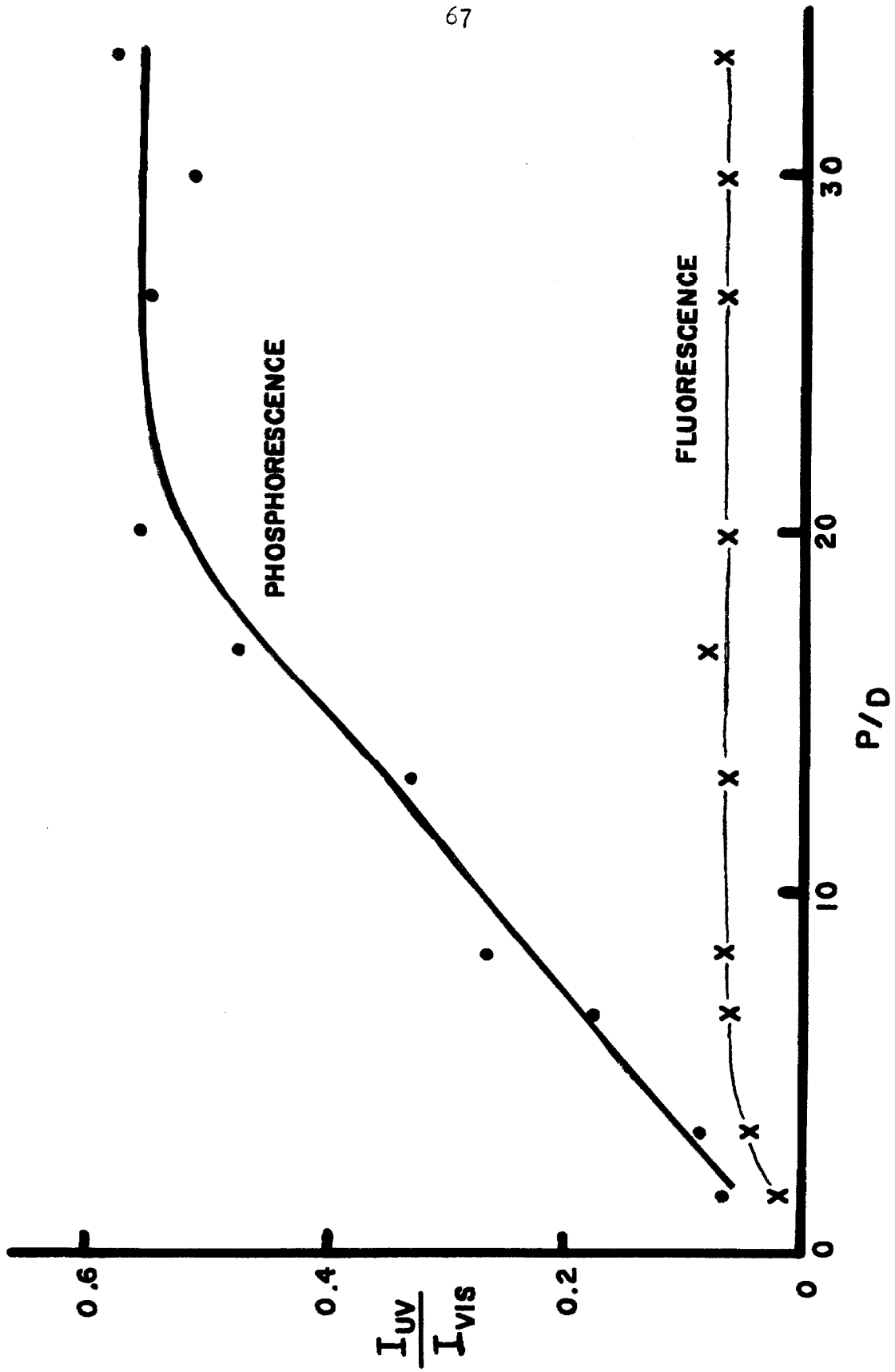
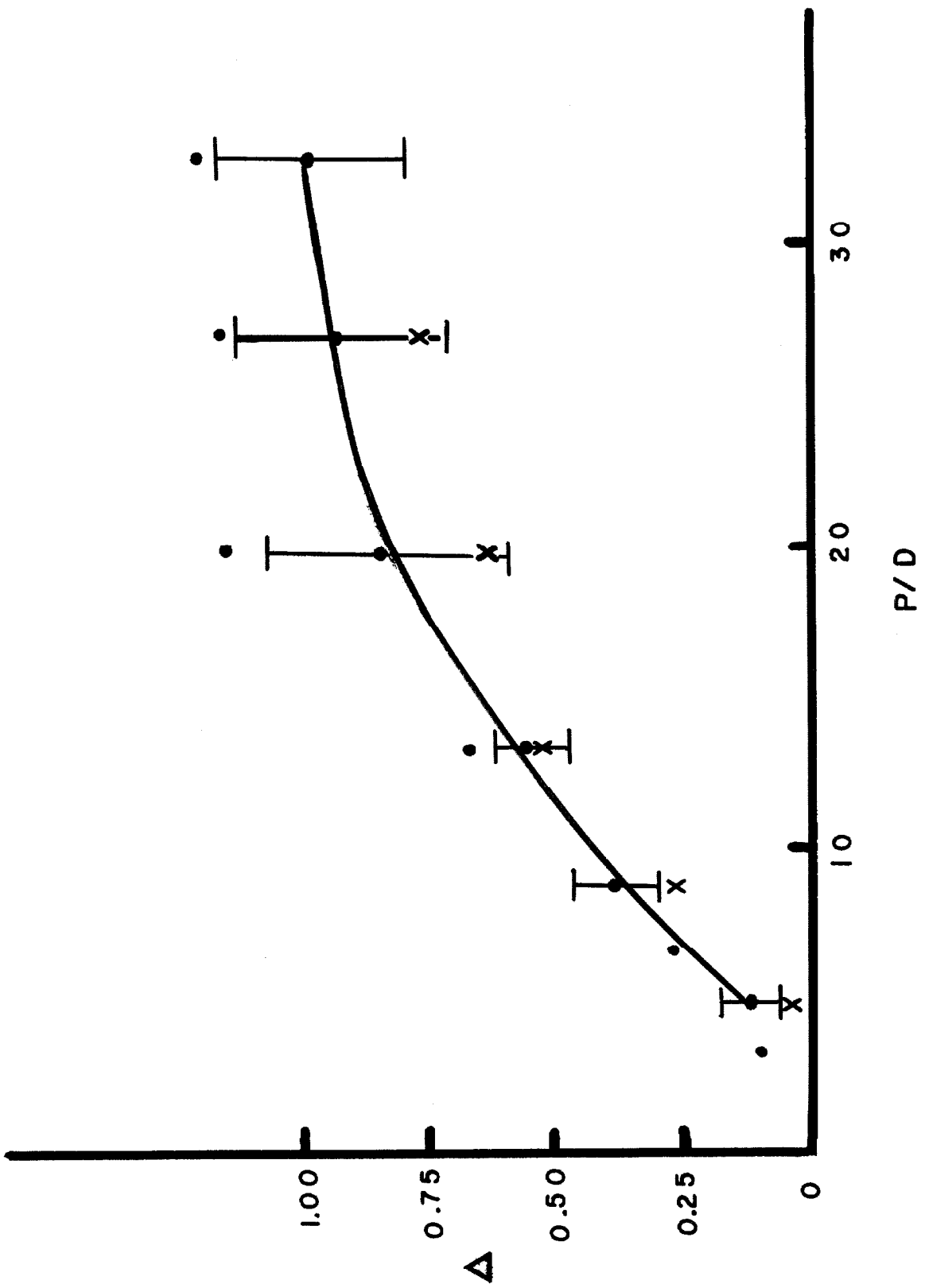

PHOSPHORESCENCE
FLUORESCENCE
$\frac{I_{UV}}{I_{VIS}}$
0.6
0.4
0.2
0
0
10
20
30
P/D

Fig. 10 Relative change in the amount of triplet energy transferred to a dye*. The error bars indicate $\pm$ the standard deviation. The additional points are from two individual experiments.+

* In view of the arbitrary nature of Δ, the function was normalized in all cases by the maximum value in the native DNA complex.

\+ The points for the individual experiments indicate that the large standard deviation arises from a comparison of results from experiment to experiment, and that variations within a given experiment are much smaller. The variations from one experiment to another parallel the variations in R.


1.00
0.75
0.50
0.25
0
Δ
10
20
30
P/D

except for very low P/D values. The difference in these ratios given by Δ is seen to increase with P/D and reach a limiting value at a P/D of about 20-25. This observation is consistent with the hypothesis that the rate of transfer of triplet excitation to a dye increases with an increasing number of bases available per dye. It has not been proven, however, that an increase in triplet energy transfer to a dye is occurring. The increase in Δ with P/D could arise from: a) a variation in intersystem crossing efficiency ϕ_i in the dye with P/D, which results in an increase in Δ [cf. eq. (II.12)] or, b) an increase in the fraction of the dye bound with increasing P/D. If the bound dye were to possess a characteristic Δ then, irrespective of whether it was the result of triplet energy transfer or due to intersystem crossing among higher excited states, the increasing fraction of the dye bound would increase Δ.

It can be shown that these two hypotheses cannot account for the increase in Δ with P/D.

It was indicated earlier that the optical properties of 9-aminoacridine do not remain constant with P/D. The fluorescence spectrum is red shifted and in addition the dye phosphorescence to fluorescence ratio with visible excitation is enhanced on going to low P/D. These changes, however, are confined to P/D values below about 7. Both the fluorescence spectrum and the p_{vis}/f_{vis} ratio remain fixed for P/D values greater than 7, and the absorption spectrum of these solutions at room temperature is unaltered over this range, indicating that a single type of complex in terms of optical behavior is present. The Δ function, on the other hand, increases by a factor of 3.5 - 4.0 X in going from

P/D = 7 to its maximum value at high P/D.

With some reservations, one can, in addition, investigate the low P/D region. The increase in the p_{vis}/f_{vis} ratio that occurs at low P/D cannot unequivocally to be interpreted as an enhanced intersystem crossing in that, in view of the low phosphorescence intensity, all of the excitation in the dye probably does not appear as emission, i.e., $\phi_f + \phi_p \neq 1$. However, enhanced intersystem crossing as a result of dye-dye interactions is not unexpected (49). The change in the p_{vis}/f_{vis} ratio was assumed, therefore, to occur as a result of intersystem crossing and the relative change in the p_{vis}/f_{vis} ratio was taken as the relative change in ϕ_i. If the Δ values in Fig. 9 are divided by the relative change in ϕ_i at the corresponding P/D value, the shape of the Δ curve is essentially unaltered. This indicates that, even in the low P/D region where enhanced intersystem crossing probably occurs, the increase in Δ cannot be accounted for by this effect.

The dye is totally bound to DNA at all P/D ratios. This is indicated by: a) The position of the fluorescence maxima remain fixed for P/D $>$ 5, but are red shifted from the free dye spectrum. The magnitude of the shift on binding, however, as indicated earlier, is small and this argument in itself is not very convincing. b) The magnitude of R, which depends on the P/D ratio, is constant at a fixed P/D over at least a ten-fold change in the DNA and 9-aminoacridine concentrations, as is evident from table VII*. If the experiments were carried out in a range of

* The Δ values tend to decrease at high concentrations of polymer and dye at high P/D. Due to the high O.D. of the solutions, self absorption of the uv light by the polymer is expected to be significant even in the 1 mm cells and is probably the cause. This effect is removed in calculating R values in that the decrease appears both in the uv-induced fluor-

escence as well as phosphorescence. For this reason, measurements of were made under conditions where the polymer concentration remained constant and the dye concentration was varied.

Table VII

P/D	DNA conc.(M)	R
5.3	1.2×10^{-3}	2.20
5.3	2.0×10^{-4}	2.09
12.8	1.2×10^{-3}	4.30
12.8	1.2×10^{-4}	4.25

concentrations where free dye was present, and the increasing R and values with P/D were simply the result of an increased fraction of the dye bound at high P/D, then a ten-fold dilution at a fixed P/D would certainly be expected to result in a marked reduction in R or Δ. At room temperature the fractional decrease in the dye fluorescence which occurs on binding is also invariant to at least a six-fold dilution from the concentrations used in these studies. This again illustrates that the dye, at the concentration used here, is essentially all bound even at room temperature.

The fact that the R and Δ values increase with increasing P/D, under conditions where the emission properties of the dye which is totally bound remain constant, excludes the possibility that the large R values in the 9-aminoacridine - native DNA complex arise as a result of an enhancement in intersystem crossing between higher excited states in the dye on binding. In such an event the enhancement would not be expected to increase further, simply by increasing the P/D ratio.

It is concluded, on the basis of the above evidence, therefore,

that the increase in Δ with P/D for the 9-aminoacridine - native DNA complex results from an increase in the population of polymer triplet which can contribute to triplet DNA to triplet dye energy transfer.

The increase in the probability of triplet transfer to a dye with increasing P/D undoubtedly arises as a result of nucleotide-nucleotide triplet energy transfer within the DNA helix. With increasing P/D a greater number of bases per dye become available to transfer energy to bases adjacent to bound dye molecules, from which the excitation can be trapped by transfer to the triplet state of the dye. These data support the conclusion of Bersohn and Isenberg (51) that triplet migration is occurring in DNA, based on the observation that the fraction of the DNA phosphorescence quenched by Mn^{++} was much greater than the fraction of the binding sites occupied by the ion.

The observation that the amount of triplet excitation being transferred to a dye tends to a limiting value for P/D $\sim$ 20 indicates that there is a finite pathlength for energy migration within the native DNA structure. It was anticipated in the original hypothesis that such a saturation would occur as a result of the finite lifetime for triplet excitation within the DNA (65). A diffusion model for migration of triplet excitation in DNA based on this same premise has been derived by Bersohn and Isenberg (51). Their equation (14) for the fraction of the DNA phosphorescence in the presence of quenchers, which is represented by "Q", is rearranged in Appendix III to give Δ', a function which represents the intensity of phosphorescence quenched per dye. The Δ' function was fitted to the experimental values of Δ with an adjustable parameter τc, the reciprocal of the nearest neighbor jumping frequency,

on the assumption that transfer to a dye molecule from nearest neighbor bases is 100% efficient. While the fit is not particularly good, the τ_c value is in agreement with the values roughly calculated from the data of Isenberg et al (54) and Shulman et al (55) on quenching of the DNA phosphorescence by paramagnetic ions. The comparison is shown in table VIII.

Table VIII

Values of τ_c for native DNA calculated on the Basis of the Diffusion Model of Bersohn and Isenberg

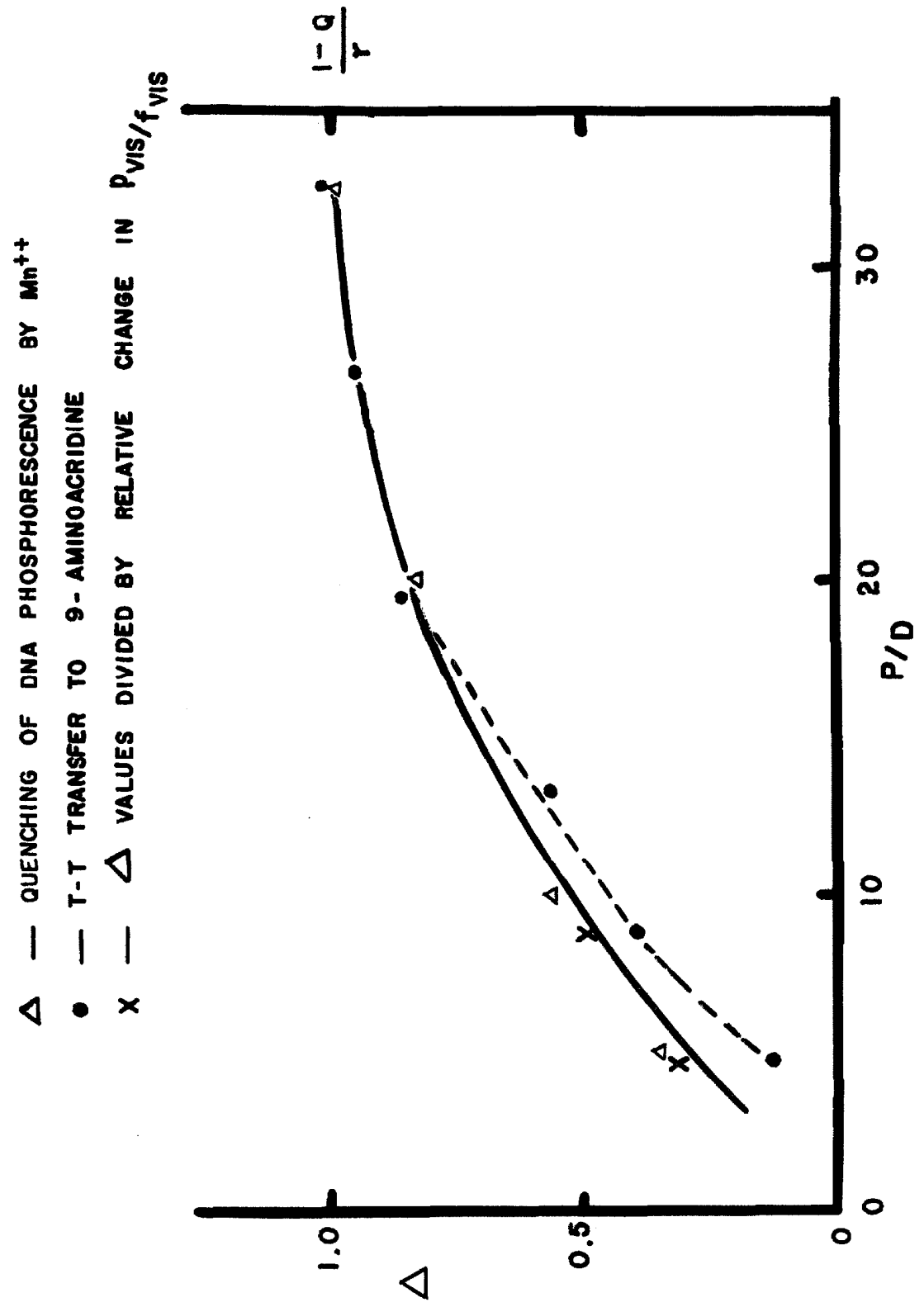
Method	τ_c (sec)
T-T transfer to 9-aminoacridine	4.2×10^{-3}
Quenching by Mn^{++} (Isenberg et al)	7.7×10^{-3}
Quenching by Mn^{++} (Shulman et al)	3.9×10^{-3}
Quenching by Cu^{++} (Isenberg et al)	2.0×10^{-3}

Calculated using τ (DNA triplet lifetime) = 0.3 sec.

The agreement in the data is seen in fig. 11 where the expression $(1-Q)/r$ (r= the number of Mn^{++} bound per phosphate) was roughly calculated from the data of Isenberg et al. and was normalized to coincide with Δ at high P/D. The similar behavior for the amount of "quenching" per guest atom or molecule indicates that, irrespective of whether the diffusion model is correct or not, the efficiency of trapping the DNA triplet excitation is similar for 9-aminoacridine and paramagnetic ions.

The values of τ_c imply that triplet energy transfer in DNA is very slow. A slow transfer rate in DNA is not unexpected. Following

Fig. 11 Comparison of the P/D dependence of the relative change in the amount of triplet transfer per dye, and the amount of quenching per paramagnetic ion.


Δ — QUENCHING OF DNA PHOSPHORESCENCE BY Mn++
● — T-T TRANSFER TO 9-AMINOACRIDINE
X — Δ VALUES DIVIDED BY RELATIVE CHANGE IN p_{VIS}/f_{VIS}
$\frac{1-Q}{r}$
Δ
1.0
0.5
0
0
10
20
30
P/D

vibrational relaxation to the lowest triplet state, triplet migration, as a consequence of the heterogeneity of the polymer, must occur by transfer through intervening bases with higher triplet energies. This should result in a considerable decrease in the rate of migration in comparison with the situation in which the monomers are identical. This point has been previously discussed by Bersohn and Isenberg in considering the very low hopping frequencies which arise from the fit of the data to the diffusion model (66). An important feature of a random walk model in which the pathlength for the excitation is limited by the lifetime of the polymer triplet state is that it predicts that triplet energy transferred to a dye at high P/D will have an average lifetime approaching that of the polymer in the absence of transfer. This is because a fraction of the excitation transferred to a dye at high P/D must migrate over a considerable distance in the polymer before being transferred to a dye. Kinetic experiments in which the DNA triplet state was populated by pulsed excitation were performed with a view to: a) measuring this slow transfer process at high P/D, and, b) providing a lower limit for the rate of excitation transfer to a dye at low P/D.

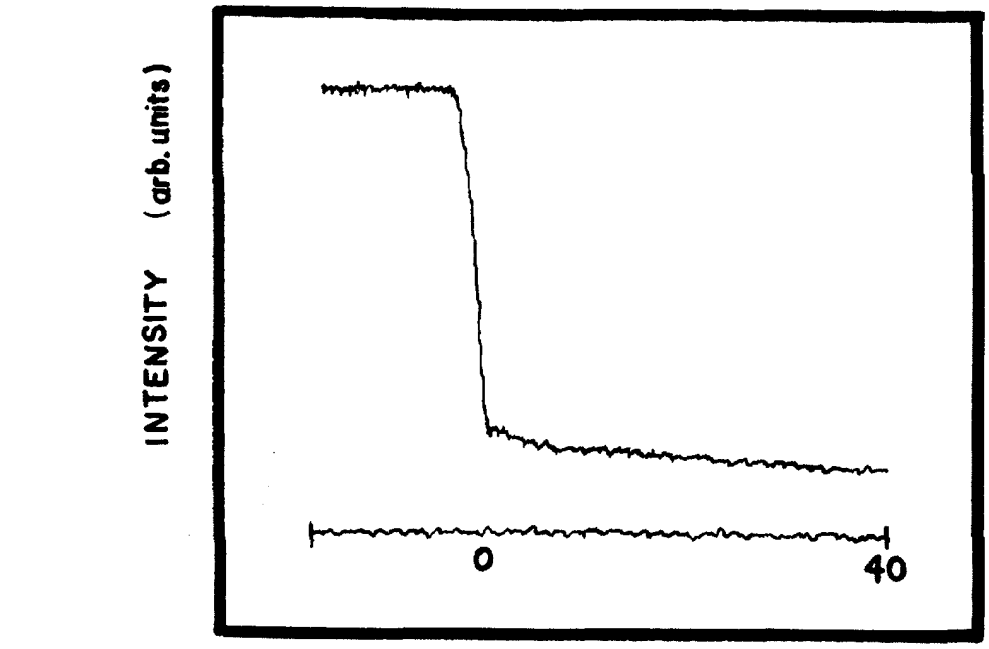
Decay of the Dye Fluorescence Following Pulsed UV Excitation.

