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OPTICAL PROPERTIES OF CHROMOPHORE-MACROMOLECULE COMPLEXES:

ABSORPTION AND FLUORESCENCE OF ACRIDINE DYES BOUND TO POLYPHOSPHATES AND DNA^{**}

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Chromophore-macromolecule complexes represent an important class of structures of biological interest. The regular ordering of chromophores on a polymeric substrate is thought to be a key to the mechanism of energy transfer, both in the primary steps of photosynthesis and in the process of vision. The present work was undertaken as part of an effort to understand the optical properties of such an array of chromophores. The results also have a significance in terms of the structure of the polymeric substrate. The polyanions chosen for this work were polyphosphate and DNA, the latter because its gross structure is well known and it possesses chromophores of its own which could interact with the added cationic dyes, proflavine and acridine orange. The results reported here deal with the changes in absorption and fluorescence spectra, quantum yield and polarization of fluorescence due to the binding of the dye.

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1) Experimental techniques:

Absorption: The absorption spectra were measured on a Cary 14 Spectrophotometer. Careful corrections for base line deviations were carried out. The complexes were prepared directly in the 1 cm path length quartz cell by addition of microvolumes of a concentrated polymer solution to the dye solution and magnetic stirring. Measurements on concentrated solutions of the pure dye were possible by reducing the path length with quartz blocks of known thickness. A check on the relative thickness is provided by measurements on compounds obeying Beer's law over a wide range of concentration. Turbidity corrections have been calculated and are generally negligible. Turbidity effects are, in any case, compensated by the use of a polymer solution in the reference cell.

Fluorescence: The fluorescence measurements were carried out on identically prepared solutions on an Aminco Bowman Spectrofluorometer. The design of the instrument allows the observation of the emission only in a direction perpendicular to the incident beam; therefore the range of useful concentrations is limited to values of the optical density sufficiently low to avoid a reabsorption of the fluorescent light. A correction for absorption of the incident beam before reaching the small observed volume in the middle of the square 1 cm cell must be carried out, and can be very important when the polymer itself absorbs. The correction factor is the anti-logarithm of half the optical density.

Two gratings monochromators allow the choice of the excitation and fluorescence wave length. A series of slits define the band width which was in our conditions approximately 400 cm^{-1} . A typical measurement involves recording of intensities for the 40 to 100 combinations of 6 to 10 wave lengths of excitation and of fluorescence. The results can be

set in the form of a matrix which is used for direct enumeration of the number of fluorescent species in solution, according to the method of Weber¹.

The construction of the true action and fluorescence spectra require a series of corrections which have been carefully studied by Parker for the case of unpolarized fluorescence². As we were primarily interested in the relative values for the free and bound dye, there was no need for a calibration of the wave length dependence of the number of quanta reaching the cell. An approximate curve can be obtained by matching the apparent action spectrum with the absorption spectrum for a dye known for the constancy of its quantum yield as a function of the excitation frequency³.

The true fluorescence spectrum is expressed as the number $dQ/d\nu$ of the quanta Q emitted at a frequency ν with a band width $B\nu$. It is deduced from the output $A\nu$ of the photomultiplier through the relation:

$$dQ/d\nu = A\nu(B\nu L\nu P\nu)$$

where $L\nu$ is the transmittivity of the optical system at a frequency ν and $P\nu$ the sensitivity of the photomultiplier at frequency ν in amp/quantum. With a grating monochromator $B\nu$ is constant and $L\nu$ is generally assumed to be constant in the range of an emission band. $P\nu$ is deduced from the sensitivity curve supplied by the manufacturer.