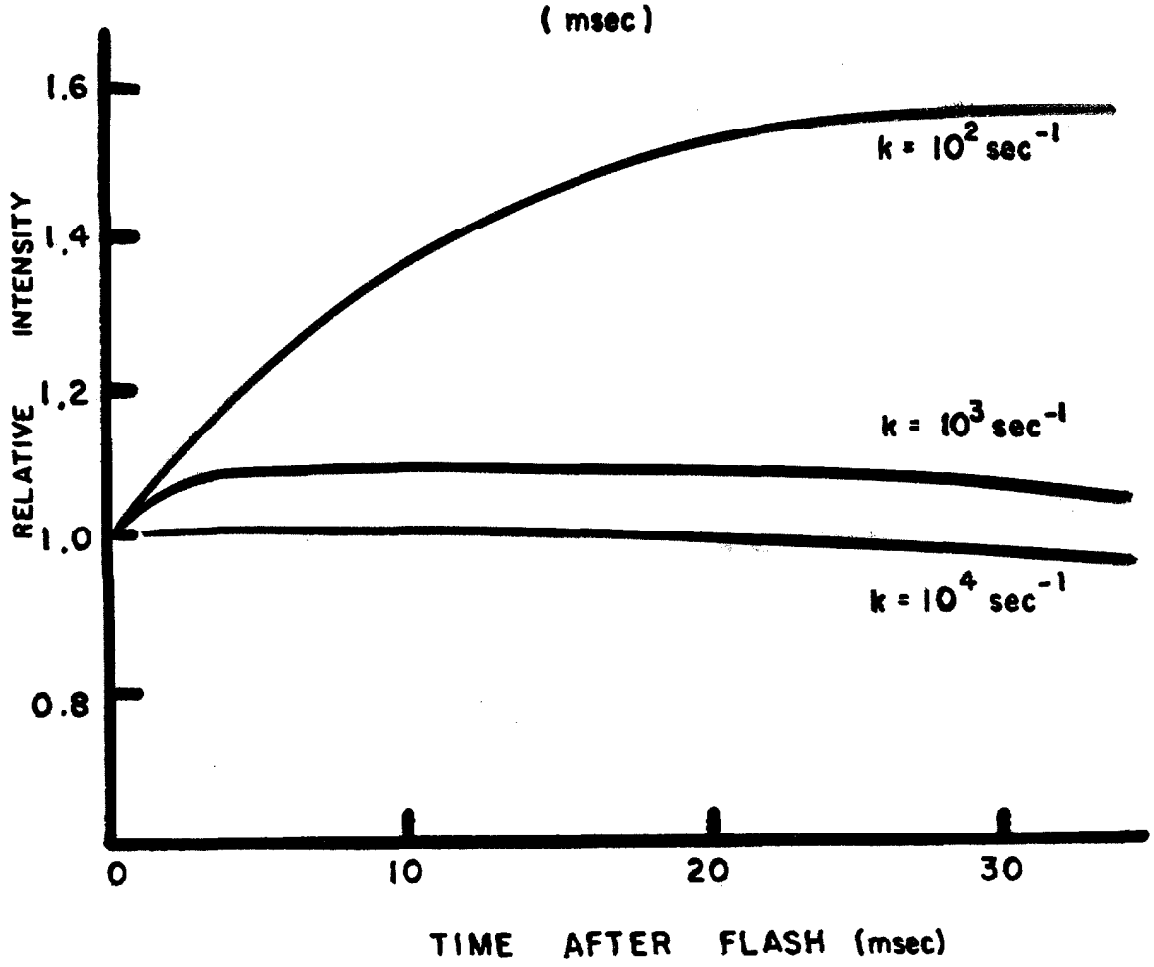
The decay of the dye phosphorescence with a 17 msec. pulse of excitation is shown in Fig. 12 with excitation at 280 mμ. The flash period is distinguished by an intense fluorescence. The decay in Fig. 12 is a result of accumulation of many pulses of excitation which were spaced at 10 sec. intervals to allow the triplet dye population to decay back to the ground state between subsequent flashes. Following the

FIG. 12 Decay of the uv-induced phosphorescence from 9-aminoacridine following pulsed excitation in the native DNA - dye complex.

(a) Experimental decay of emission at 550 mμ (dye phosphorescence) with a 17 msec. flash at 280 mμ. The decay curve is the result of 40 flashes accumulated in a multichannel analyzer. DNA concentration = 5.0×10^{-4} M, P/D = 13

(b) Theoretical decay curves for dye phosphorescence calculated from equation (7) for various average values of k. The intensity is expressed relative to the intensity at the end of the flash period.


INTENSITY (arb. units)
0
40
TIME AFTER FLASH
(msec)


RELATIVE INTENSITY
1.6
1.4
1.2
1.0
0.8
k = 10^2 sec^-1
k = 10^3 sec^-1
k = 10^4 sec^-1
0
10
20
30
TIME AFTER FLASH (msec)

flash period the delayed emission measured at the phosphorescence wavelength maximum is seen to immediately decay with excitation at 280 mμ. This occurs in both the high and the low P/D complex. No transient rise in the dye phosphorescence intensity is seen following pulsed uv excitation in spite of the fact that the steady state emission studies indicate that 80% of uv-induced dye phosphorescence arises from triplet-triplet transfer in the high P/D complex.

Theoretical curves for the phosphorescence behavior following a 17 msec. flash with light absorbed by the polymer are shown in Fig. 12. The curves in Fig. 12 are given by the following equation for the dye triplet population T_D, following pulsed excitation in the polymer.

$$T_D = \left[\frac{I_D}{k_D} + \frac{k\, I_p}{k_D(k_p - k_D)}\right](1-e^{-k_D t_f})\; e^{-k_D t} + \frac{k\, I_P}{k_p(k_p - k_D)}\,(1-e^{-k_p t_f})\,(e^{-k_D t} - e^{-k_p t}) \qquad (9)$$

where T_D = population of the dye triplet state, I_P and I_D are the rates of intersystem crossing in the dye and the polymer respectively, k_D is the rate constant for dye triplet state decay, and $k_p \equiv k_p' + k$ the rate constant for polymer triplet decay, which is given by the rate constant for polymer triplet decay in the absence of transfer, and the triplet energy transfer "rate constant" respectively, and t_f is the duration of the flash. The assumption has been made that the triplet energy transfer from polymer to dye can be represented with a single rate constant k*. The derivation of equation (7) for the triplet energy transfer

---

* The "rate constant" k for triplet energy transfer from polymer to dye is only equal to k_{t-t}, the jumping frequency from a nearest neighbor base to a dye, when either, 1) a dye is located at every base, or

2) base-base transfer is fast compared to base-dye transfer, so that the latter process (trapping) is rate limiting. If neither of these conditions are fulfilled, then k represents an "average rate constant" for transfer over a number of bases into a dye.

---

process is not presented here in that essentially the same equation has been published by Smaller et al. (59).

From a comparison of the experimental decay curve following pulsed 280 mμ excitation with the corresponding theoretical decay curves, the absence of a rising component in the dye phosphorescence following the flash period indicates that the rate constant for polymer to dye triplet energy transfer is greater than 10^2 sec.$^{-1}$ both in the high and low P/D complex. The failure to detect transfer in the low P/D case is not surprising in view of the observation of a nearest-neighbor triplet excitation jump frequency in benzene crystals of the order of 10^{12} sec.$^{-1}$. On the other hand, the finding that the transfer rate at high P/D is greater than 10^2 sec.$^{-1}$ indicates that a model for triplet energy migration in which the time available for diffusion of the excitation is the lifetime of the polymer triplet state in the absence of dye is not correct. A component of the transferred energy should have a rate constant 30 times slower than the slowest possible rate observed in these experiments. The conclusion that this simple diffusion model is incorrect has also been reached by Shulman, Rahn and Longworth, who found that on quenching with Mn^{++}, the lifetime of the residual DNA phosphorescence was not shortened (67).

Transfer rates are only measurable by flash spectroscopy when the flash period is the same order of magnitude or smaller than the reciprocal of the transfer rate, however for the flash period given by

$t_f = 1/k$ the maximum intensity of the signal is roughly 1/k of its steady state value. Shortening the flash period, therefore, must be compensated for by an increase in sensitivity or detection or by an increase in the excited light intensity.

The use of a flashlamp putting out for example 100 joules of energy in 10 μsec., on the assumption of equal efficiency of uv output as the 200 watt steady state lamp, should give 25 X as much dye phosphorescence as in the above experiment in spite of the shortened flash time. Such experiments could possibly be extended down to 10^{-8} sec with the use of lasers and frequency doubling. The principle disadvantage in the use of high intensity sources is that triplet annihilation within the polymer may drastically reduce the polymer triplet population from what is expected from low intensity measurements.

The sensitivity of detection might be increased by following the triplet ESR signal of the dye rather than the phosphorescence in view of the fact that the radiative lifetime of 9-aminoacridine appears to be longer than the radiationless lifetime resulting in a low phosphorescence efficiency. Another distinct advantage pointed out by Smaller et al. in the use of ESR is the ability to detect a single signal free from the interference which arises from overlapping emission bands. In addition, the problem of having to deal with a large signal during the flash period is eliminated.

A second alternative method of following the dye triplet state population as a function of time would be by triplet-triplet absorption, which would again have the possible advantage of yielding a unique signal free of interference. The technical problems of obtaining clear

glasses with long pathlengths would have to be overcome however.

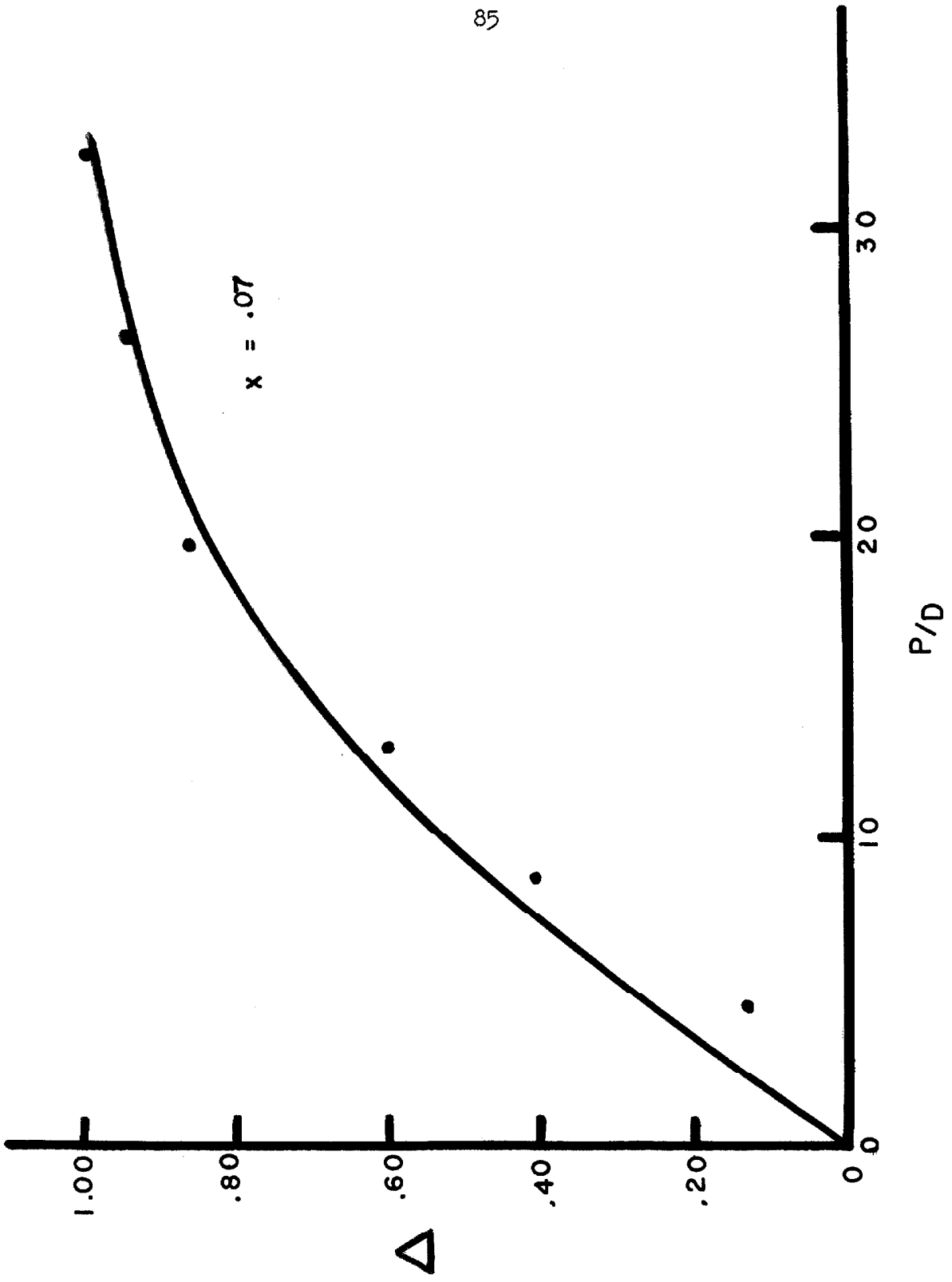
A Model Based on Trapping of Migrating Excitation

An alternate model for triplet migration in polymers is derived in Appendix III. The model is based on the assumption that there is trapping of the excitation within the polymer and that, in the absence of such trapping, the spread of excitation over the pathlengths of interest, either by a random walk or a coherent process, is rapid. According to the trapping model then, following absorption of a light quanta into the DNA, the excitation once in the triplet state migrates rapidly and is trapped within the polymer. Once in a trap the excitation may appear as phosphorescence or undergo a radiationless transition to the ground state but does not migrate. In the presence of dye or paramagnetic ion, quenching of the migrating polymer triplet reduces the probability of excitation reaching the traps, but does not shorten the lifetime of emission which does occur from the traps, as long as the migration time is short compared with the emission lifetime.

On the assumption that transfer of triplet excitation to dye, from bases adjacent to a dye, occurs efficiently, and that the probability of excitation being trapped at any one site is a constant, x, then the number of triplets contributing to energy transfer to a dye is given by equation (III-5). Equation (III-5) was adjusted to fit the experimental curve △ by computation with trial values of x, and is shown in Fig. 13. The fit to the experimental data is not impressive*, but it is

* The experimental curve appears somewhat sigmoidal. If one arbitrarily takes P/D = 3 as the origin, then the model gives a better fit to the experimental data. The sigmoidal shape to the curve may arise from

Fig. 13 The fit of equation (III.5) to the experimental data for the relative rate of triplet energy transfer per dye. The curve is calculated for x, the probability of trapping at any one site, equal to 0.07.


x = .07
Δ
1.00
.80
.60
.40
.20
0
0
10
20
30
P/D

as a result of singlet-singlet energy transfer at low P/D. The inter-relationships among the excited states of the polymer have been ignored, but it is obvious that in the event that singlet-energy transfer is 100% efficient from N bases, there will be zero triplet population in those N bases, and the Δ function will only have a non-zero value for $P/D > N$.

---

at least as good as the random walk model.

The model included here is presented, not because it is expected to be rigorous*, but simply to illustrate what is intuitively obvious, that migration may occur rapidly and be limited by trapping rather than by slow diffusion. The assumption that x, the probability of excitation being trapped on any base, is independent of N, is valid when trapping occurs with 100% efficiency at specific trapping sites. Then x is just the fraction of the sites (bases) which are traps. The model is not rigorous when trapping may occur at any site with an efficiency < 1.0. In that case, x must depend on the probability density distribution of the polymer triplet excitation and, in general, is not constant; for example, in a random walk process, allowing the excitation to hop back results in a higher probability density near the point of absorption and, therefore, a greater probability of being trapped on sites at that point. In this second case, it is apparent that the spread of excitation and trapping are intimately connected; "slow" transfer means a high probability density (at a given time, t), and, therefore, a high probability of trapping, near the point of absorption.

Triplet energy migration and trapping in DNA probably fall in this latter category, i.e., trapping can occur at a large number of

---

* Because of the assumptions used in the model, averaging over a random distribution of dyes was not undertaken.

sites with a low probability of trapping at any one site. Evidence has been presented indicating that the phosphorescence in DNA is emission from thymine in which the N- proton has been transferred to adenine (53). This proton transfer probably traps the excitation at that site. The probability of trapping the migrating triplet excitation at any one base (or base pair) may be just the probability of forming such a tautomeric structure.

Slow diffusion of the excitation in itself, then, does not give rise to the limit in the transfer pathlength. Due to heterogeneity in the molecule triplet energy transfer in DNA over 10 or more base pairs must involve tunneling and/or activation and, therefore, must be slower than transfer between identical molecules (monomers). Slow transfer probably plays an indirect role in increasing the probability of trapping.

We know that the "slow" transfer must be fast compared with the triplet lifetime. The question then of how fast is "slow" is an interesting one and is justification for extending the flash experiments to the μ sec region.

Transfer of excitation in DNA at temperatures above $0^{\circ}K$ is expected to involve activation as well as tunneling if the difference in the excitation energies of the bases is of the order of kT. A temperature dependence then is expected for the pathlength of the migration. Cooling the samples to $4^{\circ}K$ should result in a shorter pathlength than at $77^{\circ}K$.

TRIPLET ENERGY TRANSFER IN 9-AMINOACRIDINE-DENATURED DNA COMPLEXES

The phosphorescence and fluorescence intensities from 9-amino-

acridine in the denatured DNA complex were measured. Ratios of intensities were calculated and the results are shown in table IX.

Table IX

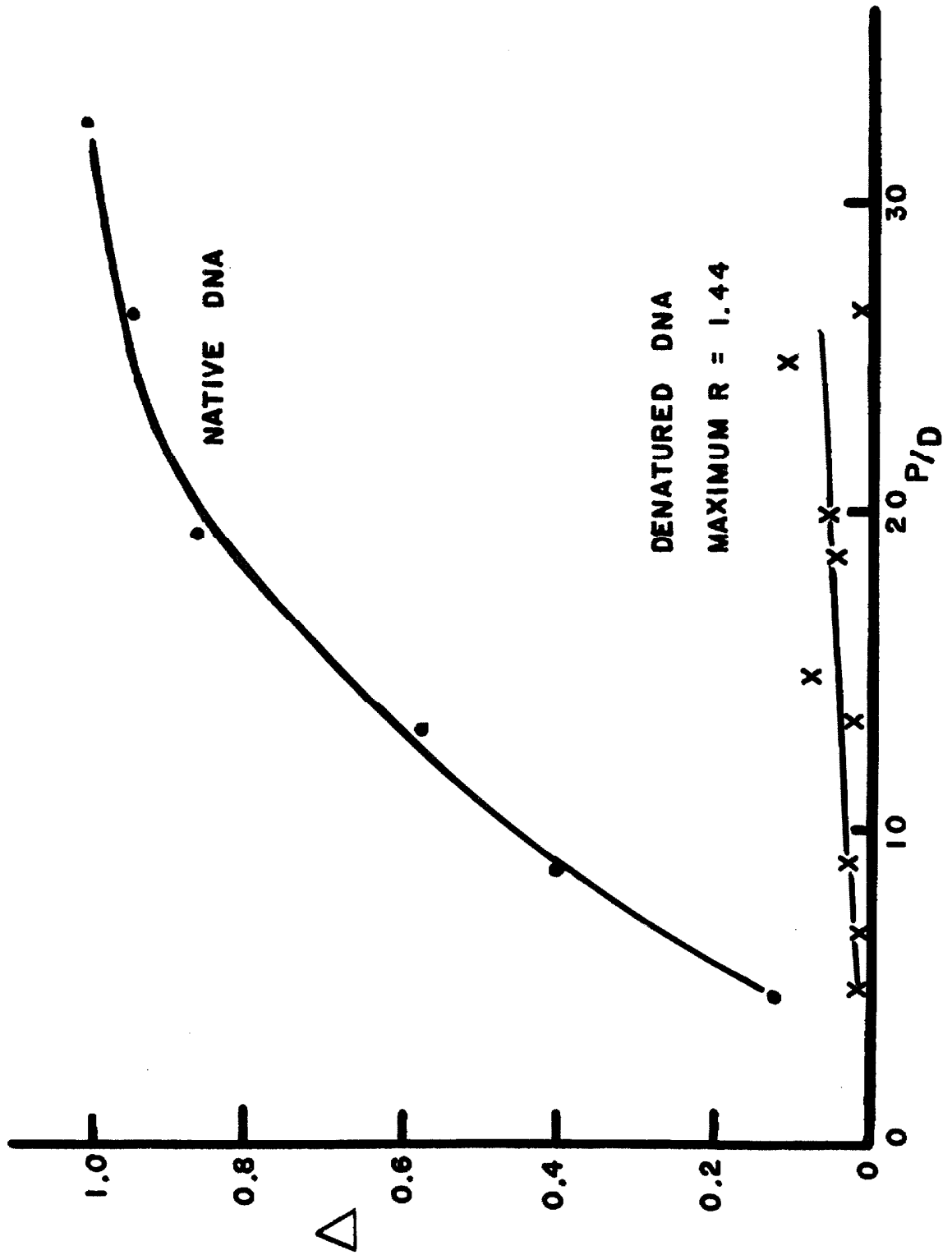
R Values for Denatured DNA

P/D	R
6.7	1.08
13.3	1.16
20	1.37

Polarization corrections were not made in this case. In view of the small corrections in the native case, however, the maximum R values given in table IX are not expected to be in error by more than 30% and clearly indicate that triplet excitation transfer from denatured DNA to 9-aminoacridine is at least an order of magnitude lower than in the native DNA complex. The fact that this decrease in R, relative to the native complex, is observed, in spite of a known enhancement in the total phosphorescence of DNA on denaturation (55), indicates that a structural change in the complex which makes triplet energy transfer less probable occurs on denaturation.

Values of Δ are plotted in Fig. 14 as a function of P/D for 9-aminoacridine bound to denatured DNA along with the average values of Δ for the native DNA-dye complex. As expected from the low maximum R value, there is very little absolute change in Δ with P/D. There appears to be a tendency for Δ to increase with P/D; however, the conclusion that triplet energy transfer from base to base is occurring

Fig. 14 Variation of the triplet energy transfer per dye with P/D for the 9-aminoacridine - denatured DNA complex.


NATIVE DNA
DENATURED DNA
MAXIMUM R = 1.44
Δ
1.0
0.8
0.6
0.4
0.2
0
10
20
30
P/D

in denatured DNA is not warranted on the basis of the low uncorrected R or Δ values.

The failure to observe triplet-triplet energy transfer to the dye could arise as a result of an unfavorable relationship between the bound 9-aminoacridine and the purines and pyrimidines of denatured DNA, or it could be due to the failure of a number of bases to contribute triplet energy to a dye, which would be expected if triplet energy transfer between bases is not possible in denatured DNA. In the latter case, the efficiency of energy transfer into the dye should be equal in the native - and denatured DNA - dye complexes at very low P/D. At P/D=5 the difference between the R values in the native and denatured DNA complex appears to be significant. However, at lower P/D ratios the errors become larger than the small difference which is being looked for, and it is not possible to decide between the above alternatives.

While quenching of the phosphorescence of denatured DNA by Mn^{++} has been observed (55), it has not been reported whether the relationship between the degrees of quenching and binding indicate delocalization of the triplet excitation. If that experiment indicates delocalization of the excitation, then the low R values seen here could only result from a difference in the structural relationship of dye to bases in the native and denatured DNA complexes. Evidence suggesting that the latter interpretation is correct will be presented in a later section which deals with the P/D dependence of delayed fluorescence arising by transfer from the triplet state of the polymer.

TRIPLET DNA-TRIPLET DYE ENERGY TRANSFER AND DYE BINDING

Many of the difficulties encountered in determining quantum efficiencies of emission as a method of demonstrating energy transfer have been eliminated in this work by measuring only ratios of emission intensities. While the large relative enhancement in uv-induced dye phosphorescence clearly demonstrates the presence of triplet-triplet energy transfer in native DNA the actual base-dye transfer efficiency is not obtained. The quenching of the DNA phosphorescence is not informative in this regard in that it does not specifically demonstrate transfer at the triplet level. The flash experiments, however, provide a lower limit for the energy transfer rate constant, i.e., $>10^2 sec^{-1}$.

On the bases of the evidence presented for transfer of triplet energy from the purines and/or pyrimidines of native DNA to bound 9-aminoacridine it can be concluded that there is some degree of overlapping of the π-electron clouds (wavefunctions) of the acridine dye with the bases of native DNA. In view of the orbital overlap requirement for triplet-triplet energy transfer with its strong intermolecular distance dependence, the presence of base-dye triplet energy transfer can be taken as evidence for association of the dye with the purines and pyrimidines of native DNA as opposed to binding at the phosphate groups on the outside of the helix.

Binding of the dye at the phosphate groups would involve a separation of ~ 9 Å between the peripheries of the dye and bases. In view of the present uncertainties in the calculation of atomic orbitals far from the nuclei (35),(68), it is not possible to obtain an estimate of the interaction over such distances. While "active radii" of 12 Å

for triplet-triplet energy transfer between hydrocarbons in rigid glasses have been reported (69),(70), it has been pointed out that a model based on the concept of an active radius breaks down for large concentrations of planar molecules (70) as a result of the shape and relatively large size (25) of the molecules, and the uncertainty in assigning a statistical distribution of intermolecular distances. The active radius represents a maximum value for the distance between centers of the molecules, and the actual distances are probably less. The independence of the transfer radii on the nature of the solvent argues in favor of the premise that the molecules involved in transfer are in essentially Van der Waals contact (70). For a donor molecule at a distance from an acceptor equal to the transfer radius the probability of intramolecular decay is supposedly equal to the probability of transfer. In the native DNA-9-aminoacridine complex the rate of transfer is > 30 x the rate of radiationless decay in the absence of dye. Furthermore this minimum for the transfer rate includes transfer through a number of non-identical bases.

In addition to the large distance between base and dye in an external binding model, a phosphate and sugar group intervene, and transfer would have to take place through these groups. It is considered highly improbable that an indirect interaction through the virtual states of these groups can occur.

While transfer of triplet excitation through virtual states of host molecules has been observed in π-electron systems (40), the triplet energy levels of the host and guest in such systems are separated by only ~ 200 cm^{-1}. In DNA the intervening states between the π-electron

systems of the base and dye bound at the phosphate group, would be excited triplet states of a number of saturated bonds. The difference in excitation energies ΔE of the dye (or base) and these excited states is expected to be $> 20{,}000\ cm^{-1}$, and there would be a minimum of 6-7 bonds between a base and a dye located at a phosphate group. In view of the presence of the term ΔE^{-N} in equation (3) for the energy of interaction through N hosts, it is apparent that both these factors contribute to trapping the excitation in the bases. It is impossible to make reliable estimates of the base-dye interaction matrix element β_N from equation (3) in this case as it is difficult to estimate the trap-host ($\beta_o f_o$) and host-host ($\beta_o' f_o'$)interaction matrix elements. If they are as high as $200\ cm^{-1}$, then for N=7, $\beta_N = 2 \times 10^{-12} cm^{-1}$. For resonance interactions, the average time for trap to trap transfer is given by:

$$\tau_N = \frac{h}{4\beta_N}$$

and for $\beta_N = 2 \times 10^{-12} cm^{-1}$, $\tau_{N=7} = 1$ sec. However, as the interaction β_N is small in comparison with molecular vibrational-lattice coupling ($\sim 1\ cm^{-1}$) the base-dye transfer would occur irreversibly, and therefore the transfer rate will be even slower, varying with β_N^2 rather than β_N.

In addition it has been shown experimentally that triplet transfer between hydrocarbon molecules through host molecules for which ΔE is greater than $1{,}200\ cm^{-1}$ does not occur (70).

It must be pointed out that the quenching of the phosphorescence of DNA by Mn^{++} introduces an element of uncertainty in this regard. It has been demonstrated by NMR that Mn^{++} binds primarily to the phosphate groups of DNA (81) and yet it is effective in quenching the phosphorescence. For the reasons given above the possibility that the quenching

occurs by excitation transfer through a sugar and phosphate group seems rather remote, but this piece of evidence cannot be ignored and requires explanation..

Finally the observation that denaturation of the DNA results in a marked decrease in the base to dye transfer in spite of a 4 fold increase in the polymer phosphorescence (55) argues in favor of a base-dye association. It has been pointed out that on the basis of the evidence presented so far it is not possible to determine whether an increased nucleotide-nucleotide or an increased nucleotide-dye separation is responsible for the decreased transfer efficiency in the denatured complex. However denaturation results in a maximum nucleotide-nucleotide separation of $\sim$11 Å. Therefore it is apparent that intermolecular distances of these sizes are sufficient to markedly decrease the transfer efficiency. The argument is more convincing when it is realized that at 77°K the denatured molecule is not expected to be fully extended and the average distance between bases is less than 11 Å. Evidence will be presented in a later section suggesting that base-base triplet energy transfer does occur in denatured DNA. By default, then, this means that 9-aminoacridine bound to denatured DNA is not as closely associated with the purines and pyrimidines as with native DNA. Therefore it is possible to have binding without triplet transfer.

While the data, then, indicate an association of the acridine dye with the bases of native DNA, it is not possible to determine the extent of the association. The data are consistent with a model in which the dyes are intercalated in the native structure, but a model in which only partial overlapping of the acridine rings with the bases

occurs (17) cannot be excluded.

TRIPLET ENERGY TRANSFER IN POLYNUCLEOTIDES

Poly (A+U) and Poly (A+2U)

It was anticipated that in the native DNA molecule the extent of delocalization of triplet excitation could be due in part to "slow" energy transfer from base to base, which increases the probability of the excitation being trapped close to the point of absorption. The slow transfer is expected by virtue of the heterogeneity of the polymer. In copolymers of the poly (A+U) type, this heterogeneity is removed at least in the individual strands, and it is expected that the pathlength for the excitation (number of bases transferring energy to a dye) should be increased. Complexes of 9-aminoacridine with poly (A+U) and poly (A+2U) were investigated, therefore.

Mixtures of equimolar amounts of poly rA and poly rU showed an absorption at 260 mu which was 70-75% of that expected from the sum of the absorptions of the individual homopolymers in the 1:1 glycerol-H_2O solvent at $\sim 0^{\circ}C$, indicating a high degree of complex formation. Absorption measurements were not made in the presence of dye in view of the strong absorption of the dye at 260 mμ. At $77^{\circ}K$ the phosphorescence of poly A was quenched on formation of the hydrogen bonded complex, confirming the earlier observations of quenching of poly A emission on complexing with poly U (71). The quenching was not complete in this case, however, with about 5-10% of the poly A phosphorescence remaining. The degree of quenching is estimated from experiments carried out at different times and was not determined in a single critical experiment and is, therefore, subject to considerable uncertainty. The residual emission was completely quenched on formation of the low P/D complex

with 9-aminoacridine.

The phosphorescence and fluorescence intensities from 9-aminoacridine in the poly (A+U) complex were determined and ratios of intensities calculated in the usual way. The behavior of Δ with P/D is shown in Fig. 16 along with the maximum R value. The relative "intensity" of triplet polymer to triplet dye energy transfer, expressed as R, is seen to be enhanced at least 4 times over its value in the native DNA complex. The R values at P/D $<$ 20 are similar to those for DNA; the larger maximum R, therefore, arises as a result of the increased number of bases in the poly (A+U) helix which can transfer energy to a dye. This is seen from the increase in Δ which only saturates at a P/D = 80-100, rather than at P/D = 25 as in native DNA.

It has been shown that in ethylene glycol H_2O and 10^{-2} F salt the 2:1 complex poly (A+2U) is formed (72). It has been pointed out that in 1:1 mixtures of poly A and poly U the 2:1 complex plus free poly A may be present (73). The quenching of the poly A phosphorescence to 10% on complex formation with poly U rather than to 50% argues against this possibility, but cannot be taken as <u>proof</u> that free poly A is not present.

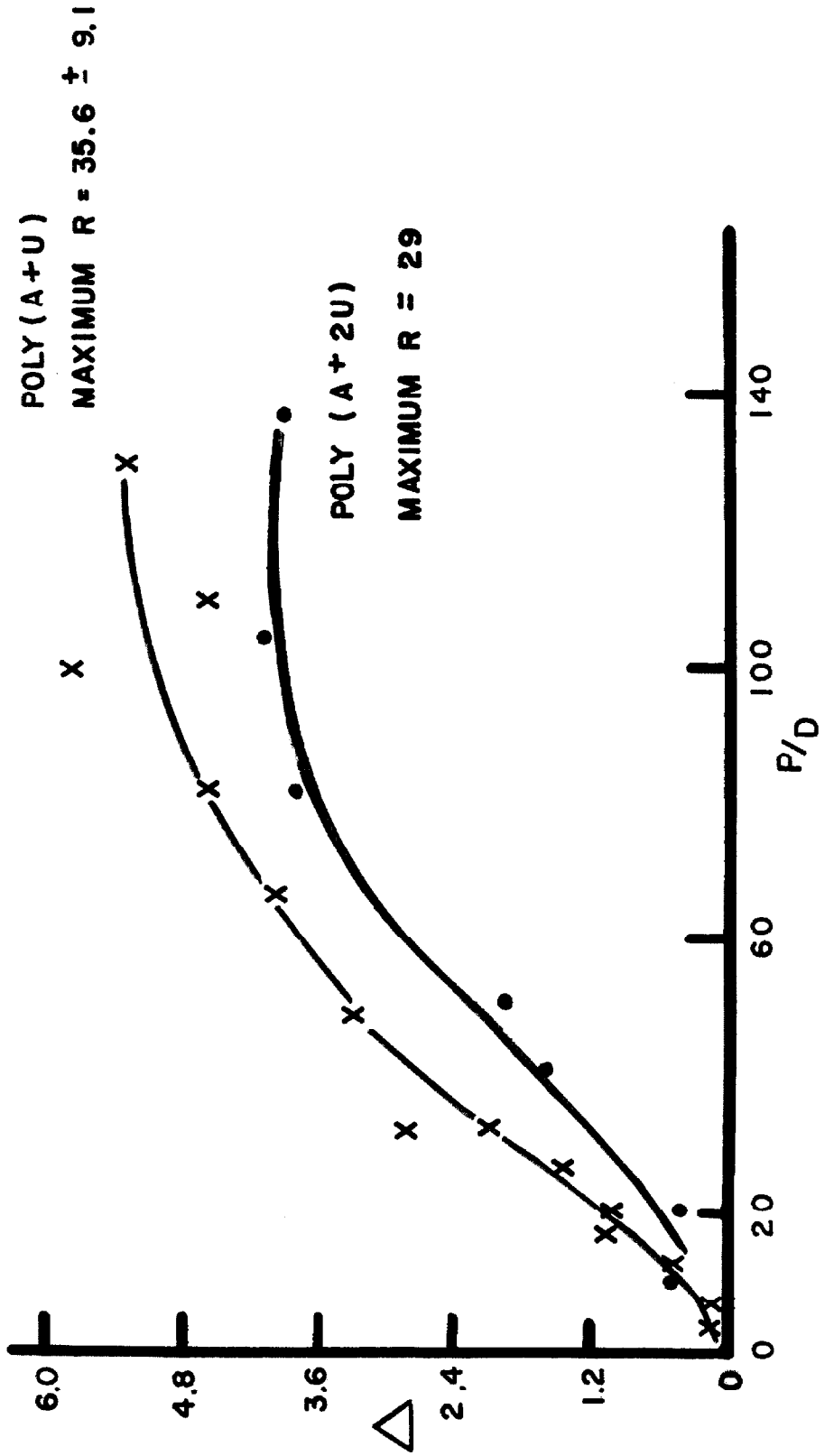
Solutions containing 2 parts poly U to 1 part poly A were made. In the absence of dye, the solutions showed about a 20% hypochromism at 257 mμ at $\sim 0^{\circ}C$. The degree of polymer emission at $77^{\circ}K$ was variable, and it should be pointed out that <u>convincing evidence</u> as to the degree of complex formation and the nature of the complexes in both these cases has not been presented.

Emission intensities from 9-aminoacridine bound to the "poly

(A+2U) complex" were determined and the resulting Δ and maximum R values are included in Fig. 16. The results are seen to be very similar to those obtained in poly (A+U). On the basis of the 20% hypochromism seen in these solutions in the absence of dye it appears unlikely that free poly A is present in these solutions, and, therefore, indicates that triplet energy migration from base to base is occurring in either the 2-stranded structure or the 3-stranded structure or both. In the absence of reliable absorption data, indicating a sharp minimum in the absorption at either 50% or 67% poly U in the solvent used, it is not possible to decide between these alternatives or, even, whether complexing is complete (in view of failure to find complete quenching of the emission). The uncertainty in the degree of complexing of poly A to poly U arises because of the low salt (10^{-3}F) which is known to decrease the melting temperature of the complex (74). These uncertainties in the data are still to be completely resolved.

The question of triplet energy transfer in poly (A+U) is important in understanding the photochemistry of the poly (A+U) complex and is of theoretical interest. It is not as useful as a model compound for understanding triplet energy transfer in DNA, as are the poly dAT complexes. Comparison of the amount of triplet energy transfer to 9-aminoacridine as a function of P/D for the alternating crab dAT, and the synthetic non-alternating copolymer promises to be an interesting experiment. Triplet energy transfer in the helix in the latter case, where the monomers are identical in a single chain, should be more efficient than base-base transfer in the former case where tunneling of the excitation through a barrier would have to occur.

Fig. 16 The dependence of the amount of triplet energy transfer per dye (9-aminoacridine) on P/D for Poly (A + U) and Poly (A + 2U). The values of Δ are expressed relative to the maximum value in native DNA.


POLY (A+U)
MAXIMUM R = 35.6 ± 9.1
POLY (A+2U)
MAXIMUM R = 29
6.0
4.8
3.6
2.4
1.2
0
Δ
0
20
60
100
140
P/D

Poly A-Acridine Dye Complex

Accurate determination of the uv-induced phosphorescence and fluorescence intensities from 9-aminoacridine is made more difficult in the poly A complex by the appearance of phosphorescence from the polymer. This can be seen from the spectra in Fig. 17. The phosphorescence appears not only in the delayed emission spectrum* (Fig.17) but the 416 mu peak can also be seen in the uv-induced fluorescence spectrum. Correction for this emission, however, should be fairly reliable in that one is more certain of the shape of the underlying emission. Emission from the dye was measured and the R and Δ values were calculated and are given in Fig. 18. The large maximum R value clearly indicates that triplet energy transfer can occur from the bases in a homopolymer to bound dye. The large enhancement in the uv-induced dye phosphorescence arises in part from the fact that energy transfer occurs from base to base as evidenced by the fact that Δ saturates only at a P/D $\sim$ 70. On the other hand, at P/D $<$ 20 the R values are $>$ 2 times the corresponding values in native DNA indicating a higher efficiency of transfer to dye in poly A. This may result from the higher population of the triplet state in poly A.

The observation of base-base triplet energy transfer in poly A is in agreement with the original observations of Bersohn and Isenberg (52). The pathlength of energy transfer in these experiments appears

* Delayed fluorescence from the dye contributes to the emission in the 400-500 mμ region. The dye fluorescence peaks at 437 and 460 mμ coincide with the poly A phosphorescence peaks at 438 and 464 mμ. It is evident from the width of the 437 mu band and the ratio of the height of the 437 to 413 mμ peaks in Fig. 17, in comparison with the spectra in Fig. 5 in the absence of dye, that all of the emission in the 437 mμ band is not poly A phosphorescence.

Fig. 17 Emission Spectra of the 9-Aminoacridine - Poly A Complex. Poly A concentration = 5.2×10^{-4} M.

(a) Delayed emission.

1 - λ_{ex} = 405 mμ

2 - λ_{ex} = 280 mμ, rel. gain = x 0.1

Lower spectrum = - x 0.05

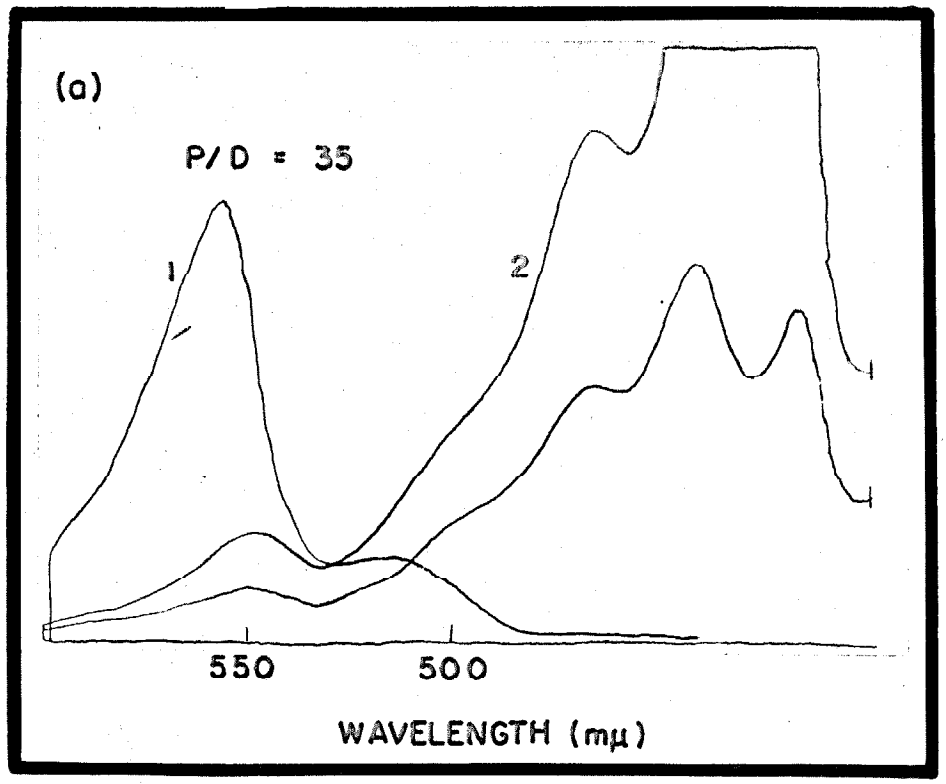
(b) Total emission.

1 - λ_{ex} = 405 mμ

2 - λ_{ex} = 280 mμ, rel. gain x 10

The gain of spectrum (a) -1 relative to (b) -1 is

The reader can verify that there is a large selective enhancement in the uv-induced dye phosphorescence. The uv-induced fluorescence intensity is seen to be about 4% that of the fluorescence excited with visible light. On the other hand, uv-induced phosphorescence is of the order of 1.5 x the visibly excited phosphorescence intensity, a ratio 40 x the fluorescence ratio. (Calculated R = 41)


(a)
P/D = 35
1
2
550
500
WAVELENGTH (mμ)

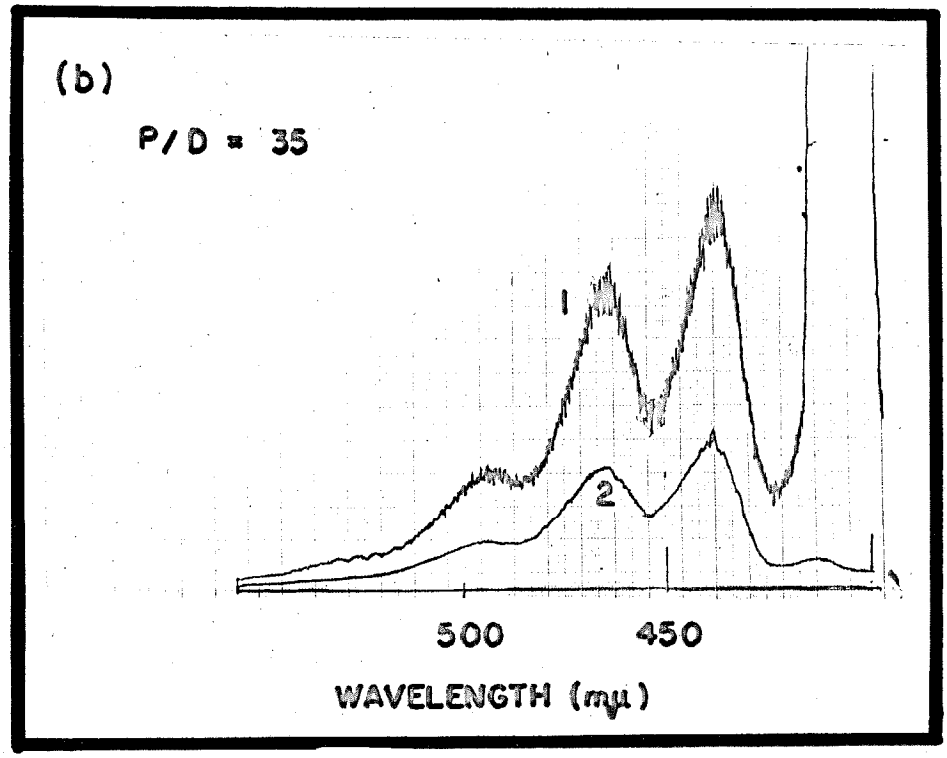
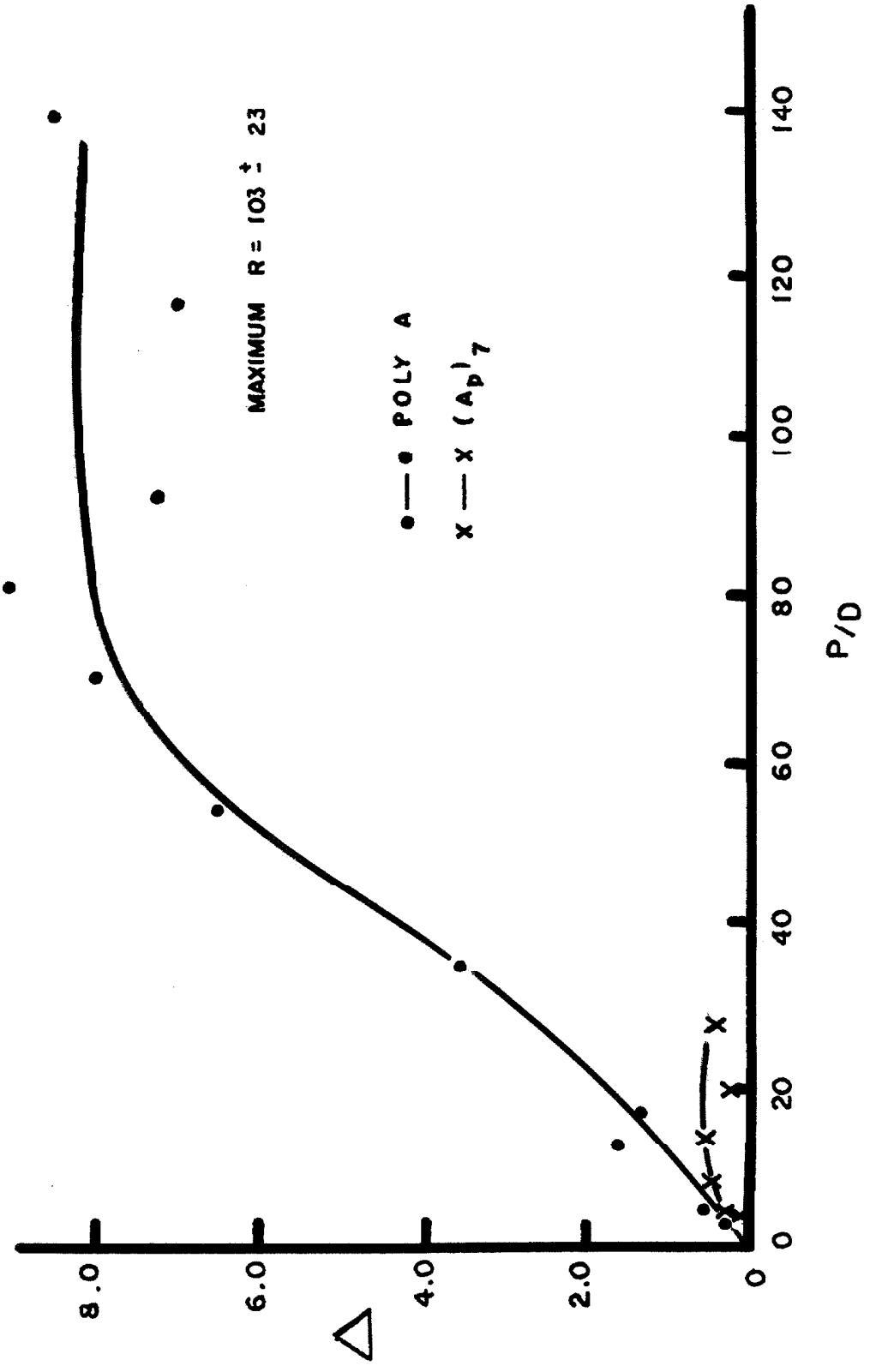

(b)
P/D = 35
1
2
500
450
WAVELENGTH (mμ)

Fig. 18 Triplet energy transfer per dye (9-aminoacridine) for the Poly A and $(A_p)_7$ complexes. The Δ values are expressed relative to the maximum value in native DNA. Poly A concentration = 5.23×10^{-4} M, $(A_p)_7$ concentration = 1.8×10^{-3} M.