Polarization: The insertion of two Glan polarizing prisms in the incident and fluorescent beam allow a separate measurement of the four components of fluorescent light, H_V , V_V , H_H and H_V , where the capital letters indicate the polarization of the fluorescent light and the subscript that of the incident light. Vertical polarization (V) indicates the electric vector perpendicular to the plane of the incident and observation beams. The polarization of fluorescence is classically defined by:

$$p = \frac{V_V - H_V}{V_V + H_V}$$

The observation of dilute solutions of free dye in a non viscous solvent reveal a large dependence of the transmittivity of the gratings according to the polarization of the light. The ratio of these transmittivities $t = (L_V)_V / (L_V)_H$ is experimentally determined from the comparison of actual values with the theoretical equality : $H_V = V_H = H_H$. In the following t_1 will mean the value of t for the monochromator selecting the excitation wave length, t_2 the value for the monochromator selecting the emission wave length. t_1 and t_2 are slightly dependent on the wave length, so that the assumption that L_V is a constant does not hold any more. More complicated correction formulas are needed to obtain the true fluorescence spectrum and the true polarization. Their derivation is given in an appendix.

Accessory techniques: The concentration of the polymer was determined from analysis of phosphorus by the method of Chen et al.⁴. Molecular weight determinations were done by light scattering.

2) Handling of the data:

Absorption spectra: The changes in absorption when the ratio of polymer (expressed as concentration in monomer units) to dye P:D increase from 0 to a value where no further change of absorption occurs are used to calculate: the absorption spectrum of the free and bound dye; the percentage q of free dye for each value of P:D; the actual ratio of polymer to dye in the complex.

Fluorescence measurements: For each value of P:D we obtain a set of values of intensities I' , V_V' , H_V' (for the notations see the appendix) corresponding to different couples of excitation and fluorescence wave lengths. The true polarization is derived, (equation 2, appendix) and used

to obtain the corrected intensity I (equation 1). For one excitation wave length the multiplication of I by PV leads to the true fluorescence spectrum. If two species are present this spectrum will depend on the excitation frequency. Spectra at $P:D = 0$ and $P:D \rightarrow \infty$ do not depend on the excitation and represent the true fluorescence spectrum of the free and bound dye.

If a fraction q of free dye remains in solution the intensities of fluorescence due to the bound dye are derived from the I' and I'_0 (value obtained for the free dye for the same couple of wave lengths and the same amplification of the photomultiplier) through the relation: $I'_1 = I' - I'_0 q$. The polarization of fluorescence of the bound dye p_1 is obtained from equation (4) of the appendix.

The ratio of the quantum yields can now be calculated for each wave length pair:

$$\frac{\phi_1}{\phi_0} = \frac{I' - I'_0 q}{I'_0} \frac{(1 + t_1)(1 + t_2)}{4 [1 + t_1 + t_2 + t_1 t_2]} \frac{1 - p_1}{4 - 2p_1} \frac{c_0}{a_1(1-q)}$$

$$\frac{k_1}{k_0} \frac{3 + p_1}{3}$$

a_0 and a_1 are the molar absorptivities of the free and bound dye at the wave length of excitation, k_0 and k_1 are the ratio of whole area to the height of the fluorescence spectrum at the emission wave length and $3 + p_1/3$ is a factor which arises from the anisotropy of emission for polarized fluorescence⁵.

The calculation for several wave length pairs allows one to estimate the inaccuracies introduced by the difference of band width in absorption and fluorescence measurements.

Study of the Free Dyes

Purification: From a solution of proflavine hydrochloride (K and K Laboratories) in a 1:1 water-ethanol mixture, the free base was reprecipitated with 0.1 N KOH. The precipitate was filtrated, washed and dried and recrystallized from a 9:1 water-ethanol mixture. The molecular weight was determined by titration with 0.02 N HCl and agrees with the predicted value within 2%. Stock solutions of the ionic form are prepared by adding the exact quantity of HCl to a given weight of proflavine and addition of buffer to a known volume.

The acridine orange base is precipitated identically from a solution of its zinc double chloride. The precipitate is redissolved in chloroform and passed through an alumina column. A dark band of impurities remains fixed upon elution with chloroform. The eluate is rapidly evaporated and the residue is recrystallized, characterized and put in solution in the monocationic form as for proflavine.

Absorption spectra: Deviations of Beer's law attributed to the formation of dimers and higher aggregates in water solutions have been extensively studied in the acridine derivatives, mostly in the visible region⁶. The spectra of Fig. 1 and 2 agree with other published data⁷. The extension of the measurements in the U.V. region reveal marked differences between the two dyes. With the increase of concentration, the molar absorptancy of proflavine decreases and the maximum of the U.V. band is shifted about the same value in wave numbers as is that for the visible band. Acridine orange under the same conditions shows an enhancement of its U.V. absorption and a very little shift compared to the one in the visible. These

observations can be of interest for the theory of absorption in dimer molecules if the different electronic transitions are well identified.

Polarization of fluorescence: The wave length dependence of p as a function of wave length in a very viscous solvent allows the recognition of different electronic transitions. In the absence of rotational depolarization and for totally anisotropic transition dipoles, p is related to the angle α between the absorption and the emission transition dipoles through:

$$p = \frac{3 \cos^2 \alpha - 1}{\cos^2 \alpha + 3}$$

Measurements in glycerine (Fig. 1 and 2) show that for both dyes the main U.V. and visible transition moments are parallel and the long wave length U.V. transition is differently polarized. Measurements at very low temperatures show that it is in fact perpendicular to the other³.

Fluorescence spectra: True fluorescence spectra of Fig. 1 and 2 are satisfactory mirror images of the absorption if the broadening due to the wave length scale and to the larger band width are taken into account. Both dyes show only one fluorescent species when their emission is analyzed according to Weber's sensitive method⁴.

Quantum yield: The quantum yield of proflavine in 10^{-3} acetate buffer is three times larger than the one of acridine orange. In glycerine the quantum yield of proflavine is enhanced by a factor of 1.5, the one of acridine orange by a factor of 3. Some published data⁹ suggest that concentration quenching occurs in solutions of proflavine at concentrations as low as 10^{-6} . The results of Table 1 show no effect of this type in our conditions if the absorption correction is made.

TABLE I

Change of Fluorescence of Proflavine Solution as a Function of Concentration. Excitation 4400 Å, Emission 5100 Å.

10^6 molar conc.	Intensity of fluorescence	Optical density at 4400 Å	Correction for absorption	10^{-6} I/c
1	21.5	0.036	1.04	21.3
2	40	0.072	1.08	21.8
5	91	0.180	1.23	22.4
10	140	0.360	1.52	21.4

Study of the Complexes with Polyphosphates

Characterization of the polyphosphate: The sample was a Kuroll salt prepared at 680-690° C. Its molecular weight measured in 0.35 N NaBr was $1.9 \pm .1 \cdot 10^6$ and its radius of gyration 600 Å.

Absorption spectra: The formation of a complex between the polymer and the dye in the presence of inorganic cations represents a complex equilibrium which takes in account both the degree of dissociation of the phosphate groups and the competition between organic and inorganic cations. Fig. 3 shows the change of absorption spectrum with addition of polyphosphate at constant concentration of dye c ($2 \cdot 10^{-5}$ M). The equilibrium constant for the binding reaction is related to the ratio r of bound dye to the total number of binding sites

$$k = r/c \cdot q (1 - r)$$

Calculations taking the total number of phosphate groups as binding groups give a limiting value of $r = 0.66$ for low P:D ratio. k decreases with increasing P:D ratio from 5 to $1 \cdot 10^{-5}$. For the ten fold dilution used in fluorescence studies, total binding is practically impossible.