MAXIMUM R = 103 ± 23
● — ● POLY A
X — X (Ap)7
Δ
8.0
6.0
4.0
2.0
0
0
20
40
60
80
100
120
140
P/D

to be $\frac{1}{2}$ that found in the quantitative work of Eisinger and Shulman (56). This difference may arise as a result of a shorter polymer length in the poly A, as Eisinger and Shulman have shown that polymer length limits the pathlength below S=4.

The availability of oligo-A with a degree of polymerization of 7, $(Ap)_7$, provides a critical test of the base-base energy transfer model, in that, with increasing P/D, triplet energy cannot be transferred to a dye from more than 7 bases. The Δ values for the 9-aminoacridine $(Ap)_7$ complex are included in Fig. 18, and it is apparent that the expected limit occurs. The errors in determining the intensity of uv-induced dye phosphorescence are very large in these samples because of this limit, i.e., the uv-induced dye phosphorescence is not enhanced, and interference from the polymer phosphorescence is more marked, above a P/D = 5-10. One can reliably conclude, however, that, a) triplet energy transfer to dye occurs, and, b) the amount of transfer per dye does not increase beyond P/D = 10.

It was indicated earlier that triplet energy transfer from native DNA to acridine orange could not be detected and that this failure could be due in part to a higher intersystem crossing efficiency in acridine orange. On the basis of p_{vis}/f_{vis} ratio the R value was expected to be at least 1/10th of the value for 9-aminoacridine. In the presence of poly A where the maximum R with 9-aminoacridine is equal to 100, the uv-induced phosphorescence in acridine orange should be selectively enhanced ~ 10 X. Several samples of poly A - acridine orange at high P/D were observed. The R value ranged from 5-9. It is evident then that triplet energy transfer to acridine dyes other than 9-aminoacridine can

be observed. Without an accurate measure of the relative intersystem crossing efficiencies in these two dye molecules, the possibility that the lower R values are the result of a lower efficiency of triplet energy transfer to acridine orange, rather than simply a decreased sensitivity of detecting triplet-triplet transfer due to a higher intersystem crossing efficiency, cannot be excluded.

Quenching of the Poly A Emission

The occurrence of significant intensities of phosphorescence and fluorescence from poly A (neutral) makes it possible to follow the reciprocal events in the polymer in this case. While selective quenching of the polymer phosphorescence does not prove that triplet energy transfer is occurring, the presence of a significant amount of triplet energy transfer must be accompanied by a decrease in the ratio of polymer phosphorescence to fluorescence. That this is in fact occurring can be seen from the spectra of part of the total polymer emission seen in Fig. 19. The poly A phosphorescence peaks at 390 and 413 mμ are seen to decrease relative to the poly A fluorescence centered at ~ 340 mμ with decreasing P/D. The emission goes off scale above 425 mμ dye to the presence of dye fluorescence. No marked decrease in the poly A fluorescence occurs for P/D > 10, however it is apparent from Fig. 19 that for P/D < 5.0 a significant quenching of the poly A fluorescence occurs, presumably due to singlet energy transfer to the 9-aminoacridine. The fluorescence intensities were determined from the total emission spectra and the poly A phosphorescence intensities with the phosphoroscope*.

* Poly A phosphorescence was measured as the height of the 413 mμ

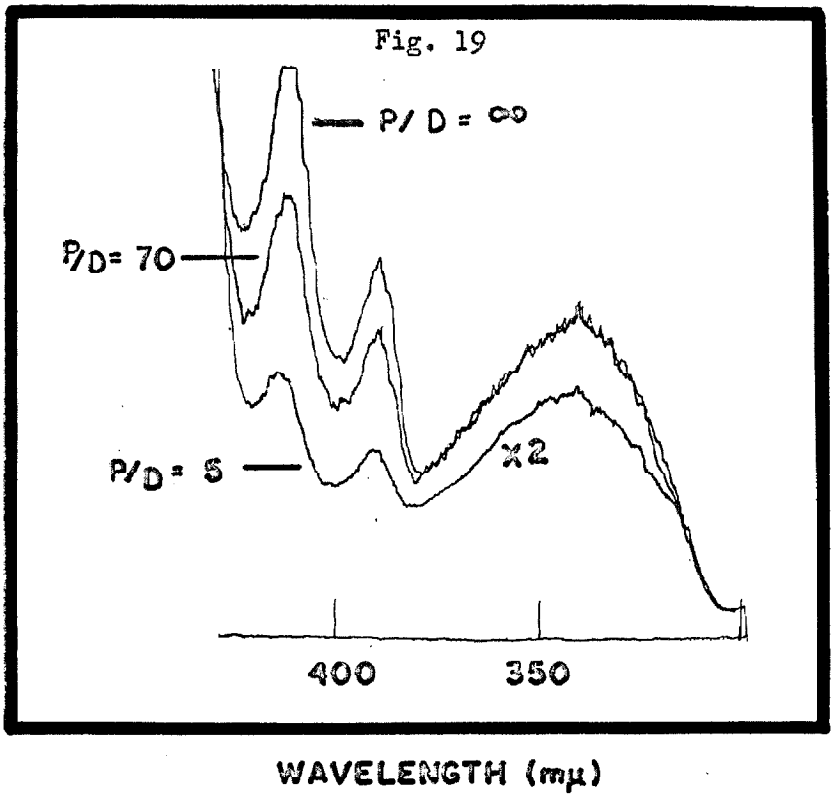


Fig. 19 Part of the Total Emission from the 9-amino-acridine - Poly A Complex.

Poly A concentration = 5.2×10^{-4} M.

peak where delayed fluorescence from the dye does not contribute to the emission.

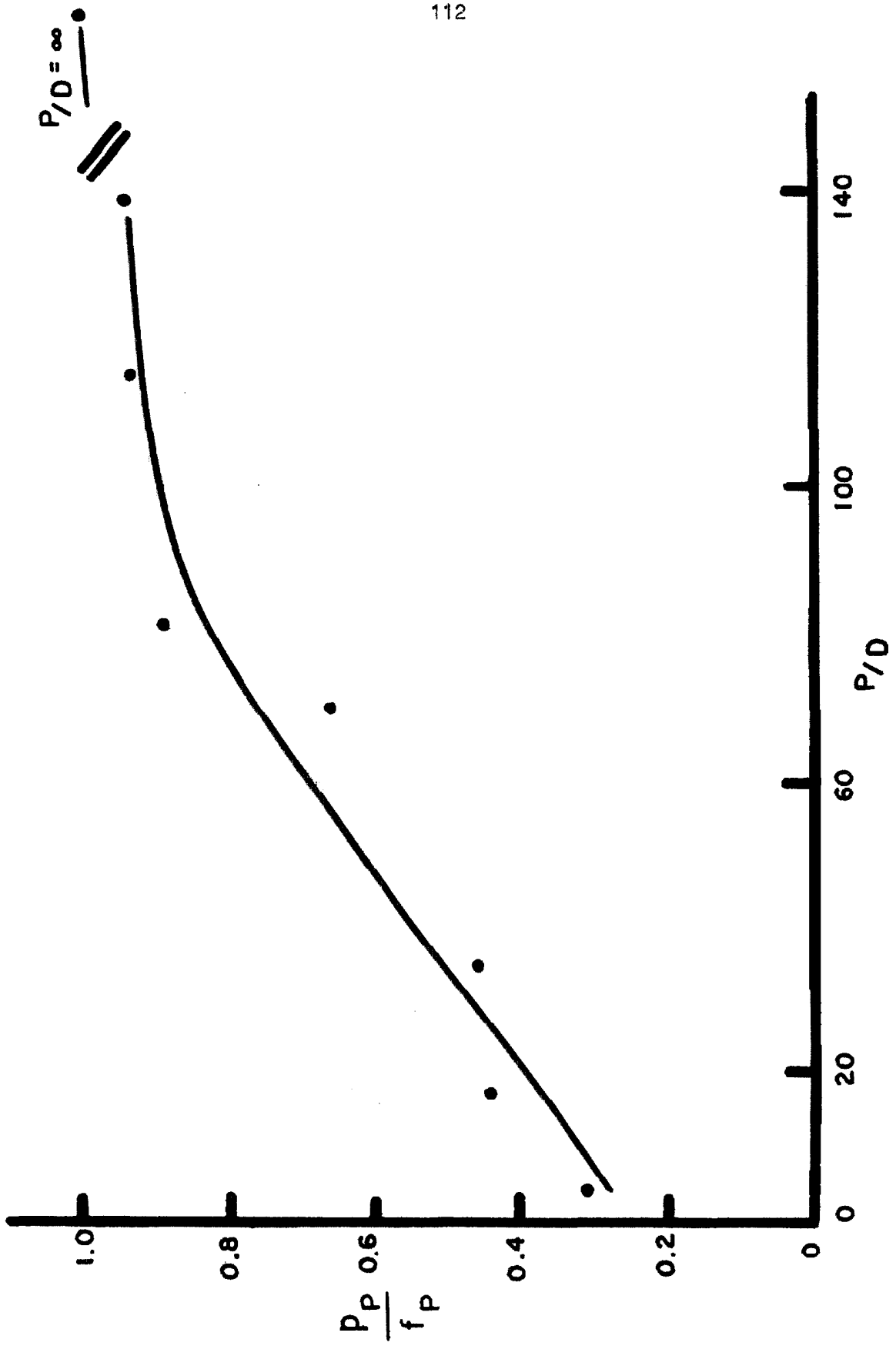
The resulting ratios are plotted in Fig. 20 as a function of P/D, and the results are seen to be complimentary to the result on enhancement of the dye phosphorescence. The p/f ratio is reduced to $\frac{1}{2}$ its maximum value at a P/D = 40-50. This, again, is considerably less than the value of 1/r for half quenching observed by Eisinger and Shulman for poly A (S=11). The observation that the p/f ratio is only reduced to 0.3 times its maximum value at a P/D = 4* is somewhat disturbing. This may arise from cooperative binding (preference for nearest neighbors) of the 9-aminoacridine to the poly A at low P/D. The emission that is observed from DNA and poly (A+U), where nearest neighbor interactions are less favorable (see later section), appears to be completely quenched to the level of glycerol emission at low P/D. The results, due to the low emission, however, are not conclusive. An alternate explanation of the above result is that dyes which bind at low P/D are not as readily available for triplet energy transfer from the poly A. The emission of poly A at P/D values lower than 4 has not been examined as yet.

The lifetime of the poly A phosphorescence (2.1 sec.) is not significantly affected by quenching to 10% of its maximum intensity. This is in agreement with the observation made by Eisinger and Shulman (56) on quenching with paramagnetic ions, and, as pointed out by them, illustrates that the diffusion model for excitation migration cannot be correct in poly A. They have stated that their data fit a model derived

---

* The poly A phosphorescence intensity is quenched to about 10% of its maximum value at P/D = 4, but this in part arises from quenching at the singlet state.

Fig. 20 Selective quenching of the poly A (neutral) phosphorescence in the presence of 9-aminoacridine.
Poly A concentration = 5.2×10^{-4} M.


P/D = ∞
1.0
0.8
0.6
0.4
0.2
0
$\frac{P_P}{f_P}$
0
20
60
100
140
P/D

by R. Bersohn in which the pathlength of migration is limited by barriers. Details of this model have not been published as yet.

9-Aminoacridine - Poly U Complex

Phosphorescence and fluorescence from 9-aminoacridine in the poly U - dye complex were determined and the intensity ratios are given in table X.

Table X

Triplet Transfer Data in Poly U-9-Aminoacridine

P/D	P_{vis}/f_{vis}	R
5.4	3.5×10^{-4}	2.20
21	5.5×10^{-4}	1.14
84	9.3×10^{-5}	1.26

In the poly U complex, R values at low P/D appear somewhat lower than at high P/D. This is probably a complication arising out of the fact that there is not a single species of dye molecule present.

The low R values indicate that essentially no triplet energy is transferred from polymer to dye. From a comparison of the maximum R-1 values in the poly A and poly U complexes triplet energy transfer to dye is at least $\frac{100}{0.2}$ = 500 times lower in poly U than in poly A.

The failure to observe triplet energy transfer in poly U is not unexpected. There are a number of observations which would lead to such a prediction: 1) phosphorescence efficiency of poly U is very low, and the failure to detect a triplet ESR signal from poly U (72) indicates that the low efficiency arises as a result of a short non-radiative life-

time, rather than a long radiative one. This in itself, however, does not prove that energy transfer from the triplet state of poly U cannot occur. In view of rapid transfer times found in organic crystals and the fact that triplet energy can be transferred to dye or trapped in the polymer, in DNA and poly A, in a time which is short compared to the triplet lifetime indicates that transfer to dye cannot be ruled out, a priori. 2) Poly U is known to exist in solution, at room temperature, as a random coil (75) and absorption (76) and ORD data (77) indicate that the bases are not stacked. Triplet energy transfer from base to base, then, would undoubtedly be impossible. It is not known, however, whether the bases in poly U are stacked or not at 77°K in 50% glycerol. 3) The lack of a red shift in the dye fluorescence spectrum on binding to poly U suggests that Van der Waals interactions between pyrimidine and dye may be weaker than between purine and dye, and, therefore, the dye is not as intimately associated with the pyrimidine. The failure to observe triplet polymer to singlet dye energy transfer (uv-induced delayed fluorescence at high P/D) tends to support the first two mechanisms over the third in that T-S transfer should not require the close association of dye to base. The possibility exists that all three factors may be cooperating to make triplet polymer to triplet dye transfer inefficient.

Triplet Transfer to Dye as a Probe for the Polymer Triplet State.

Following triplet energy transfer in DNA and polynucleotides by the observation of triplet energy transfer to a dye (trap) compliments the experiments on quenching of the polymer phosphorescence with para-

magnetic ions, and conclusively establishes the presence of triplet excitation migration in DNA and some polynucleotides. The dye method has the disadvantage that singlet energy transfer from the polymer and dye-dye interactions can distort the shape of the experimental transfer curves. On the other hand, the dye method has the advantage that the energy that is transferred to dye migrates, on the average, over an increasing number of bases with increasing P/D, and, therefore, measurements on the rate of appearance of phosphorescence in the dye by fast kinetic experiment should yield information on the rate of base-base transfer. The quenching technique does not promise to be useful in this regard. It is difficult to study the quenching of polymer emission when the efficiency of polymer phosphorescence is very low. Initial observations on the acid form of poly A in which the phosphorescence and fluorescence are markedly quenched indicate, however, that triplet energy transfer to dye is almost $\frac{1}{2}$ as efficient as in neutral poly A, and that long range base-base transfer can be detected. Therefore it is apparent that it is possible to study the delocalization of triplet excitation in polymers in which emission is not observed. This is, of course, not surprising to the photochemists, who know that molecules which do not phosphoresce at room temperature due to their short radiationless lifetimes, can nevertheless transfer energy and carry out photochemistry through the triplet state efficiently in the short triplet lifetime.

Following base-base transfer by monitoring the intensity of delayed fluorescence (see following section) may prove more generally useful than the observation of triplet-triplet transfer in that the experiments are easier to carry out, and the results do not depend on the

availability of an acceptor with a low intersystem crossing efficiency.

TRIPLET DNA - SINGLET DYE ENERGY TRANSFER

It was indicated that the observation of uv-induced delayed fluorescence from acridine dyes bound to DNA (32) has been confirmed in the case of acridine orange and has also been observed in 9-amino-acridine. On the assumption that the delayed fluorescence does arise as the result of a triplet DNA to singlet dye transfer, then the kinetic analysis in Appendix II gives for the intensity of delayed fluorescence relative to the normal dye fluorescence induced with visible light:

$$\frac{f_d}{f_{vis}} = \frac{k_{t\text{-}s} T_p'}{I_d} \qquad (II.15)$$

where f_d is the intensity of delayed fluorescence (the other symbols are the same as defined earlier). If it is assumed that the population of polymer triplet that contributes to the triplet-singlet transfer process (T_p'), is the same as that which contributes to triplet-triplet transfer (T_p), then f_d/f_{vis} should have the same P/D dependence as the $\triangle$ function for triplet-triplet energy transfer. This provides a test then of the interpretation of the uv-induced fluorescence as arising from the triplet state of the DNA.

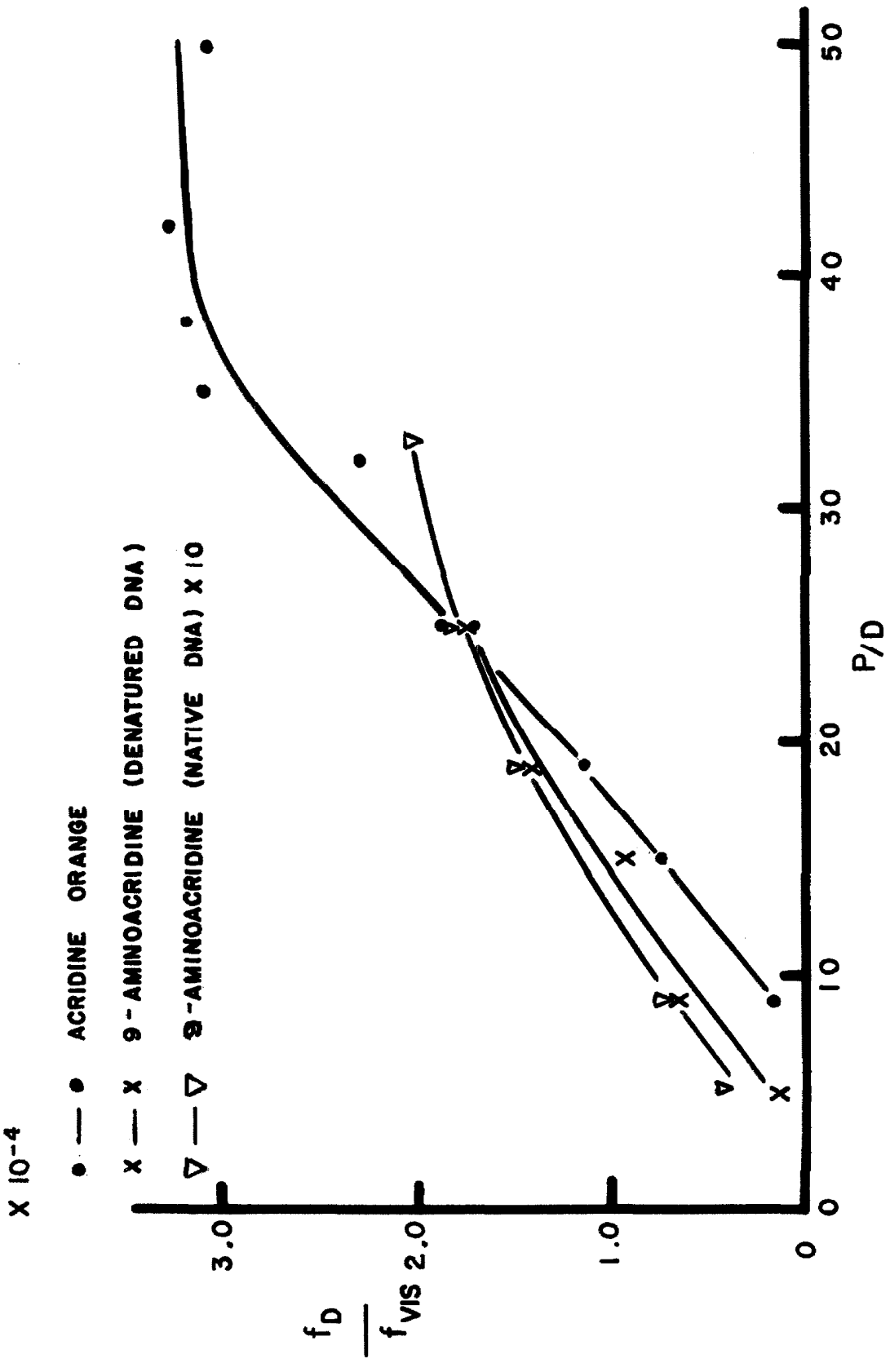
The intensity of delayed fluorescence from 9-aminoacridine cannot be reliably measured because of the complexity of the spectrum in the region in which it appears. Estimates of the intensity were made, however, from peak to saddle differences. The results are probably good to ±50%. The intensities were normalized by the corresponding intensities of normal fluorescence induced with visible

light and the results (f_d/f_{vis}) are plotted as a function of P/D in Fig.20(a). The f_d/f_{vis} ratio is seen to increase with P/D and there appears to be a tendency for the curve to saturate. In view of the errors involved in determining f_d the same experiment was carried out with acridine orange and native DNA. The fluorescence of acridine orange appears at a wavelength in which there is no interference from glycerol and DNA emission. The f_d/f_{vis} values are included in Fig.20(a). The relative increase in f_d/f_{vis} with P/D is the same as with 9-amino-acridine but saturation appears to occur at a somewhat higher P/D. The result clearly indicates that the delayed fluorescence is not arising from photo-ionization and recombination due to excitation to the higher excited singlet state of the dye. In that case f_d/f_{vis} would vary with P/D as f_{uv}/f_{vis}; that is, it would remain constant. The results indicate that the delayed fluorescence is arising from the triplet state of the DNA, confirming the interpretation of Isenberg et al. (32).

The possibility that the transfer takes place, however, via the radiative field, phosphorescence from the DNA being reabsorbed by the dye, has not been excluded. With constant DNA concentration, in the presence of triplet DNA-triplet dye transfer (quenching), the DNA phosphorescence should have the same P/D dependence as Δ, i.e., it should become constant at high P/D. If the experiment were performed at constant dye and increasing DNA, with radiative transfer, the f_d/f_{vis} should not saturate but continue to increase with P/D because the DNA phosphorescence would continue to increase. That experiment has not been performed.

The ratio of delayed fluorescence to normal fluorescence was

Fig. 20(a) The P/D dependence of the intensity of uv-induced delayed fluorescence in the acridine orange - and the 9-aminoacridine - DNA complexes. The absolute values for the 9-aminoacridine - native DNA complex are plotted on the same scale but are a factor of 10 lower.