The spectra obtained at P:D values are, according to Bradley and Wolf¹⁰ characteristic of molecules bound in neighbouring positions, leading to a large hypochromicity and a large hypsochromic shift attributed to strong coupling between dye molecules. Extension of the measurements in the U.V. region shows a large hypochromicity of the main U.V. transition and a blue shift of the maximum, comparable to the visible shift in the case of proflavine, but considerably reduced in the case of acridine orange. In the spectrum of proflavine there is some indication that the long wave length U.V. transition is more sensitive to P:D ratio.

the main band does not allow any definite conclusion for this band nor for the short wave length U.V. band which seems unchanged in the spectrum of acridine orange. As in the case of dimer molecules, it is important to observe the change in several electronic transitions to try an experimental verification of the theoretical calculations of hypochromicity and shift of maximum absorption in geometrical arrays of chromophores^{11,12,13}.

Fluorescence: The intensities of fluorescence at all wave lengths of excitation and emission decrease when P:D increases, exactly as the fraction q of free dye deduced from the absorption measurement. A careful study of the polarization of fluorescence and the use of the matrix method show that the quantum yield of fluorescence of the bound dye is 0, with a possible error of 25% of the quantum yield of the free dye.

Study of the Complexes with DNA

Changes of absorption of dye-DNA complexes in the visible region of the spectrum have been extensively studied with the aim of understanding the metachromatic phenomena and of finding the nature of the binding sites¹⁴. There are very few studies of the fluorescence of the complex^{15,16,17}, especially for high polymer to dye ratios. The recent results of Lerman¹⁸ and his model of intercalation of the acridines in the DNA structure give a new interest to these studies.

Characterization of the DNA: Johnson sperm DNA was obtained from California Corporation for Biochemical Research. Its molecular weight determined by light scattering, was 5×10^6 with a radius of gyration of 2200 Å. Its sedimentation coefficient was only 17 S., but its molecular absorptancy per gram of phosphate $\epsilon_p = 5709$ is characteristic of native DNA.

Absorption spectra: The results given in Fig. 4 were obtained on very dilute solutions ($2 \cdot 10^{-6}$ M) of dye in 10^{-3} M acetate buffer, pH 5.5. Under these conditions the ratio of phosphate groups to bound dye molecules is higher than 5 when P:D is higher than 1. We are therefore dealing with the "second type" of binding for which the intercalation model has been proposed. The spectra present a well-defined isobestic point. For values of P:D higher than 20 no more change of absorption occurs and the quasi totality of the dye is bound. The limiting spectrum with a red shift of 100 to 150 Å in the visible region is similar to those already published.^{7,10} The U.V. spectra obtained as difference spectra with DNA solutions of equal concentration were not as accurate, due to the absorption by the polymer itself. The differences with the spectrum of the free dye are striking: large hypochromicity of the main band, hyperchromicity of the weak long wave length transition. The proflavine spectrum shows an additional anomaly in the short wave length band. Possible shifts are difficult to evaluate precisely.

It may be asked whether these effects represent an alteration of the absorption properties of the dyes or of the bases of the DNA molecule. However, the absolute value of the change of oscillator strength corresponds roughly to 50% for the dye but would correspond to the total contribution of at least 2 to 3 purines or pyrimidines. In addition the comparison with the spectra obtained in the case of dye-dye interaction as well as the frequency dependence of the effect for the two dyes, strongly suggest that they represent an alteration of the absorption characteristic of the bound dye, due to coupling with the bases. The analogy with the hypochromicity observed in DNA alone as a result of helix formation, cannot however be taken, alone, as a proof of the validity of the intercalation model.

Fluorescence spectra: The fluorescence spectra reveal, markedly in the case of proflavine (Fig. 5), a shift of the maximum of the fluorescence band of the bound dye towards shorter wavelength, and a narrowing of the band. This blue shift must be related to the red shift observed in the absorption spectrum, which shows a similar narrowing. If the latter was to be explained by an environmental effect perturbing the gap between the ground and the first excited state, the former should be in the same direction, i.e. towards red. Shifts of opposite direction in absorption and fluorescence spectra, which bring the maxima closer, strongly suggest an alteration of the vibrational pattern, with a higher probability of population of the low vibrational levels, with the subsequent shifts and narrowing of the bands. A similar effect is observed when comparing the free dye in water and glycerine.

Quantum yield and polarization of fluorescence. Discussion of the energy transfer in the DMA molecule: So far we have only compared the properties of the free dye to the properties of the dye bound to a very large excess of polymer. Fig. 6 and 7 show that the quantum yield of fluorescence of the bound dye varies with the actual P:D ratio in the complex. The results present four interesting features: a) The relative quantum yield at high P:D ratio is different from one; b) the relative quantum yield at low P:D ratio is zero; c) the variation of the relative quantum yield as a function of log P:D is sigmoidal in shape; d) the quantum yield is a function of the wavelength of excitation and is higher when the excitation takes place in a region of absorption of the DMA itself.

Assuming that the limiting quantum yield at high P:D ratio (with no dye-dye interaction) is a characteristic of an "isolated bound dye" (discussed later) the three last features can be interpreted

as evidence for energy transfer both from dye to dye and from the purines and pyrimidines to the dye.

Excitation in the visible region and dye to dye transfer: The shape of the variation of the relative quantum yield as a function of $\log P:D$ (the actual ratio in the complex) is very similar to that given by concentration quenching in solutions of free dyes. The value of $P:D$ corresponding to half quenching in the complex is for both dyes approximately 20, which corresponds to an average separation of 34 \AA between the two binding sites along the DNA structure. This value is very similar to the half quenching values obtained for concentration quenching¹⁹. It is not possible to relate this value to the distance of half probability of energy transfer in the absence of information on the mechanism of quenching and on the distribution of dye to dye distances in the complex. Assuming some necessary number of "jumps", N , of the excitation energy in order to reduce to half the probability of radiative transition, a gaussian distribution of dye to dye distance with adjustable width, ΔR , and a dipole-dipole mechanism of energy transfer with a value R_0 for the distance at which the probability of transfer is equal to $1/2$, it is possible to construct a curve which fits the experimental results. Since it contains three adjustable parameters, N , ΔR , and R_0 , it does not shed much light on the physical mechanisms involved. If, however, different dyes with largely different values of R_0 determined from concentration depolarization¹⁹ of the free dye could be used, such a mathematical model would be of notable interest. A distance for half quenching so similar to those obtained when the relative positions of the molecules are at random might appear contrary to an expected increase of probability of transfer due

to some regular relation in orientation between dye molecules intercalated according to the Lerman model. It must be remembered that the angle between transition moments lying at a distance n times 3.4 \AA would be $2\pi n/10$ in this model so that the average factor $\cos^2 2\pi n/10$: $(3.4 \cdot n)^6$ which governs the probability of transfer in Forster theory¹⁹ will not be larger than in the absence of some relation between distance and orientation.

An explanation alternative to energy transfer might be found in a redistribution of the dye molecules along the polymer between sites of different quenching power as a function of loading. However, the results of the measurements of the polarization of fluorescence provide a satisfactory proof of the proposed energy transfer mechanism.

Fig. 8 and Table 2 give the results of measurements of polarization for excitation of the complex in the visible region. The polarization, being related to the angle between the absorption and emission transition moment, decreases with transfer of energy from one molecule to the other so that a parallel change in polarization and quantum yield provides good proof of the validity of the mechanism.

TABLE 2

Polarization of Fluorescence of the Dye-DNA Complex.