X 10⁻⁴
• — • ACRIDINE ORANGE
X — X 9-AMINOACRIDINE (DENATURED DNA)
▽ — ▽ 9-AMINOACRIDINE (NATIVE DNA) X 10
f_D / f_VIS
3.0
2.0
1.0
0
0
10
20
30
40
50
P/D

calculated from an experiment with 9-aminoacridine on denatured DNA and the results also appear in Fig. 20(a). The f_d/f_{vis} ratio is seen to increase with P/D, suggesting that the tendency for Δ to increase with P/D in the denatured DNA complex seen earlier is real, and that base to base triplet energy transfer occurs in denatured DNA. This implies that the low efficiency of transferring triplet excitation to dye arises as a result of poor π-electron overlap between the dye and the bases of denatured DNA. The experiment on the quenching of the phosphorescence of denatured DNA by Mn^{++} becomes even more interesting in that if this analysis is correct, the phosphorescence of denatured DNA is expected to be quenched by small quantities of Mn^{++}.

Not only does the delayed fluorescence per dye (f_d/f_{vis}) increase with P/D, but the relative increase in f_d/f_{vis} is the same as the relative increase in Δ except at P/D > 20. This can be seen from table XI

Table XI

Ratio of UV-Induced Delayed Fluorescence to Triplet Energy Transfer in DNA-9-Aminoacridine Complexes

P/D	$f_d/f_{vis}/\Delta$	
	native	denatured
5.0	11.0×10^{-5}	2.5×10^{-3}
9.0	5.0×10^{-5}	3.1×10^{-3}
15	6.5×10^{-5}	1.3×10^{-3}
18.7	4.4×10^{-5}	3.0×10^{-3}
25	6.6×10^{-5}	2.5×10^{-3}

Data are from 1 experiment

where the ratio f_d/f_{vis} is divided by the corresponding Δ value. The results are constant. On the assumption that T_p and T_p' are equal, or are related by a constant, K, then the kinetic analysis yields:

$$\frac{\frac{f_d}{f_{vis}}}{\Delta} = \frac{\Phi_i \, k_{t-s}}{K \, k_{t-t}} \qquad (10)$$

and the ratio is expected to be constant.

The flash experiments have indicated that triplet energy transfer to dye occurs in a time < 10 msec., that is to say the average lifetime of the migrating DNA triplet was < 10 msec. Isenberg et al. have observed, on the other hand, that the lifetime of the delayed fluorescence from acridine orange on DNA was of the order of 100 msec. indicating an average DNA triplet lifetime of 100 msec. This apparent discrepancy implies that either triplet DNA to singlet dye transfer is occurring by: a) reabsorption by the dye of phosphorescence from the DNA, or, b) the T-S transfer occurs non-radiatively, at a distance, from traps which happen to be close to dye molecules, and, therefore, the delayed fluorescence lifetime is not expected to be limited to a time < 10 msec. Non-radiative transfer to acridine orange is expected to be more efficient than transfer to 9-aminoacridine and this would explain the saturation of the delayed fluorescence curve at higher P/D with acridine orange.

DYE-DYE INTERACTIONS

Singlet State Interactions

It has been pointed out that the emission properties of 9-aminoacridine are altered on going from the high to the low P/D complex. In

view of the nature and incompleteness of the data, in particular the lack of absorption spectra, the interpretation of observations in this region is highly speculative. However, a number of new observations have been made in this work and a discussion of the results is perhaps not unwarranted.

A marked blue shift in the absorption-spectrum of acridine orange bound to DNA and polynucleotides has previously been observed on formation of the low P/D complex and has been interpreted in terms of stacking of the dye molecules in a card pack fashion on the polymers (4). Exciton theory for allowed singlet states has been partially successful in explaining this absorption shift and the quenching of the fluorescence which occurs concomitantly. With strong coupling and the transition movements of the monomers in a parallel stack, a blue shift in the absorption spectrum and a quenching of fluorescence is predicted on the basis of exciton theory. If fluorescence does occur from the aggregates, it is predicted to be red shifted. While a red shift in the fluorescence spectrum accompanied by quenching occurs in free acridine orange with increasing concentration, quenching occurs without a red shift on formation of the low P/D complex on DNA (78). The explanation of the failure to observe a red shift in terms of a very low fluorescence efficiency for stacked molecules is not entirely satisfying in that quenching saturates at P/D = 1 with 20% of the fluorescence remaining (78).

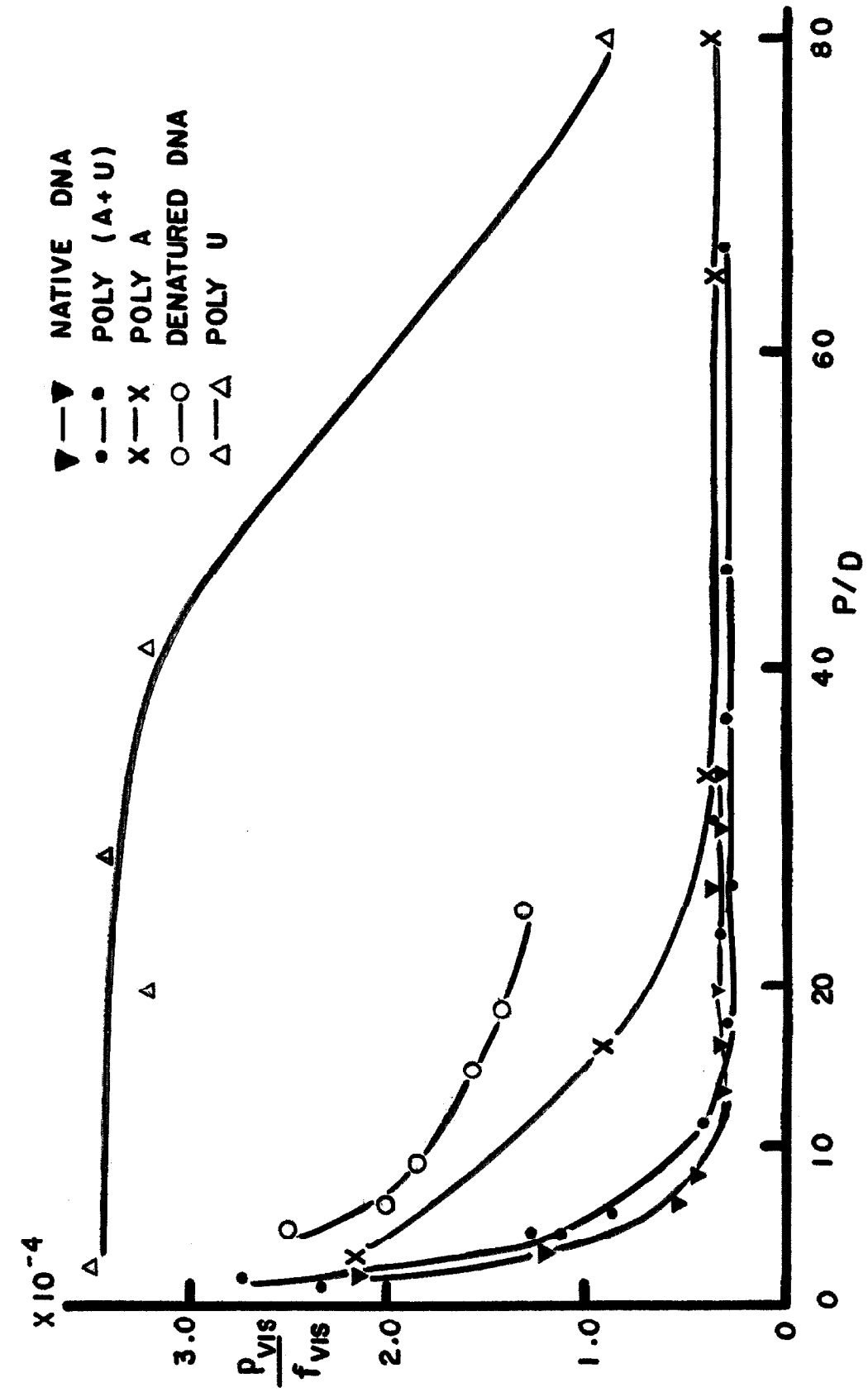
The failure to observe similar shifts in the absorption spectrum of 9-aminoacridine at low P/D (8) is probably due at least in part to the much lower oscillator strength of the lowest singlet-singlet absorp-

tion band in 9-aminoacridine than in acridine orange. The splittings (spectral shifts) in exciton theory for allowed transitions are proportional to the oscillator strength of the transition and, therefore, much smaller shifts are expected. A small blue shift ($\sim$ 7 mu) relative to the free dye has, in fact, been observed in the 9-aminoacridine absorption spectrum at room temperature, and the red shift in the fluorescence spectrum seen here is of a similar magnitude.

While the efficiency of fluorescence was not determined, the fluorescence intensity of 9-aminoacridine is not markedly quenched at low P/D in comparison with acridine orange. This is understandable in terms of excited singlet state interactions if the transition moments do not lie parallel but form an angle between them, making the lower exciton level at least partially allowed. In view of the low intersystem crossing efficiency in 9-aminoacridine even a small relative decrease in the fluorescence efficiency could explain the relatively large increase in the p_{vis}/f_{vis} ratio at low P/D. The observation of an increased p_{vis}/f_{vis} ratio is <u>consistent</u> with the predicted enhanced intersystem crossing as a consequence of formation of a lower singlet exciton state with a long radiative lifetime (49).

The magnitude of the red shift in the fluorescence spectrum of 9-aminoacridine on formation of the low P/D complex is dependent on the polymer to which the dye is bound. In addition, the P/D at which both the shifts in the fluorescence spectrum and the increases in the phosphorescence to fluorescence ratio (p_{vis}/f_{vis}) occur, depends on the polymer involved. The p_{vis}/f_{vis} ratio in 9-aminoacridine bound to a number of polymers is plotted as a function of P/D in Fig. 21. It is

Fig. 21 The phosphorescence to fluorescence ratio (excited with visible light) for 9-aminoacridine bound to a number of polymers.


NATIVE DNA
POLY (A+U)
POLY A
DENATURED DNA
POLY U
X 10⁻⁴
3.0
2.0
1.0
0
$\frac{P_{VIS}}{f_{VIS}}$
0
10
20
40
60
80
P/D

obvious from Fig. 21 that dye-dye interactions become apparent at higher P/D in the less rigid polymers. These results are in agreement with the observations of Bradley and co-workers who found that the tendency for acridine orange to stack was dependent on the nature of the polymer. They concluded that there is a greater preference for nearest neighbor binding in polymers with less rigidly fixed binding sites (4). It is not possible to analyze these p_{vis}/f_{vis} curves in terms of stacking coefficients, however, without knowledge of the relative contribution of dimers, trimers, etc., to the increase in p_{vis}/f_{vis}.

Triplet Dye - Triplet Dye Interactions

In experiments concerned with uv-induced delayed fluorescence in acridine orange bound to DNA, it was observed that, at low P/D, delayed fluorescence was also present with visible excitation. The low P/D spectra in which visibly induced delayed fluorescence was seen were ignored at that time. The appearance of delayed fluorescence with excitation into the lowest excited singlet state at low temperature cannot be explained by mechanisms which involve excitation of the polymer, photo-ionization and recombination, or thermal activation from the dye triplet to the excited singlet state. On the other hand, the delayed fluorescence can be explained in terms of a triplet-triplet annihilation mechanism.

Under conditions where triplet-triplet annihilation is not the principle pathway for depopulating the triplet state of the dye, the intensity of delayed fluorescence varies with the square of the exciting light

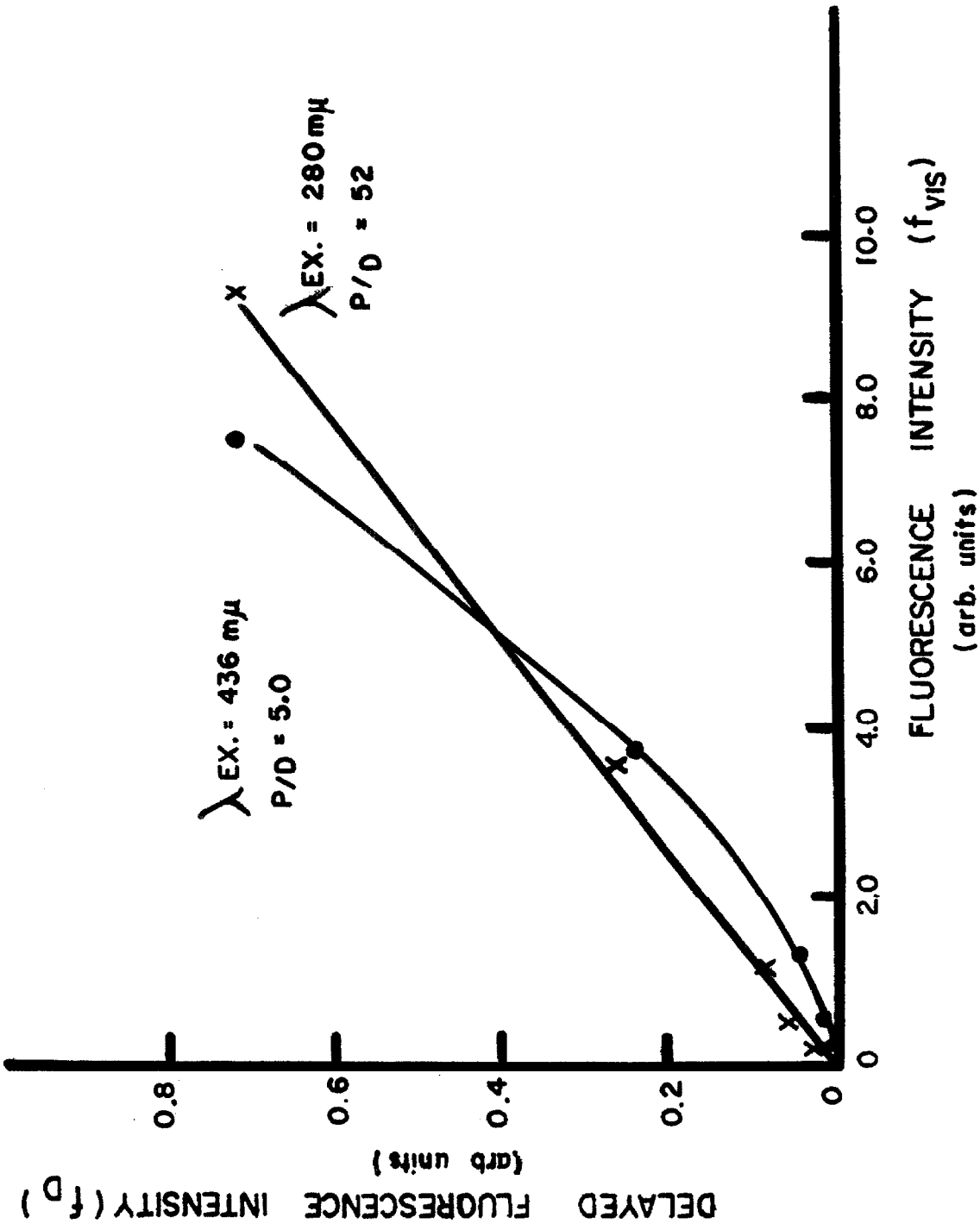
intensity (79). The intensity of the exciting light was varied with an aperture over the quartz lens in the exciting beam. The variation of delayed fluorescence intensity from acridine orange bound to native DNA, induced with visible light is plotted in Fig. 22 as a function of the normal dye fluorescence intensity which was determined simultaneously and is used as a measure of the exciting light intensity. The intensity of the uv-induced delayed acridine orange fluorescence at high P/D which was studied earlier was determined in the same manner and is included in Fig. 22. It is apparent that while the acridine orange delayed fluorescence observed only with uv excitation at high P/D varies linearly with exciting light intensity confirming the observation of Isenberg et al., the delayed fluorescence intensity induced with visible light* is not a linear function of the exciting light intensity. The dependence is not exactly a square dependence but goes as the 1.8 power of the exciting intensity.

As the probability of dye-dye interaction increases with decreasing P/D, the probability of observing delayed fluorescence from a dye with visible excitation should increase with decreasing P/D. The intensity of delayed fluorescence relative to that of normal fluorescence $(f_d/f)_{vis}$ is plotted as a function of D/P in Fig. 23. The intensity of delayed fluorescence relative to normal fluorescence increases almost linearly with D/P. This is in contrast to the delayed fluorescence observed at high P/D which was predicted and observed to increase with

---

* Delayed fluorescence arising as a result of triplet-triplet annihilation is expected to be also present with uv excitation. Reference to that component of the delayed fluorescence which is excited with visible light is made simply to differentiate from delayed fluorescence arising from the triplet state of the polymer.

Fig. 22 The dependence of the intensity of delayed fluorescence of acridine orange bound to native DNA on the exciting light intensity. The normal dye fluorescence intensity was determined as a measure of the exciting light intensity.


DELAYED FLUORESCENCE INTENSITY (f_D)
(arb units)
0.8
0.6
0.4
0.2
0
0
2.0
4.0
6.0
8.0
10.0
FLUORESCENCE INTENSITY (f_{VIS})
(arb. units)
$\lambda_{EX.}$ = 436 mμ
P/D = 5.0
$\lambda_{EX.}$ = 280 mμ
P/D = 52

P/D. These data indicate that delayed fluorescence observed in acridine orange DNA complexes is the result of two mechanisms; one at high P/D which involves the triplet state of the DNA, and triplet-triplet annihilation between interacting dyes at low P/D.

Triplet-triplet annihilation involves the migration of triplet excitation to a second molecule in a triplet state resulting in annihilation. Triplet energy migration then must be able to occur between dyes to give rise to delayed fluorescence. On the assumptions that, a) triplet energy migration occurs efficiently in stacks of dye molecules, b) the dye molecules are randomly oriented on the DNA and, c) annihilation and delayed fluorescence occur in such stacks, the function $(f_d/f)_{vis}$ which is derived in Appendix IV, was computed for a number of D/P values. The slope of the resulting curve is arbitrary to within a constant. The fit of the resulting curve to the experimental points is shown in Fig. 23.

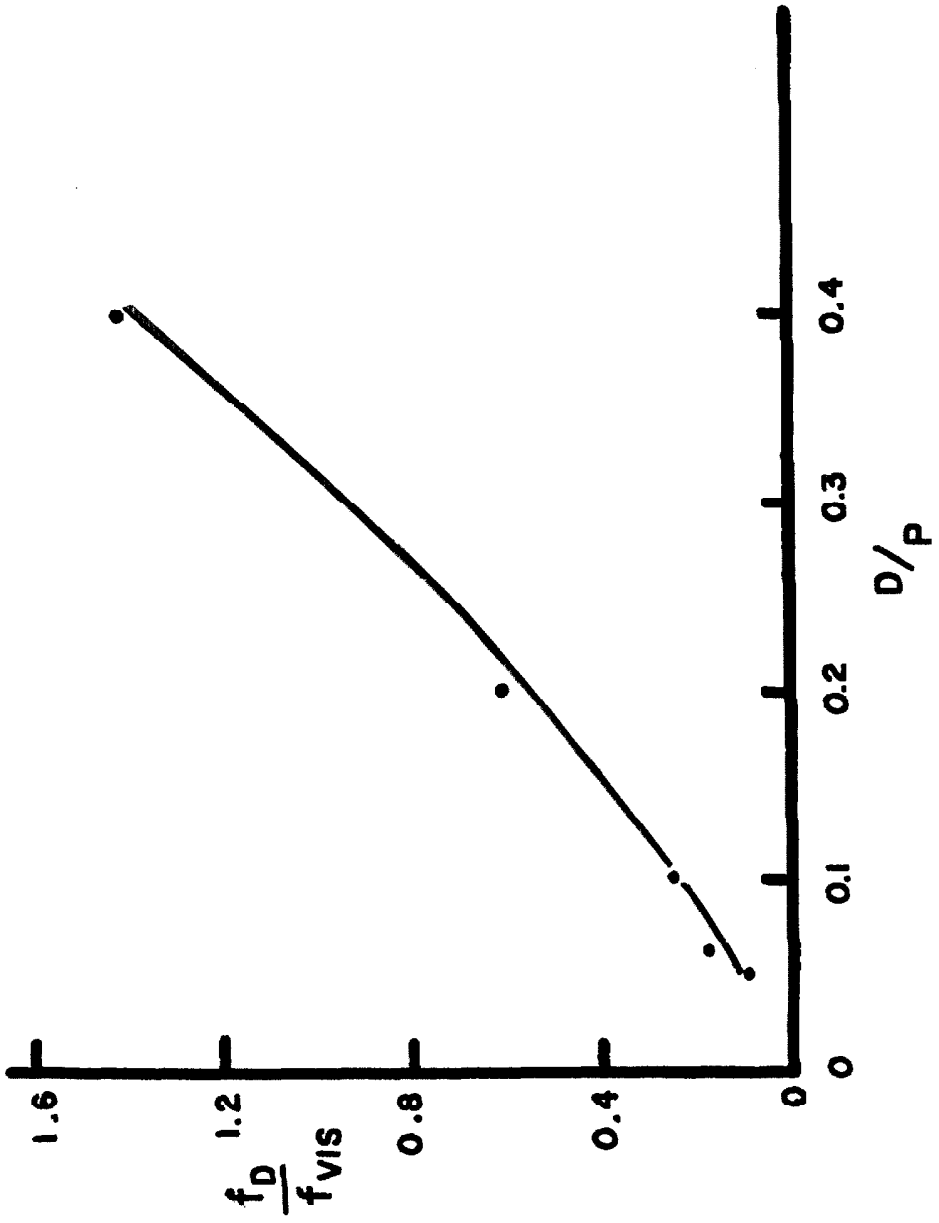
The agreement is seen to be quite good and there is little doubt that increasing the degree of interaction between dyes in the D/P region studied increases the probability of annihilation. On the other hand, there are reasons for seriously questioning the assumptions involved.

If the emission arises from stacked dyes, then it would be expected that the delayed fluorescence would have a spectrum which is different from the monomer spectrum.

The second objection is the fact that the delayed emission is observed at all. If the emission is in fact occurring from stacks of dyes, then, with the nearest neighbor triplet migration frequency of the order of 10^{12} sec.$^{-1}$ observed in organic crystals, migration and

Fig. 23 Variation of the intensity of delayed fluorescence excited with visible light in acridine orange bound to native DNA, with the number of dyes bound per phosphate.

The points are experimental and the line is given by equation (IV.4).


1.6
1.2
0.8
0.4
0
$\frac{f_D}{f_{VIS}}$
0
0.1
0.2
0.3
0.4
D/P

annihilation in such stacks is expected to occur rapidly and the resulting fluorescence would decay with its normal lifetime. The fact that the delayed fluorescence is observed with the phosphoroscope means that it must have a lifetime in the millisecond range. As the lifetime of the delayed fluorescence is determined primarily by the triplet migration time, it must be concluded that the triplet migration is, in fact, very inefficient.

The low efficiency of the dye to dye triplet energy migration could arise in one of two ways: 1) the direct interaction between dyes on adjacent binding sites is small because the overlapping of the electron clouds (wavefunctions) is reduced as a consequence of the nature of the binding; 2) dyes which are not stacked directly over one another interact indirectly through the virtual states of intervening bases. This kind of interaction is quite conceivable in this case in that the intervening states are pi-electron systems, and the difference in the triplet excitation energies of the bases and dye is about 5000 cm^{-1}. Interaction through one or more intervening bases, however, would considerably decrease the transfer rate. This is consistent with the fact that the emission appears in the form of the monomer spectrum. On the basis of this the presence of stacks would be expected to decrease the efficiency of the delayed fluorescence that is observed as a consequence of rapid annihilation. This predicts that the ratio of delayed to normal fluorescence should saturate and decrease with D/P as more stacks are formed, and not increase indefinitely as predicted by the model. No saturation is observed up to D/P = 0.4. It was found to be impossible to investigate D/P values above 0.4 in that the complexes

always precipitated on addition of the dye beyond this region.

Having detected triplet-triplet annihilation in the acridine orange - DNA complex, the delayed emission spectra of the 9-aminoacridine complexes were looked at with some care. The delayed emission spectrum of the 9-aminoacridine-DNA complex show a small amount of emission in the 450-540 mu region on visible excitation. This was originally thought to originate from an impurity in the dye, and, as it was small in comparison with the phosphorescence, it was ignored. It did, however, appear to increase somewhat with low P/D. In the poly U-9-aminoacridine complex at low P/D, shown in Fig. 24, it is obviously no longer possible to ignore this emission in that, in this case, it is large in comparison with the phosphorescence. The presence of the emission makes it impossible to accurately determine the phosphorescence intensity.

In the poly U complex the normal fluorescence spectrum of 9-aminoacridine undergoes the most marked red shift and this red shift occurs at higher P/D's than in the presence of the helical polymers. It was hypothesized that the delayed emission observed was delayed fluorescence from this aggregate state (exciton state). If this in fact were true the normal fluorescence spectrum should appear identical with the delayed emission at very low P/D where only stacked dyes are present. At the same time, if our assumption that the fluorescence spectrum excited with uv and visible light differ at low P/D because there is more than one complex present is correct, then in terms of a stacking model the spectra should merge to a single spectrum again at P/D=1. A comparison of uv and visible induced fluorescence spectrum of

the dye at very low P/D in Fig. 24 indicates that this occurs. The normal fluorescence spectrum at P/D=2 looks very much like the delayed emission suggesting that the hypothesis is correct.

The intensity of the delayed emission does not have a quadratic dependence on the excited light intensity but is distinctly non-linear with a 1.3 power dependence. This is not surprising if the annihilation process is significantly affecting the triplet population. The lifetime, and the exciting light dependence of the phosphorescence, however, was not determined in view of the interference from the delayed fluorescence.

It would appear that the same two mechanisms for delayed fluorescence as observed in the acridine orange-DNA complex are operating. In the 9-aminoacridine poly U complex, however, the delayed fluorescence arising as a consequence of triplet-triplet annihilation and triplet-singlet energy transfer corresponds to the normal stacked and monomer fluorescence spectra respectively. In this case, then, delayed fluorescence induced with visible light arises from dyes in which strong singlet-singlet interactions are present.

The intensity of delayed fluorescence normalized by the normal fluorescence intensity was determined as a function of D/P and the results are plotted in Fig. 25. The f_d/f_{vis} ratio is seen to initially increase with D/P.

Therefore, on one hand, while the evidence suggests that the delayed fluorescence is arising from stacked dyes, it is anticipated that the annihilation rate and, therefore, the resulting fluorescence should be fast for pairs of triplets within a stack.

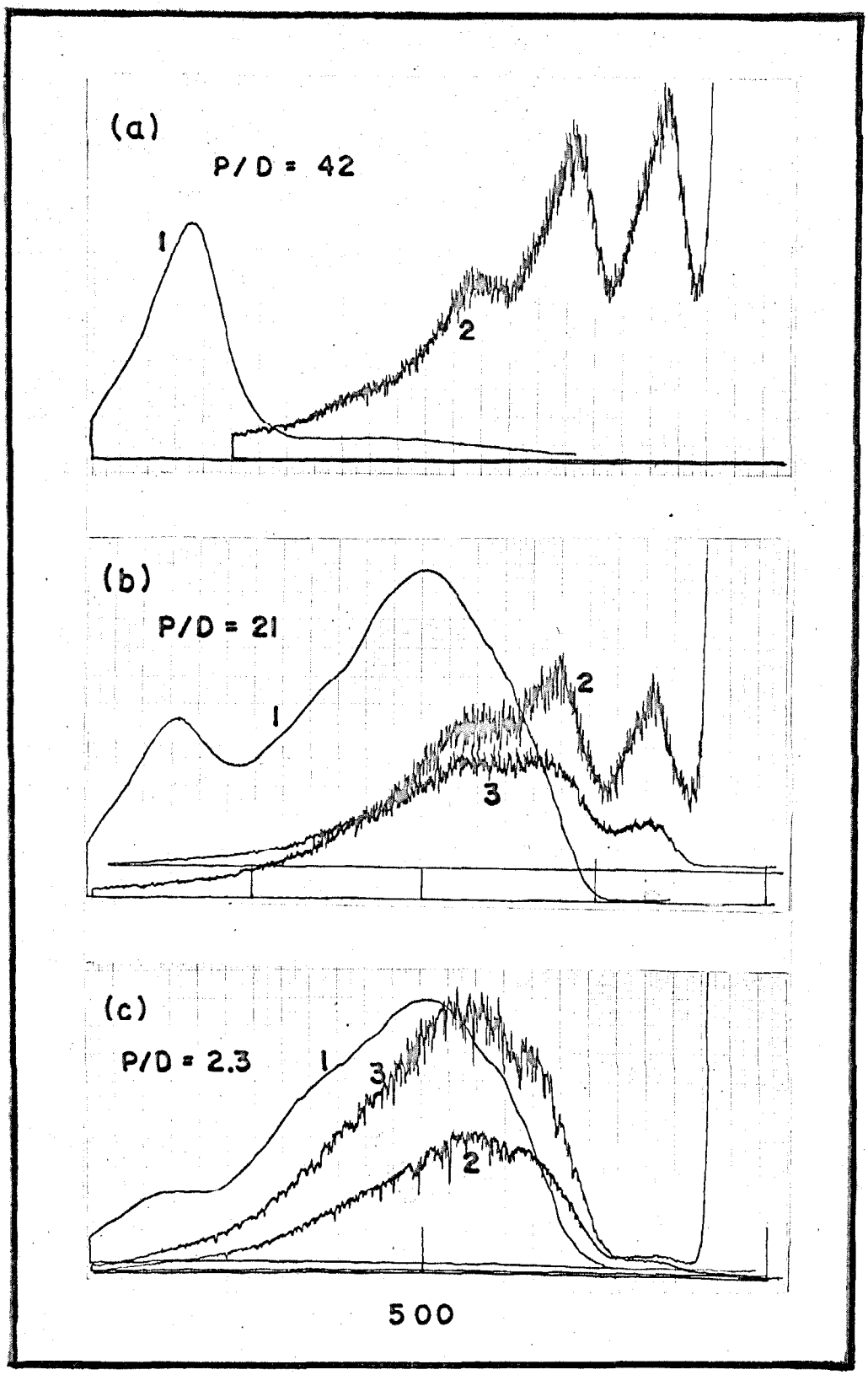
A model, however, in which triplet migration occurs between

Fig. 24 Delayed emission from the 9-aminoacridine - poly U complex at "low" P/D.

1 - Delayed emission, λ_{ex} = 405 mμ

2 - Total emission (fluorescence), λ_{ex} = 405 mμ

3 - Total emission, λ_{ex} = 280 mμ


(a)
P/D = 42
1
2
(b)
P/D = 21
1
2
3
(c)
P/D = 2.3
1
3
2
500
WAVELENGTH (mμ)

stacks of 9-aminoacridine molecules through a small number of intervening bases is probably not inconsistent with the data. Therefore, while pairs of triplets within a stack annihilate fast and are not observed, with one member of a pair occurring in each of two stacks separated by a small number of bases, migration can occur slowly from one stack to the other and give rise to delayed fluorescence. Emission is not observed from monomer dyes then, because, 1) there is a statistical factor which favors the probability of finding two triplets in adjacent stacks as opposed to two isolated monomers, and, 2) the lower p_{vis}/f_{vis} ratio in monomer dye suggests that the triplet population is much lower and, as the intensity of delayed fluorescence goes as the square of the triplet population, the failure to observe emission from monomer dye is not surprising. On the basis of this model it is expected that at very low P/D, where the intervening sites between stacks become occupied, the ratio of the "observed" delayed fluorescence relative to normal fluorescence will in fact decrease due to rapid annihilation.

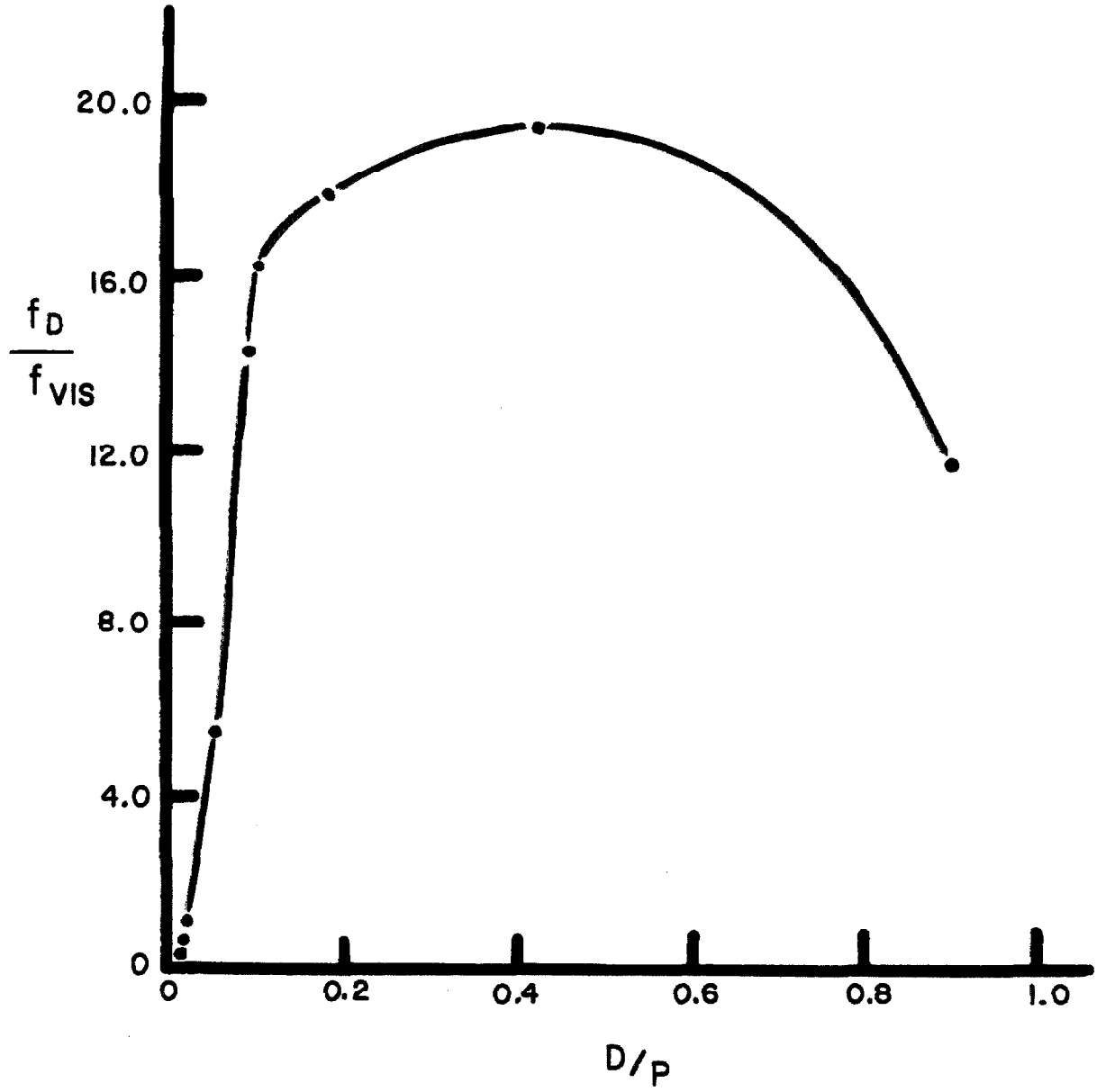
It can be seen from Fig. 25 that the f_d/f_{vis} ratio at D/P=0.9* decreases from the maximum value. While the saturation seen at intermediate D/P values would not be surprising in terms of a mechanism in which slow direct transfer occurred between adjacent dyes, it is difficult to explain the <u>decrease</u> at high D/P, which is anticipated, however, in a model in which intervening bases are required. This decrease is shown by only one point on the graph and requires further confirmation.

Determination of the lifetime of the delayed fluorescence as a

---

* High D/P solutions can be readily made without precipitation with poly U, presumably because of the much lower molecular weight of the polymer relative to DNA.

Fig. 25 The variation of the visibly induced 9-amino-acridine delayed fluorescence intensity in the poly U complex with the number of dyes per phosphate.


20.0
16.0
12.0
8.0
4.0
0
$\frac{f_D}{f_{VIS}}$
0
0.2
0.4
0.6
0.8
1.0
D/P

function of P/D would yield the average triplet transfer time directly. If triplet transfer and annihilation are occurring only within stacks, then one would anticipate that the lifetime of delayed fluorescence would remain essentially constant with increasing P/D or perhaps decrease as the stacks and, therefore, the migration distance, becomes smaller. On the other hand, if the observed delayed fluorescence only arises by transfer through intervening bases, then the lifetime of the delayed fluorescence should increase with increasing P/D, because transfer would have to occur through a greater number, on the average, of intervening bases. It was pointed out by Professor G. W. Robinson, in reference to the latter case, that with a random distribution of dyes the lifetime of the delayed fluorescence could extend from the nanosecond region out to times of the order of seconds.

These data represent preliminary observations of triplet-triplet annihilation in polymer-dye systems. Further investigations of the intensity and lifetime of delayed fluorescence as a function of the polymer to dye ratio promises to be useful in understanding the nature of dye-dye interactions and transfer through intervening polymer states.

REFERENCES

(1) Brenner, S., Barnett, L., Crick, F. H. C., Orgel, A. J. Mol. Biol., 3, 121 (1961)

(2) Albert, A., The Acridines, (London, 1951)

(3) Acheson, R. M., Acridines, Interscience Pub. Inc. (New York, 1956)

(4) Bradley, D. F., and Wolf, M. D., Proc. Natl. Acad. Sci, U.S., 45, 944 (1959)

(5) Lawley, P. D., Biochim. Biophys. Acta, 22, 451 (1956)

(6) Stone, A. L., and Bradley, D. F., J. Am. Chem. Soc., 83, 3627, (1961)

(7) Yew, F. F.-H., personal communication

(8) Peacocke, A. R., and Skerrett, J. N. H., Trans. Far. Soc., 52, 261 (1956)

(9) Schneider, F., personal communication

(10) Tubbs, R. K., Ditmars, W. E. Jr., and Van Winkle, Q., J. Mol. Biol. 9, 545 (1964)

(11) Weill, G., Biopolymers 3, 567 (1965)

(12) Le Pecq, J.-B., Paoletti, P. T., and Paoletti, C., Compt. Rend. Sc., 259, 1786 (1964)

(13) Gellert, M., Smith, C. E., Neville, D., and Felsenfeld, G., J. Mol. Biol. 11, 445 (1965)

Kersten, W., Biochim. Biophys. Acta, 47, 610 (1961)

(14) Boyland, E., and Green, B., Brit. J. Cancer, 16, 507 (1962), Liquori, A. M., De Lerma, B., Ascoli, F., Botre, C., and Trasciatti, M., J. Mol. Biol., 5, 521 (1962)

(15) Steiner, R. F., and Beers, R. F., Arch. Biochem. Biophys, 81, 75 (1959)

(16) Lerman, L. S., J. Mol. Biol., 3, 18 (1961)

(17) Drummond, D. S., Simpson-Gildemeister, V. F. W., and Peacocke A. R., Biopolymers, 3, 135 (1965)

(18) Albert, A., Rubbo, S. D., Goldacre, R. J., Davey, M.E., and Stone, J. D., Brit. J. Exptl. Pathol., 26, 160 (1945)

(19) Lerman, L. S., J. Mol. Biol., 10, 367 (1954)

(20) Lerman, L. S., Proc. Natl. Acad. Sci., U.S., 49, 94 (1963)

(21) Ohnishi, S., and McConnell, H. M., J. Am. Chem. Soc., 87, 2293 (1965)

(22) Chan,S. I., Bangerter, B. W., and Peter, H. H., Proc. Natl. Acad. Sci., 55, 720 (1966)

(23) Frenkel, L., Phys. Rev., 37, 17 (1931)

(24) Davydov, A. S., The Theory of Molecular Excitons, McGraw-Hill, 1962. References to the early Russian Experimental work on crystals are given.

(25) Windsor, M. R., Luminescence and Energy Transfer, In Physics and Chemistry of the Organic Solid State. Vol. II (D. Fox, M. M. Labes, A. Weissberger, Ed.) Interscience Pub., New York (1965) p. 343. A recent review.

(26) Cairo, G., and Franck, J., Z. Physik, 17, 202 (1923)

(27) Forster, T., Discuss. Far. Soc., 27, 7 (1959). References to earlier work are given here.

(28) Weber, G., Trans. Far. Soc., 50, 552 (1954)

(29) Forster, T., Comparative Effects of Radiation. (M. Burton, J. J. Kirby-Smith, and J. L. Magee, Eds.), p. 300, Wiley, New York, 1960

(30) Forster, T., Modern Quantum Chemistry, part iii: Action of Light and Organic Crystals, (O. Sinanoglu, ed.) p. 93, Acad. Press, New York, 1965

Robinson, G. W., Excitation Transfer and Trapping in Photosynthesis, in press. Both these recent articles illustrate the possible variation in transfer mechanism and the corresponding transfer rate equations.

(31) Bennett, R. G., Schwenker, R. P., and Kellogg, R. E., J. Chem. Phys., 41, 3040 (1964)

(32) Isenberg, I., Leslie, R. B., Baird, S. D., Rosenbluth, R., and Bersohn, R., Proc. Natl. Acad. Sci., 52, 379 (1964)

(33) Terenin, A. N., and Ermolaev, V. A., Trans. Far. Soc. 52, 1042 (1956)

(34) Ermolaev, V. H., Optics and Spect., 6, 417 (1959)

(35) Nieman, G. C., and Robinson, G. W., J. Chem. Phys., 37, 2150 (1962)

(36) Hochstasser, R., Proc. Org. Cryst. Symp., Ottawa, Canada, 1962

Hochstasser, R., J. Chem. Phys. 39, 3153 (1963). References appearing since this early work are too numerous to cite.

(37) Porter, G., and Wilkinson, F., Luminescence of Organic and and Inorganic Materials, p.132, Wiley, New York, 1962

(38) Dexter, J., J. Chem. Phys., 21, 836 (1953)

(39) Merrifield, R. E., J. Chem. Phys., 23, 402 (1955)

(40) Nieman, G. C., and Robinson, G. W., J. Chem. Phys., 39, 1298, (1963)

(41) Sternlicht, H., Nieman, G. C., and Robinson, G. W., J. Chem. Phys., 38, 1326 (1963)

(42) McConnell, H. M., J. Chem. Phys., 35, 508 (1961)

(43) Weill, G., and Calvin, M., Biopolymers, 1, 401 (1963)

(44) LePecq, J.-B., in press

(45) Lim, E. C., and Wen-Yang Wen, J. Chem. Phys., 39, 847 (1963)
Lim, E., Lozzara, C. P., Yang, M. Y., and Swenson, J. Chem. Phys., 43, 970 (1965)

(46) Basu, S., and Greist, J., J. Phys. Chem., 67, 1394 (1963)

(47) Tinoco, I., J. Am. Chem. Soc., 82, 4785 (1960)
Rhodes, W., J. Am. Chem. Soc., 83, 3609 (1961)

(48) Tinoco, I., J. Am. Chem. Soc., 83, 5047 (1961)

(49) McRae, E. G., and Kasha, M., J. Chem. Phys. 28, 721 (1958)
A simple illustration is given here.

(50) Steele, R. H., and Szent-Gyorgyi, A., Proc. Natl. Acad. Sci., 43, 477 (1957)

(51) Bersohn, R., and Isenberg, I., J. Chem. Phys., 40, 3175 (1964)

(52) Bersohn, R., and Isenberg, I., Biochem. Biophys. Res. Comm. 13, 205 (1963)

(53) Rahm, R. C., Shulman, R. G., and Longworth, J. W., Proc. Nat. Acad. Sci, 53, 893 (1965)

(54) Isenberg, I., Baird, S. L., Jr., Rosenbluth, R., Science 150, 1179 (1965)

(55) Rahm, R. O., Shulman, R. G., and Longworth, J. W., J. Chem. Phys. 45, 2955 (1966)

(56) Eisinger, J., and Shulman, R. G., Proc. Natl. Acad. Sci., 55, 1387 (1966)

(57) Helene, C., Douzou, P., and Michelson, A. M., Biochim. biophy. Acta. 109, 261 (1965)

Helene, C., Douzou, R., and Michelson, A. M., Proc. Natl. Acad. Sci., 55, 376 (1966)

(58) Helene, C., Biochem. Biophy. Res. Comm., 22, 237 (1966)

(59) Smaller, B., Avery, E. C., and Remko, J. R., J. Chem. Phys. 43, 922 (1965)

(60) Azumi, T., and McGlynn, S. P., J. Chem. Phys. 37, 2413 (1962)

(61) Weber, G., and Teale, F. W. J., Trans. Far. Soc., 54, 640 (1958)

(62) Baer, M., and Lunguet-Higgins, H. C., J. Chem. Phys., 23, 1390 (1955)

(63) O'Dwyer, M. F., Ashraf El-Bayoumi, M., and Strickler, S. J., J. Chem. Phys. (Comm.) 36, 1395 (1962)

(64) Robinson, G. W., and Frosch, R. P., J. Chem. Phys., 38, 1187 (1963)

(65) Galley, W., Research Report, Caltech, 1964

(66) Isenberg, I. and Bersohn, R., Triplet States in DNA, in Electronic Aspects of Biochemistry (Pullman, B., ed.)

Acad. Press, New York, 1964, p. 3.

(67) Rahn, R. O., Shulman, R. G., and Longworth, J. W., Unpublished results.

(68) McGlynn, S. P., Armstrong, A. T., and Azumi, T., in Modern Quantum Chemistry part III: Action of Light and Organic Molecules. (Sinanoglu, O., ed.) Academic Press, New York, 1965, p. 203

(69) Terenin, A. N., and Ermolaev, V. L., Acad. of Sci. USSR., Bull., Phy. Ser. 26, 21 (1962)

(70) Siegel, S., and Judeikis, H., J. Chem. Phys., 41, 648 (1964)

(71) Rahm, R. O., Longworth, J. W., Eisinger, J., and Shulman, R.G., Proc. Natl. Acad. Sci., 51, 1299 (1964)

Douzou, P., Francq, J., Hanss, M., and Ptak, M., J. Chim. Phys.. 58, 926 (1961)

(72) Rahn, R. O., Yamane, T., Eisinger, J., Longworth, J. W., and Shulman, R. G., J. Chem. Phys. 45, 2947 (1966)

(73) Eisinger, J., private communication.

(74) Steiner, R. F., and Beers, R. F., Jr., Polynucleotides, Elsevier Pub. Co., Amsterdam, 1961, p. 223

(75) Richards, E. G., Flessel, P. C., Fresco, J. R., Biopolymers 1, 431 (1963)

(76) Warmer, R. C., J. Biol. Chem. 229, 711 (1957)

(77) Fresco, J. R., Trans. N. Y. Acad. Sci., 21, 653 (1959)

(78) Van Duuren, B. L., in Fluorescence and Phosphorescence Analysis (D. M. Hercules, ed.) Intersciences Pub., New York,

1966, p. 195

(79) Sternlicht, H., Nieman, G. C., and Robinson, G. W., J. Chem. Phys. 38, 1326 (1963)

(80) Albert, A., and Ritchie, B., Org. Syn. 22, 5 (1942)

(81) Shulman, R. G., Sternlicht, H., and Wyluda, J. Chem. Phys. 43, 3116 (1965)

APPENDIX I - EMISSION ANISOTROPY CORRECTION

Imagine a coordinate system whose origin is situated at the center of an emitting sample. The sample is excited with light whose direction of propagation is along the x-axis. If the exciting light is unpolarized then it can be resolved into two equal components polarized along the Y and Z directions. The total emission at O is given by:

$$E_t = Z_z + Z_y + Y_z + Y_y + X_z + X_y \qquad (I.1)$$

where E_t is the total emission intensity. The large symbols denote the component of the emission, and the subscripts represent the polarization of the excitation. For unpolarized exciting light:

$$Z_z = Y_y \ , \ Z_y = Y_z = X_z = X_y$$

and therefore

$$E_t = 2\ Z_z + 4\ X_z \qquad (I.2)$$

If emission is observed at right angles to observation, i.e., along the y-axis, then the emission components polarized along Y are not observed, and the total observed emission is given by:

$$E_t' = Z_z + 3\ X_z \qquad (I.3)$$

or, the ratio of total to observed emission is

$$\frac{E_t}{E_t'} = \frac{2\ Z_z + 4\ X_z}{Z_z + 3\ X_z} \qquad (I.4)$$

From the definition of the degree of polarization p in equation (4) it is found:

$$Z_z = X_z \ \frac{(1 + p)}{(1 - p)}$$

$$\therefore \qquad \frac{E_t}{E_t'} = \frac{2(1 + p) + 4}{\frac{(1 + p)}{(1 - p)} + 3}$$

or $$\frac{E_t}{E'_t} = \frac{(3 - p)}{(2 - p)} \qquad (I.5)$$

It is apparent that when $p = 0$, 2/3 of the total intensity is observed at right angles to excitation. To correct all observed emissions to zero polarization, equation (I.5) is multiplied by 2/3 to give

$$Q_A = \frac{2\,(3 - p)}{3\,(2 - p)} \qquad (I.6)$$

If the degree of polarization is determined with unpolarized exciting light and is given by:

$$p' = \frac{Z - X}{Z + X}$$

where $Z = Z_z + Z_y$, and $X = X_z + X_y$

then $$Z_z + X_z = 2\,X_z\,\frac{(1 + p')}{(1 - p')} \qquad (I.7)$$

substituting for Z_z in equation (I.4) gives:

$$\frac{E_t}{E'_t} = \frac{3 + p'}{2} \qquad (I.8)$$

Multiplying again by the 2/3 factor to correct to zero polarization gives the corresponding expression when polarizations are determined with unpolarized exciting light.

$$Q'_A = \frac{3 + p'}{3} \qquad (I.9)$$

APPENDIX II - KINETIC ANALYSIS

a) Triplet - Triplet Transfer in the Polymer - Dye Complex

In a polymer-dye complex under conditions of constant illumination the steady state populations of the excited states of the dye with visible excitation are given by:

$$(S_d^1)_{vis} = \frac{I_d}{k_f + k_i + k_y} \qquad (II.1)$$

$$(T_d)_{vis} = \frac{\Phi_i}{k_p + k_r} I_d \qquad (II.2)$$

where (S_d^1) and (T_d) represent the populations of the lowest excited singlet state of the dye, and the triplet state respectively. I_d is the rate of absorption of visible light by the dye, k_f, k_i and k_y represent the rate constants for dye fluorescence, intersystem crossing, and $S_d^1 \rightsquigarrow S_d^o$ radiationless transitions *, k_p and k_r the rate constants for phosphorescence and $T_d \rightsquigarrow S_d^o$ radiationless transitions respectively. The corresponding populations with uv excitation are given by:

$$(S_d^1)_{uv} = \frac{I_d^1 + k_{s-s}\,(S_p^1) + k_{t-s}\,(T_p)}{k_f + k_i + k_r} \qquad (II.3)$$

$$(T_d)_{uv} = \frac{\Phi_i}{k_p + k_r}\left[I_d^1 + k_{s-s}\,(S_p^1) + k_{t-s}\,(T_p)\right] + \frac{k_{t-t}}{k_p + k_r}\,(T_p) \qquad (II.4)$$

where I_d^1 is the rate of uv absorption by the dye, k_{s-s}, k_{t-s} and k_{t-t} represent the rate constants for singlet polymer-singlet dye, triplet

* This pathway is usually considered to be negligible but is included here for the sake of completeness.

polymer-singlet dye, and triplet polymer-triplet dye energy transfer respectively; (S_p') and (T_p) denote the populations * of the lowest excited singlet and triplet state of the polymer respectively.

The steady state emission from the dye is given by, in general:

$$f = k_f (S_d') \qquad (II.5)$$

$$p = k_p (T_d) \qquad (II.6)$$

where f and p are the dye fluorescence and phosphorescence intensities respectively. Substitution of equations (II.1) and (II.3) in turn into equation (II.5), and equations (II.2) and (II.4) into (II.6) results in the following expressions for the steady state dye emission intensities:

$$f_{vis} = \Phi_f I_d \qquad (II.7)$$

$$p_{vis} = \Phi_p' \Phi_i I_d \qquad (II.8)$$

$$f_{uv} = \Phi_f \left[I_d' + k_{s-s}(S_p') + k_{t-s}(T_p) \right] \qquad (II.9)$$

$$p_{uv} = \Phi_i \Phi_p' \left[I_d' + k_{s-s}(S_p') + k_{t-s}(T_p) \right] + \Phi_p' k_{t-t} (T_p) \qquad (II.10)$$

in which

$$\Phi_f \equiv \frac{k_f}{k_f + k_i + k_r}$$

is the fluorescence efficiency, and

$$\Phi_p' \equiv \frac{k_p}{k_p + k_r}$$ *

---

* (S_p') and (T_p) are in fact the populations of the excited polymer states which can interact directly with dye. The reason for this distinction is apparent in the text.

* The prime superscript is used here to distinguish the quantity defined here from the customary use of "phosphorescence efficiency", $\Phi_p = \frac{k_p}{k_f + k_i + k_r}$ in the literature.

Two useful criteria for triplet-triplet energy transfer can be arrived at through combinations of equations (II.7) - (II.10):

$$R \equiv \frac{p_{uv}/p_{vis}}{f_{uv}/f_{vis}} = 1 + \frac{k_{t-t}(T_p)}{\Phi_i \left[I_d' + k_{s-s}(S_p') + k_{t-s}(T_p) \right]} \qquad (II.11)$$

$$\Delta \equiv p_{uv}/p_{vis} - f_{uv}/f_{vis} = \frac{k_{t-t}(T_p)}{\Phi_i \, I_d} \qquad (II.12)$$

b) Triplet Polymer-Singlet Dye Transfer:

Under conditions where triplet polymer-singlet dye energy transfer can occur, when the exciting light is turned off, then for a period of time which is short compared to the lifetime of the delayed fluorescence the population of the excited singlet state is essentially constant and is given by:

$$(S_d')_{uv} = \frac{k_{t-s}(T_p')}{(k_f + k_i + k_r)} \qquad (II.13)$$

These are the conditions under which the intensity of delayed fluorescence is determined with a phosphoroscope. The prime ' superscript in the term (I_p') is used to indicate that the population of the triplet state of the polymer that can interact directly with a dye may not be identical for triplet polymer-singlet dye and triplet polymer-triplet dye interactions. There is probably a greater number of bases which can interact directly with a dye by T-S interaction by virtue of the dipole nature of the interaction. This distinction was ignored in deriving equation (II.11).

Substituting equation (II.13) into (II.5) gives for the "steady state" delayed fluorescence intensity:

$$f_d = \Phi_f \, k_{t-s}(T_p') \qquad (II.14)$$

Normalization of eq. (II.14) by eq. (II.7) then yields

$$f_d/f_{vis} = \frac{k_{t\doteq s}(T_p')}{I_d} \qquad \text{(II.15)}$$

which is independent of the dye concentration per se.

APPENDIX III - MODELS FOR ENERGY MIGRATION IN POLYMERS

a) Energy Transfer by Diffusion (Random Walk)

From the diffusion model of Bersohn and Isenberg (31) for triplet energy migration in DNA the steady state intensity of DNA phosphorescence in the presence of quenchers, (S), relative to the phosphorescence in the absence of quenching (S_o) is given by their equation (14):

$$Q = S/S_o = 1-\alpha^2 \left[\frac{1}{\alpha} - \frac{1}{2} \Psi (1 + \frac{\alpha}{4}) + \frac{1}{2} \Psi (\frac{1}{2} + \frac{\alpha}{4}) \right]$$

where $\alpha = 2f(\tau/\tau_c)^{\frac{1}{2}}$, in which f is the fraction of the polymer sites occupied by quenching agents (f = D/P in our case). The polymer triplet state lifetime is given by τ, and τ_c is the reciprocal of the nearest neighbor jumping frequency for the excitation. Ψ is the derivative of the logarithm of the gamma function Γ .

The amount of triplet excitation transferred to a dye is directly proportional to the change in the polymer phosphorescence intensity per dye $(S - S_o)/D$. The latter expression can be expressed in terms of the variables used by Bersohn and Isenberg:

$$\frac{S_o-S}{D} = \frac{(1-Q)}{\alpha} K2(\tau/\tau_c)^{\frac{1}{2}} \qquad \text{(III.1)}$$

where K is the proportionality constant relating phosphorescence to polymer concentration. Substituting their equation (14) into (III.1) gives

$$\frac{S_o-S}{D} = K2(\tau/\tau_c)^{\frac{1}{2}}(1 - \frac{\alpha \Psi}{4}) \qquad \text{(III.2)}$$

When $\alpha \rightarrow 0$ the amount of quenching per dye tends to a limiting value:

$$\left(\frac{S_o-S}{D}\right)_{max.} = K2(\tau/\tau_c)^{\frac{1}{2}}$$

Normalizing eq.(III.2) by its maximum value then gives

$$\Delta' = \frac{S_o - S}{D} \Big/ \left(\frac{S_o - S}{D}\right)_{max.} = 1 - \frac{\Psi_d}{4} \qquad (III.3)$$

The Δ' function was fitted to the experimental Δ curve by variation of (γ_c).

b) Triplet Energy Migration Through a Polymer with Traps.

If the probability that excitation migrates over n monomer sites to a site 0 is given by p_n, then the number of excitations per second arriving at 0 from n is:

$$T_n = p_n k_t$$

when k_t is the rate of formation of triplet excitation on the site n and is assumed to be constant for all n. The total excitation arriving at 0 per second from all the sites between 0 and N is given by:

$$T_N = \sum_{n=1}^{N} T_n = k_t \sum_{n=1}^{N} p_n \qquad (III.4)$$

Let the probability of excitation being trapped on any one site n be given by x* and the probability of it not being trapped by (1-x). The probability that excitation is not trapped in migrating over monomer sites is given by:

$$(1-x)^n = p_n$$

Equation (III.4) then gives:

$$T_N = k_t \sum_{n=1}^{N} (1-x)^n \qquad (III.5)$$

With efficient transfer of excitation to a dye located at the site 0, and with N = P/D then T_N represents the rate of triplet energy transfer to dyes separated by P/D monomer sites. Equation (III.5) was calculated

---

* The assumption that x is a constant, independent of n is discussed in the text.

with trial values of x and normalized by the limiting value at high N. The best fit to the normalized experimental Δ values for the 9-amino-acridine-native DNA complex with x=.07 is shown in Fig. 13.

APPENDIX IV - THE DEPENDENCE OF TRIPLET-TRIPLET ANNIHILATION ON P/D

Delayed fluorescence is assumed to arise when 2 triplet excitations come together as a result of dye-dye interactions between dyes bound on adjacent polymer sites. If p is the probability that a dye exists as a triplet, then the probability that any dye in a stack of n dyes exists as a triplet = np. The probability that there are two triplets in a stack of n dyes is given by:

$$\rho_n = n(n-1)\, p^2 \qquad \text{(IV.1)}$$

The number of stacks of n dyes with two triplets is:

$$^2N_n = \rho_n N_n \qquad \text{(IV.2)}$$

where N_n = the number of n-mers. But the number of n-mers can be represented by:

$$N_n = \frac{f_n C}{n}$$

where C is the total number of dyes, and f_n is the fraction of the dyes existing in n-mers and is given by:

$$f_n = nf^{n-1}(1-f)^2$$

in which f is the probability that a dye has a nearest neighbor. For a random distribution of dyes on polymer sites f = D/P.

The total number of stacks with two triplets is then given by:

$$\sum_{n=2}^{\infty} {}^2N_n = C(1-D/P)^2 p^2 \sum_{n=2}^{\infty} n(n-1)(D/P)^{n-1} \qquad \text{(IV.3)}$$

On the assumption that pairs of triplets in a stack migrate efficiently, annihilate, and give rise to delayed fluorescence, the intensity of delayed fluorescence is proportional to the steady state population of stacks with pairs of triplets:

$$F_D = \Phi_f \sum_{n=2}^{\infty} {}^2N_n$$

or

$$F_D = \Phi_f c_p^2 \ (1-D/P)^2 \sum_{n=2}^{\infty} n(n-1)(D/P)^{n-1} \qquad (IV.4)$$

It is apparent that at constant dye concentration or when normalized by the normal dye fluorescence, the intensity of delayed fluorescence varies with the D/P ratio. Equation (IV.4) was computed for values of D/P up to D/P = 0.4. For D/P = 0.4 the series converges with $\sim$10 terms.

PROPOSITIONS

Proposition I

A Deuterium Effect on Radiationless Transitions Between Excited Electronic States

---

Abstract: While substitution of deuterium for hydrogen has no pronounced effect in general on radiationless transitions from the lowest excited singlet state of aromatic hydrocarbons, an influence of deuteration on internal conversion between higher excited singlet states is anticipated. It is proposed that a decrease in the rate of $S_2 \rightsquigarrow S_1$ radiationless transitions should be accompanied by a diversion of excitation to the triplet manifold. This effect is expected to appear experimentally as a decreased fluorescence efficiency from the lowest excited singlet state and an enhanced p/f ratio with excitation to the higher excited singlet state relative to excitation to the lowest excited singlet state.

It has been demonstrated that substitution of deuterium for hydrogen in aromatic hydrocarbons results in an increase in the lifetime of the triplet state of these molecules (1,2,3). This phenomenon has been interpreted by Robinson and Frosch in terms of an effect on the Franck-Condon factors governing radiationless transitions between the excited and ground states of the molecule (4). Deuterium substitution increases the number of ground vibrational states in the energy interval between the ground and excited electronic states and therefore decreases the vibrational overlap factor between the 0-vibrational level of the excited electronic state and vibrational levels

of the ground state in near resonance with it. For a small electronic energy difference no deuterium effect is anticipated. This accounts for the failure to observe a deuterium effect on $S_1 \sim\!\!\blacktriangleright T$ intersystem crossing (5,6) where the energy difference is small.

The deuterium effect on radiationless transitions is expected to be independent of the multiplicity of the initial and final electronic states of the molecule.

Deuterium substitution, however, has been shown to have little effect on the lifetime and efficiency of fluorescence of a number of organic molecules (5,6), azulene being a notable exception (7).

An increase in fluorescence efficiency of a number of organic molecules with exchangeable hydrogens in going from a H_2O to a D_2O media has recently been observed (8). This effect however has been interpreted in terms of an isotope effect on the rate of proton transfer of the excited molecules rather than an effect on radiationless transitions. Clear cut evidence in the case of 2-naphthol supports this general conclusion in these molecules.

The failure to detect a deuterium effect on fluorescence lifetime is anticipated on the basis of the theory of Robinson and Frosch. The large S_1-S_0 electronic energy difference results in very small Franck-Condon factors for the S_1-S_0 radiationless transitions and these small Franck-Condon factors make the $S_1 \sim\!\!\blacktriangleright S_0$ pathway highly improbable. Even a <u>large relative decrease</u> in the $S_1 \sim\!\!\blacktriangleright S_0$ transition probability, therefore, will go undetected.

Internal conversion from higher excited singlet states and the lowest excited singlet state, on the other hand, occurs in general with

high efficiency. Emission, with the exception of azulene (9), is only observed from the lowest excited singlet state and fluorescence efficiencies have been shown, in many cases, to be independent of exciting wavelength. Efficient internal conversion is anticipated theoretically in that the S_2-S_1 energy difference, in general, is much smaller than S_1-S_0. Very little is known, however, about the absolute rates of these S_2-S_1 radiationless transitions or their rates relative to intersystem crossing between higher excited states, or emission from the S_2 electronic state.

If the lifetime of the higher excited singlet state is increased significantly through a decrease in internal conversion, then intersystem crossing to the triplet manifold would be expected. It is proposed therefore that the effect of deuteration on the efficiencies of these excited state transitions be examined as a means of experimentally investigating radiationless processes.

If the rate constant for internal conversion to the lowest excited singlet state does not exceed the rate constant for intersystem crossing to the triplet manifold by more than 2 orders of magnitude then a decrease in the rate of internal conversion on deuteration should result in a significant fraction of the excitation being diverted to the triplet manifold. This diversion of excitation to the triplet manofold would give rise to experimentally detectable effects: 1) a decrease in the quantum efficiency of fluorescence and 2) enhanced phosphorescence to fluorescence ratio with excitation to the higher excited singlet state relative to excitation to the lowest excited singlet state, 3) possibly emission from the higher excited singlet state.

Neglecting the 3rd possibility the first 2 effects can be expressed mathematically in terms of efficiencies.

The p/f ratio with excitation to the lowest excited singlet state is given by:

$$(p/f) = \frac{\Phi_p \Phi_i}{\Phi_f} \qquad (1)$$

where Φ_p represents the efficiency of emission from the triplet state, Φ_i the intersystem crossing efficiency and Φ_f the fluorescence efficiency. With excitation to a higher excited singlet ...

$$(p/f)' = \frac{(\Phi_i' + \Phi_{ic}\Phi_i)\Phi_p}{\Phi_{ic}\Phi_f} \qquad (2)$$

where Φ_i' represents the efficiency of intersystem crossing from the higher excited singlet, and $\Phi_{ic} = (1 - \Phi_i')$ is the efficiency of internal conversion.

The ratio of equations (2) to (1) then gives:

$$\alpha = \frac{(p/f)'}{(p/f)} - 1 = \frac{\Phi_i'}{\Phi_{ic}\Phi_i} \qquad (3)$$

In the absence of deuteration, for most molecules Φ_i' and therefore α are equal to 0. The sensitivity of α as a method of detecting the presence of $\Phi_{ic} - 1$ depends on the magnitude of Φ_i. For very low Φ_i, α is sensitive to small values of Φ_i'. As phosphorescence can be detected in molecules with 1% intersystem crossing, it should be possible to detect Φ_i' efficiencies as low as 10^{-3}.

If a significant fraction of the excitation is diverted to the triplet manofold of states, then Φ_{ic} can be determined directly from the relationship:

$$\Phi_f' = \Phi_f \Phi_{ic} \qquad (4)$$

where $\Phi_f^{'}$ represents the fluorescence efficiency with excitation to higher excited singlet states.

It is apparent from relations (3) and (4) that the determination of $\Phi_f^{'}$ and α leads to an indirect measure of intersystem crossing $S^{'}$-T in these molecules:

$$\Phi_i = \frac{\Phi_f - \Phi_f^{'}}{\alpha \Phi_f} \qquad (5)$$

The technique, therefore, not only provides a tool for the study of radiationless transitions among the higher excited states but suggests a method of determining intersystem crossing efficiencies. The technique differs from previous methods of determining intersystem crossing in that it does not depend on intermolecular energy transfer with a second molecule (10,11).

For the technique to be successful deuterium substitution must make, for example, the $S_2 \rightsquigarrow T_2$ transition competitive with $S_2 \rightsquigarrow S_1$. This is expected to depend primarily on the S_2-S_1 electronic energy difference relative to the S_2-T_2 energy gap. If the S_2-T_2 energy difference is close to S_2-S_1 difference, then deuterium substitution would be expected to decrease the rate of both pathways and no effect would be observed. Investigations of the above type could be compared with data on the position of higher excited triplet states obtained by triplet-triplet absorption.

It is proposed that experiments of this type be first attempted on molecules in which there has been previous evidence, in the absence of deuterium, suggesting that intersystem crossing at higher excited state levels can occur. This includes chrysene and hexahelicene (12)

and dibromanthracene (13).

Complications in the interpretation of deuterium effects due to proton transfer in the excited state could be eliminated by working in media in which the protons cannot be transferred, or by working in H_2O, leaving only the nonexchangeable positions deuterated.

REFERENCES

(1) C. A. Hutchison and B. W. Mangum, J. Chem. Phys. 32, 1261 (1960)

(2) W. R. Wright, R. P. Frosch, and G. W. Robinson, J. Chem. Phys., 33, 934 (1960)

(3) M. S. de Groot and J. H. van der Waals, Mol. Phys., 4, 189, (1961)

(4) G. W. Robinson and R. F. Frosch, J. Chem. Phys., 38, 1187 (1963)

(5) E. C. Lim and J. D. Laposa, J. Chem. Phys., 41, 3257 (1964)

(6) J. D. Laposa, E. C. Lim, and R. E. Kellogg, J. Chem. Phys., 42, 3025 (1965)

(7) G. D. Johnson, L. M. Logan, and I. G. Ross, J. Molec. Spectr. 14, 198 (1964)

(8) L. Stryer, (in press)

(9) M. Beer and H. C. Lonquet-Higgins, J. Chem. Phys. 23, 1390 (1955)

(10) A. A. Lamola and G. S. Hammond, J. Chem. Phys. 45, 2129 (1965)

(11) R. E. Kellogg and R. G. Bennett, J. Chem. Phys. 41, 3042 (1964)

(12) M. F. O'Dwyer, M. A. El-Bayoumi, and S. J. Strickler, J. Chem. Phys. 36, 1395 (1962)

(13) J. Ferguson, J. Mol. Spectr. 3, 177 (1959)

Proposition II

The Use of Optical Polarization Data in the Determination of Crystal Structures of Enzymes.

Abstract: Binding of chromophoric groups at the active site of enzymes has been observed, and the recent demonstration of crystallization of one such complex has made optical investigation of these systems in the solid state possible. It is proposed that absorption and emission polarization measurements on crystals of this type can be used to complement x-ray diffraction data in the determination of the crystal structures of enzymes.

A number of aromatic molecules have been shown to bind covalently and non-covalently to α-chymotrypsin (1),(2). It has recently been shown by Haugland and Stryer (3) that a highly fluorescent and stable anthraniloyl group can be inserted at the active site. Fluorescence polarization and emission kinetics in the nanosecond region indicate that the chromophore has no rotational mobility independent of the motion of the enzyme molecule (3). Furthermore it has been shown that the anthraniloyl enzyme co-crystallizes with α-chymotrypsin (4).

It is proposed that studies of the polarization of absorption and emission of the chromophore in single crystals of enzyme complexes of this nature can be used in determining crystal structures of enzymes.

Perutz has used the dichroism of absorption of the haem group in determining its orientation in a number of haemoglobins (5), and predictions of crystal structures of small organic molecules have been

made on the basis of polarization data (6). However, the lack of correspondence of the emission and absorption spectra in the gas phase and solution with the solid state spectra, and the failure to observe polarization ratios predicted on the basis of a model in which the molecules do not interact ("oriented gas model") (7), makes it difficult, in general, to use optical polarization data in predicting crystal structures.

In the enzyme-chromophore crystals considered here interactions between chromophores are expected to be negligible, particularly in "dilute" crystals in which only a small fraction of the enzyme molecules carry a chromophoric group. The use of "dilute" crystals also simplifies spectroscopic investigation. The difficulties encountered in preparing crystals which are sufficiently thin for absorption measurements (6) are avoided. It should be possible, therefore, to use crystals of a convenient size and to work with more than one face on any one crystal.

Given an enzyme-chromophore crystal in which the crystal structure is unknown, rotations which transform translationally inequivalent enzyme molecules in the unit cell into one another (neglecting translations) must simultaneously transform the transition moment directions in the chromophore. From the determination of the transition moment directions the rotational symmetry of the protein molecules in the unit cell can be fixed.

The extent to which the rotational symmetry of the enzyme molecules can be defined depends on the particular relations between the transition moment directions and the observation direction. In crystal polarization measurements the intensities of absorption or emission

polarized along the principal optical directions of the crystal are measured. For a model of non-interacting molecules the intensity of absorption is given by the sum of the squares of the projections of the transition moments of the crystal along the principal directions (8). In a simple case where the n moments in a unit cell lie in a plane formed by the principal axes x and y, the intensities (absorption or fluorescence in this case) polarized along x and y are:

$$Ix = M^2 \sum_n \cos^2 \alpha_n$$

$$Iy = M^2 \sum_n \sin^2 \alpha_n$$

where M is proportional to the magnitude of the transition moment and α_n is the angle that the transition moment makes with the x-axis. We can define an angle Θ such that:

$$\tan^2 \Theta \equiv \frac{Iy}{Ix} = \frac{\sum_n \sin^2 \alpha_n}{\sum_n \cos^2 \alpha_n} \qquad (3)$$

It is apparent that $\Theta = \pm \alpha$ only when all the molecular moments in the unit cell are translationally equivalent. With 2 molecular moment directions per unit cell it follows that:

$$\sin^2 \alpha_1 + \sin^2 \alpha_2 = \frac{2 \tan^2 \theta}{1 + \tan^2 \theta} \qquad (4)$$

and for a given Θ there is a range of possible values for α_1 and α_2 which depends on the magnitude of Θ. For $\theta = 0$ or ∞, the transition moment directions are uniquely determined and lie along the x and y axis respectively. In this favourable case the only possible rotations which will transform protein molecules are: 1) a 2-fold rotation about y or x and all possible rotations about the transition moment direction. For a value of $\tan \Theta$ equal to unity, on the other hand, it is not possible to decide either the number of transition moments in the xy plane or

their directions relative to the principal axes.

An increased amount of information can be obtained if a second transition in the chromophore can be excited whose transition moment direction relative to the first is known from measurements of the degree of polarization of emission in solution (rigid media). It is not anticipated that a second higher energy singlet-singlet transition in the chromophore can be used due to the absorption of the aromatic amino acids in the protein. Determination of the degree of polarization of phosphorescence from the chromophore would give the orientation of the $T - S_0$ moment relative to the first $S_1 - S_0$ moment, and the $T - S^0$ moment could be used as a second vector. Polarization of the $T - S_0$ moment is known in some mixed crystals to be influenced by host - guest interactions (9). In the enzyme-chromophore systems the degree of polarization of the phosphorescence would be determined with the chromophore on the protein, i.e., in the presence of the host. Further perturbations on crystallization of the complexes are not expected.

To illustrate the use of a second vector, consider again the case where the absorption moments are confined to the xy plane and assume $\theta = \frac{\pi}{2}$ (y-polarized). Rotation about the y-axis was not defined. If the relative intensities of phosphorescence polarized along the x and z axes are determined, it is possible to define the $T\text{-}S_0$ moment directions to the extent of eq.(3).

For polarization ratios close to unity ($\tan^2\theta \sim 1$) it is not possible to decide by absorption whether there is one moment along the θ direction or whether a number of molecular moments exist such that the total projections on the x-axis equal those on the y-axis. This

question can be decided by measuring the polarized fluorescence on excitation with polarized light. We again dispense with a formal analysis and illustrate with the simple case above in which $\tan^2\ominus = 1$. If only one molecular moment is present, then excitation polarized along x excites the moment along $\ominus$ and fluorescence appears along x and y with equal intensity and the result is independent of dilution of the chromophore in the crystal. On the other hand if there are for example two molecular moments lying close to x and y, then in a "dilute" crystal in which energy transfer does not occur, the emission remains polarized along x and it only becomes equally polarized along y in crystals in which the chromophoric groups are sufficiently close so that energy transfer occurs. Photoselection of this type has not been previously observed in crystals; however it should be possible in these systems.

It should be emphasized that the method is expected to be useful in determining the non-crystallographic symmetry involved in the asymmetric unit rather than the crystallographic symmetry which is readily established by x-ray analysis.

REFERENCES

(1) Bruice, T. C. and Benkovic, S. J. (1966) "Bioorganic Mechanism", Vol. 1, p. 212, N. Y: W. H. Benjamin

(2) Wallace, R. A., Kurtz, A. N. and Niemann C., Biochem. 2,824 (1963)

(3) Haugland, R. P. and Stryer, L. Manuscript submitted for presentation at the International Symposium on Conformation of Biopolymers, Jan. 1967

(4) Stryer, L. (unpublished results)

(5) Perutz, M. F., Acta Cryst. 5, 323 (1952)
Perutz, M. F., Acta Cryst. 6, 859 (1953)

(6) Stewart, R. F. and Davidson, N. J. Chem. Physics. 39, 255 (1963)

(7) McClure, D. S., Solid State Physics, 8, 1 (1959)
Wolf, H. D., Solid State Physics, 9, 1 (1959)
The above reviews give many of the earlier references.

(8) Albrecht A. C. and Simpson, W. T. J. Chem. Phys., 23, 1480 (1955)

(9) Chaudhuri, N. K. and El-Sayed, M.A., J. Chem. Physics, 43, 1423 (1965)

Proposition III

An Autoradiographic Study of the Distribution of (A-T) - Rich Regions in λ -DNA

Abstract: It is proposed that autoradiography of λ -DNA labelled with radioactive methyl mercury ($Hg\text{-}C^{3}H_{3}$) will reveal the regions of the molecule which are rich in A-T. The experiment can be used to determine whether the early melting regions in λ -DNA are A-T rich.

It has recently been shown by Inman (1) that λ -DNA initially melts in selected regions of the molecule (1) It was suggested that the early melting regions in λ correspond to (A-T)-rich locations. The partial denaturation, however, was carried out in the presence of formaldehyde, and the denaturation was not allowed to come to equilibrium. Formaldehyde is known to decrease the melting temperature of DNA (2), and it has been pointed out that as formaldehyde binds to guanine, cytosine and adenine but not to thymine, the decrease in the melting temperature of G-C regions may be greater than A-T regions so that it is not obvious that the A-T regions in the molecule are the first to denature (3). An independent method for locating (A-T)-rich regions in the molecule would indicate whether the early melting regions arise because of their high A-T content.

It is proposed that the binding of methyl mercury labelled with tritium in the methyl group can be used to locate (A-T)-rich regions in the molecule. Selective binding would be indicated by location of the methyl mercury by autoradiography.

At low concentrations methyl mercury binds to denatured but not native DNA. At higher concentrations an interaction occurs with native DNA resulting in denaturation by virtue of a replacement of the protons involved in H-bonding (4). The binding occurs cooperatively; binding of a small amount of methyl mercury increases the probability of further binding, presumably due to local denaturation close to the initial site. Furthermore methyl mercury binds preferentially to (A-T)-rich DNA's. The addition of methyl mercury to native DNA at a concentration which results in only partial denaturation must be accompanied then by binding preferentially at (A-T)-rich regions of the molecule. The local denaturation in this case is not induced by heating per se but results rather from a shift in the equilibrium between native and denatured DNA due to preferential binding to the denatured molecule.

The detection of binding at selective regions of the molecule could be accomplished through a combination of autoradiography and electron microscopy or even by autoradiography alone.

Methyl mercury heavily labelled with tritium in the methyl group would be added to native DNA in varying concentrations in the concentration range where denaturation is known to occur. The complex could then be prepared for electron microscopy either by the Kleinsmidt technique (5) or by simply streaking the DNA on grids and positive staining with uranyl acetate. In the former case the methyl mercury would be held at a fixed concentration in both the concentrated and dilute NH_4SO_4 solutions. The use of cytochrome C in forming a film would give rise to binding of the $Hg-CH_3$ to sulfhydryl groups of the protein with a resulting high background radiation. This could be

eliminated by initial blocking of the -SH groups with iodoacetate. The excess Hg-CH_3 would be removed by complexing with NaCN.

The grids would be prepared for autoradiography after shadow casting or staining with uranyl acetate. Cairns (6) has described a technique for autoradiography of DNA. The resolution however was only about 1 μ. Adaptation of autoradiography to electron microscopy (7), however, has resulted in considerable improvement in resolution, and with the use of nuclear emulsions with a grain size of the order of 400 Å it is possible to achieve a resolution of around 0.1 μ(8). Following the coating of the grid with a monolayer of photographic emulsion the samples would be stored for several months to allow for the tritium decay.

A sufficient number of disintegrations are expected during a 2-3 month period to allow detection of selective binding regions. Tritium decays about 1% in 2-3 months ($t_{\frac{1}{2}}$ = 12 yr.). If we require on the average of one disintegration per binding region per molecule then it is possible to locate sites with 200 tritium atoms taking into account the fact that only one-half the disintegrations are recorded as a result of the geometry. With 100% specific activity this means it is possible to locate 60 Hg-C^3H_3 molecules. If we assume that all of the bases bind Hg-C^3H_3, due to cooperative binding, in an (A-T)-rich region, then one expects to be able to detect denatured regions of this type with 200 bases or 350 Å. It is apparent then that the ability to locate the site of disintegration by autoradiography, rather than the sensitivety of the method, limits the ability to resolve selective binding, and therefore denatured, regions.

It would not be possible to resolve "single site" regions observed by Inman, however, it should be easy to resolve the three major "multiple site" early denaturing regions which are separated by 2-4 μ, if those locations do represent regions of the molecule which have a high A-T content.

The polarity of the molecule could be determined in these experiments by labelling one half (or less) of the λ-molecule with thymine-^{3}H in some experiments. The information, with respect to methyl mercury binding, would be lost in that half of the molecule, but would allow the assignment of the early denaturing regions to one half or the other.

The use of a half labelled molecule would make it possible to carry out the above experiments in the absence of electron microscopy. The labelled half of the molecule could be used to locate the position of the molecules, and one would then look for the distribution of "spots" in the unlabelled half.

A λ-DNA molecule could be radioactively labelled in one half or the other by: 1) shearing thymine-^{3}H-DNA into half molecules, 2) separation of the halves by the technique of Wang et al, (9), 3) denaturation and renaturation of the sheared halves in the presence of whole unlabelled molecules to give "hot half-cold whole" hybrids, 4) repair of the missing quarter with unlabelled nucleotide triphosphates and DNA polymerase plus alkaline phosphatase.

REFERENCES

(1) Inman, R. B., J. Mol. Biol., 18, 464 (1966)

(2) Grossman, L., Levine, S. S., and Allison, W. S., J. Mol. Biol. 3, 47 (1961)

(3) LePecq, J-B., (private communication)

(4) Gruenwedel, D. W., and Davidson N., J. Mol. Biol. 21, 113 (1966)

(5) Kleinschmidt, A., Lang, D., Jackerts, D., and Zahn, R., Biochim. Biophys. Acta, 61, 857 (1962)

(6) Cairns, J., J. Mol. Biol. 4, 407 (1962)

(7) Caro, L. G., and van Tubergen, R. P., J. Cell. Biol. 15, 173 (1962)

(8) Salpeter, M. M., and Bachmann, L., J. Cell. Biol. 22, 469 (1964)

(9) Wang, J. C., Nandi, U. W., Hogness, D. S., and Davidson, N., Biochem., 4, 1697 (1965)

Proposition IV

The Kinetics of Ground State Recovery Following Electronic Excitation with Nanosecond Pulses

---

Abstract: It is proposed that the rate of recovery of the ground electronic state population of organic molecules following nanosecond excitation can be measured to determine the relative contributions of the excited singlet and triplet state pathways to the relaxation process.

In the majority of molecules in which emission from both the excited singlet and triplet state has been determined the number of quanta which appear as emission does not account for the number of quanta absorbed, indicating the importance of the $S_1 \rightsquigarrow S_0$ and $T \rightsquigarrow S_0$ radiationless transitions. In hydrocarbons with large S_1-S_0 electronic energy gaps, internal conversion ($S_1 \rightsquigarrow S_0$) is regarded as being unimportant (1). Experimental evidence on this point, however, is conflicting (2). Absolute intersystem crossing efficiencies have only recently been determined by a photochemical method (3). A spectroscopic method which would independently measure intersystem crossing rates in the solid or liquid state would be valuable. In many cases where both the fluorescence and phosphorescence efficiency are low even a semi-quantitative measure of the partitioning of the excitation energy between the singlet and triplet states would be informative.

It is proposed that the relative contributions of the singlet and triplet state pathways can be determined from the rate of relaxation of the ground state population following excitation of a measurable

fraction of the molecules out of the ground state.

If a steady state excitation source is shuttered, the initial (steady state) populations of the excited singlet S_1, and triplet state T, are given by:

$$S_1 = \frac{I}{k_s} \qquad (1)$$

and

$$T = \frac{k_i}{k_T} \cdot \frac{I}{k_s} \qquad (2)$$

where I is the rate of absorption $S_o \rightarrow S_1$, k_s the sum of rate constants for decay from the singlet state, k_i the rate constant for intersystem crossing and k_T the sum of rate constant for decay from the triplet state. The steady state triplet population is greater than the excited singlet by a factor of k_i/k_T, which can be of the order of 10^9 or larger. The ground state population, then, recovers exponentially with essentially the triplet state lifetime.

If a nanosecond pulse of excitation is used, however, which we assume here to be a square wave with a duration equal to the singlet state lifetime $1/k_s$ ($tf = 1/k_s$), then the populations of the excited states immediately following the flash are given by:

$$S_1 = .63 \frac{I}{k_s} \qquad (3)$$

and

$$T = .37 \, \Phi_i \frac{I}{k_s} \qquad (4)$$

when the flash time t_f is assumed to be small in comparison with $1/k_T$. In this case the triplet state has not been pumped up, and the relative populations are of a comparable magnitude. Without further calculations it is apparent that the ground state recovery rate will not, in general, be dominated by decay from the triplet state, but the excitation will be

partitioned between the pathways.

In the above approximation ($t_f = 1/k_s$) the time dependence of the excited state populations subsequent to flashing is calculated to be:

$$S_1(t) = .63 \frac{I}{k_s} e^{-k_s t} \qquad (5)$$

$$T_1(t) = \Phi_i \frac{I}{k_s} (e^{-k_T t} - .63\, e^{-k_s t}) \qquad (6)$$

The increase (recovery) of the ground state population following the flash S_o is given by:

$$\frac{dS_o}{dt} = k_R S_1 + k_T T \qquad (7)$$

where $k_R = k_f + k_r$ is the rate constant for decay of the excited singlet to the ground state, and is given by the fluorescence plus the radiationless rate constants respectively.

Substitution of eq(5) and (6) into eq(7) gives:

$$\frac{dS_o}{dt} = k_R .63 \frac{I}{k_s} e^{-k_s t} + k_T \Phi_i \frac{I}{k_s} (e^{-kTt} - .63\, e^{-k_s t}) \qquad (8)$$

Integration of eq (8) and rearrangement after taking the limits gives:

$$\frac{S_\infty - S_o^o}{S_o^\infty - S_o^o} = \frac{.63(1 - \Phi_i)}{.63(1 - \Phi_i) + \Phi_i} (1 - e^{-k_s t}) + \frac{\Phi_i}{.63(1 - \Phi_i) + \Phi_i} (1 - e^{-k_T t}) \qquad (9)$$

where S_o^o and S_o^∞ represent the ground state population at $t = 0$ and ∞ respectively. The numerical factor enters into eq (9) because of the particular flash condition chosen in this case. Eq (9) then represents the relaxation of the ground state population. It is apparent from eq (9) that one should be able to resolve the relaxation curve into 2 time dependent exponentials yielding k_s and k_T, and the pre-exponential factors provide a measure of the fraction of the excitation which

undergoes intersystem crossing to the triplet state. Independent determination of the fluorescence and phosphorescence efficiency then provides a complete description of the rate constants for all major processes which result in dissipation of electronic excitation energy.

Experiments of this type should be possible. An excitation source which is capable of lifting a detectable fraction of the molecules out of the ground electronic state in nanosecond times is required. The ground state population would be simultaneously monitored by absorption from a second source.

For a molecule with an extinction coefficient at the exciting wavelength of 10^4 l mole^{-1}cm^{-1}, and an O.D.(1 cm) of 0.3 there are 1.8×10^{16} molecules cm^{-3}. Lifting 10% of these out of the ground state giving rise to $\sim$10% increase in transmittance of the spectroscopic light, requires that 3.6×10^{15} quanta (for which $\epsilon = 10^4$) be incident on 1 ml of solution. This is beyond the capabilities of the relatively weak nanosecond flash lamps currently available (4). With some sacrifice in time resolution it is possible to increase the intensity of these flash lamps (5). Proper design of the cell could reduce the volume required to 0.1 ml, and with time averaging of the signal a 1% change in O.D. could probably be used. One could probably get by with as little as 10^{13} quanta, which then makes such an experiment feasible.

Pulsed lasers have recently been described which have pulse duration times in the nanosecond region. DeMaria (6) has described a neodymium Q-spoiled laser with a flash time of $<$ 1 nsec, and Ernest et al (7) has produced a Kerr cell Q-switched ruby laser of 100 mJ per pulse and a pulse time of 2 nsec. In the latter case this corresponds

to $\sim 3 \times 10^{17}$ photons per pulse at 6935 Å. The use of second harmonic generation would produce more than an adequate number of photons at wavelengths which are useful in organic molecules to attain a measurable decrease in the population of the ground state. The pulse intensity would probably have to be reduced to avoid too large a fraction of the molecules being excited giving rise to multiple quantum effects.

The change in the ground state population would be followed by absorption of light at a wavelength different from the exciting light. The source would have to be of sufficiently high intensity to allow: 1) careful filtering out of the exciting flash, and 2) maximum signal to noise. A CW laser with second harmonic generation could be useful as a spectroscopic source, in that use can be made of the parallel nature and polarization of the laser light in excluding scattered light.

The principle drawback in the use of lasers is in the lack of versatility in the wavelengths of the exciting light.

Experiments of the above type should be possible in solution as well as in rigid glasses.

REFERENCES

(1) Robinson, G. W., and Frosch, R. P., J. Chem. Phys., 38, 1187 (1963)

(2) Calvert, J. G., and Pitts, J. N., Photochemistry, Wiley & Sons, Inc., New York (1966)

(3) Lamola, A. A., and Hammond, G. S., J. Chem. Phy. 43, 2129 (1965)

(4) Huntley, L., Coburn, T., Garwin, E., and Stryer, L., A Nanosecond Fluorimeter (In Press)

(5) Stryer, L., (personal communication)

(6) DeMaria, A,, Reported at the Amm. Phy. Soc. Meeting. Dec.(1966)

(7) Ernest, J., Michon, M., and Debrie, J., Phy. Letters, 22, 147 (1966)

Proposition V

On the Hydrolysis of Oligosaccharides by Lysozyme

Abstract: It is proposed that the time dependence of the optical activity of substrates of lysozyme be determined during the course of hydrolysis as a method of studying the configurational change about carbon-1 on cleavage of the β (1-4) linkages.

The complete 3-dimensional structure of the lysozyme molecule has been determined (1). The solution of crystal structures of the enzyme in the presence of substrates has led Phillips to propose an active site for the enzyme and a mechanism of hydrolysis. In order to achieve a satisfactory fit of the substrate (tri-N-acetylglucosamine) to the enzyme it was necessary to postulate that the sugar ring on which carbon-1 of the glycosidic bond to be cleaved is located is distorted. This distortion with a concerted protonation of the glycosidic oxygen from a glutamic acid residue favors the formation of a carbonium ion at carbon-1. It is then postulated that hydroxyl ion addition to the carbonium ion complete the hydrolysis.

As well as can be judged from 2-dimensional representations of the enzyme substrate complex (1), an OH^- ion could approach the carbonium ion from either above or below the "plane" of the sugar. This implies that hydrolysis by the enzyme will give rise to either α or β terminal hydroxyl groups, or in other words, the enzyme will catalyze mutarotation.

It is proposed therefore that the optical activity of substrates

for lysozyme be followed during the course of hydrolysis.

The reaction could be carried out either with chitotriose I (2), or with tetra-N-acetyl-glucosamine (NAG). With the latter substrate it has been shown that hydrolysis of the first residue occurs rapidly (< 15 min.) while cleavage beyond the trimer proceeds much more slowly (3).

The (1-4) glycosidic linkages in these oligosaccharides are β, and the tetramer of NAG, for example, shows a characteristic specific rotation $[\alpha_D]$ (3). Hydrolysis by a mechanism involving a sterically unhindered carbonium ion would proceed with "racemization"* about carbon-1 to generate equal numbers of α and β ends. It is apparent then that the mixture would remain in equilibrium with respect to α and β forms. If the reaction is stopped, for example by precipitation of the enzyme with trichloracetic acid, the optical rotation will remain constant in time. It should be pointed out that the change in optical activity would not be due solely to changes in the α, β form but also as a result of the generation of new species.

On the other hand if the mechanism of hydrolysis does not involve formation of a carbonium ion but occurs rather by an Sn2 mechanism with either inversion or retention of configuration about carbon-1, then the products of hydrolysis at any time during the reaction will not be in equilibrium. If the enzymatic reaction is stopped as before, the optical rotation will continue to slowly change as a result of mutarotation in H_2O. It would not be necessary to wait for equilibrium to be

---

* The α and β forms are not mirror images in that the glucose moiety retains the D-configuration. Mixtures of equal amounts of α, and β still show optical activity.

attained. One would simply add acid or base to catalyze the reaction and determine the extent of the change.

In this latter case, the above argument only holds when the rate of enzymatic hydrolysis is fast compared to the mutarotation rate in H_2O. With an enzyme excess this should not present a problem with tetra-NAG as long as hydrolysis beyond the trimer is not attempted.

Monomeric NAG is known to inhibit the action of lysozyme. It would be interesting to determine whether binding per se to the enzyme would result in mutarotation. NAG in either the α or β form would be required. These could probably be obtained by crystallization. Catalyzed mutarotation of this type, which does not involve opening of the ring, would result in ^{18}O uptake from $H_2{}^{18}O$ in contrast to mutarotation by acid or base (for glucose) which does not involve significant oxygen exchange (4).

References

(1) Phillips, D. C., Scientific American, Nov., (1966) p. 78

(2) Dahlquist, F. W., and Raftery, M.A., Chem. and Chem. Eng. Report, California Institute of Technology, (1966) p. 108

(3) Rupley, J. A., Biochim. Biophy. Acta, 83, 245 (1964)

(4) Pigman, W., The Carbohydrates, Acad. Press, New York, (1957) p. 49.