Acridine	P:D	6	10	25	60	100
DNA	p	.175	.180	.22	.275	.35
Proflavine	P:D	20	40	80		
DNA	p	.24	.30	.33		

In the case of concentration depolarization of pure dyes, the polarization drops to zero with the quantum yield. The fact that in this case the polarization tends to a limiting value at low quantum yield provides a strong argument for the intercalation model. At low

P:D ratio, energy is indeed likely to be transferred through several dye molecules so that the angle between absorption and emission transition dipoles will be $2\pi n/10$ with an equal probability of energy transfer for each value of n. The calculation of the average square cosine gives

$$\cos^2 \alpha = \frac{1}{10} \sum_{n=1}^{10} \cos^2 2\pi n/10 = 0.49$$

leading to $p = .135$ which can be compared with the experimental value .175. Introduction of a variable probability of energy transfer as a function of n would increase the calculated polarization.

A last experiment was performed to confirm directly the possibility of energy transfer from dye to dye. Taking advantage of the relative positions of the absorption and fluorescence bands of bound proflavine and acridine orange an experiment of sensitized fluorescence has been realized with a complex of DNA and proflavine (P:D = 20) to which a small proportion of acridine orange has been added (P:D = 100). By variation of the excitation wave length from 4000 \AA , where the absorption of acridine orange is small in comparison to proflavine absorption, to 4900 \AA , where the situation is reversed, and by comparison of the intensities of fluorescence of this mixed complex with that of a pure proflavine complex, the phenomenon of sensitized fluorescence is clearly demonstrated. The difference spectrum (column 3) has its maximum near 5400 \AA which corresponds to the emission maximum of acridine orange. In the absence of the sensitized fluorescence of acridine orange one might expect the excess emission of the mixed complex, excited at different wave length, to vary as the absorption of the acridine orange, i.e. the excess emission (column 3) at 4000 \AA should be

TABLE 3

Sensitized Fluorescence of Acridine Orange by Proflavine in
the Proflavine-Acridine Orange-DNA Complex.

Wave length of excitation	Wave length of emission*											
	5200 Å			5400 Å			5600 Å			5800 Å		
	1	2	3	1	2	3	1	2	3	1	2	3
4000 Å	7	33	26	5.5	32	26.5	2.9	16	13.1	.5	6	5.5
4500 Å	27	49	22	21	48	27	11	24	13	4.5	11.5	7
4900 Å	8	37	29	7	47	40	3.6	24	21.4	1.6	11.4	9.8

*1 = Fluorescence of the proflavine-DNA complex; 2 = fluorescence of the proflavine-acridine orange-DNA complex; 3 = difference.

some twenty fold less than it is at 4900 Å. This is clearly not the case, thus demonstrating the transfer from proflavine to acridine orange. Additional proof is given by the variation of the polarization of fluorescence emitted at 5300 Å with exciting wave length :
 $p(4200) = .175$ and $p(4900) = .28$. For these wave lengths about 80% of the fluorescence is from acridine orange. The polarization of a simple proflavine-DNA complex (P:D. ca. 20) is .24.

Excitation in the U.V. and base to dye transfer: It now appears possible to attribute the change of quantum yield upon excitation in the U.V. to sensitized fluorescence, involving transfer of excitation from the purines and pyrimidines to the dye. Such an hypothesis has already been advanced by Lerman to explain relative uncorrected action spectra of quinacrine and quinacrine-DNA complexes.

Polarization measurements at very high P:D values are impossible, due to the high absorption by DNA. The values for intermediate P:D ratios reported in Fig. 4 indicate a smaller value than in the visible. Quantitative deductions are however difficult because dye to dye transfer is still effective and transfer of excitation through the long wave length perpendicularly polarized transition of the dye is likely to be important. It must, however, be pointed out that the same considerations used to explain the limiting value of polarization of the dye at low P:D ratio for visible excitation should hold for the angle between the absorption transition dipole of the bases and the emission transition dipole of the dye with modifications due to slightly different geometry of base and dye pairs and the different possible orientations of the transitions in the dye. The net result should be a positive polarization; this

provides an explanation of the inversion of polarization of the 2600 Å absorption band of quinacrine bound to DNA which appears in the curves published by Lerman. Table 4 gives some data obtained with proflavine and acridine orange complexes in the U.V. region.

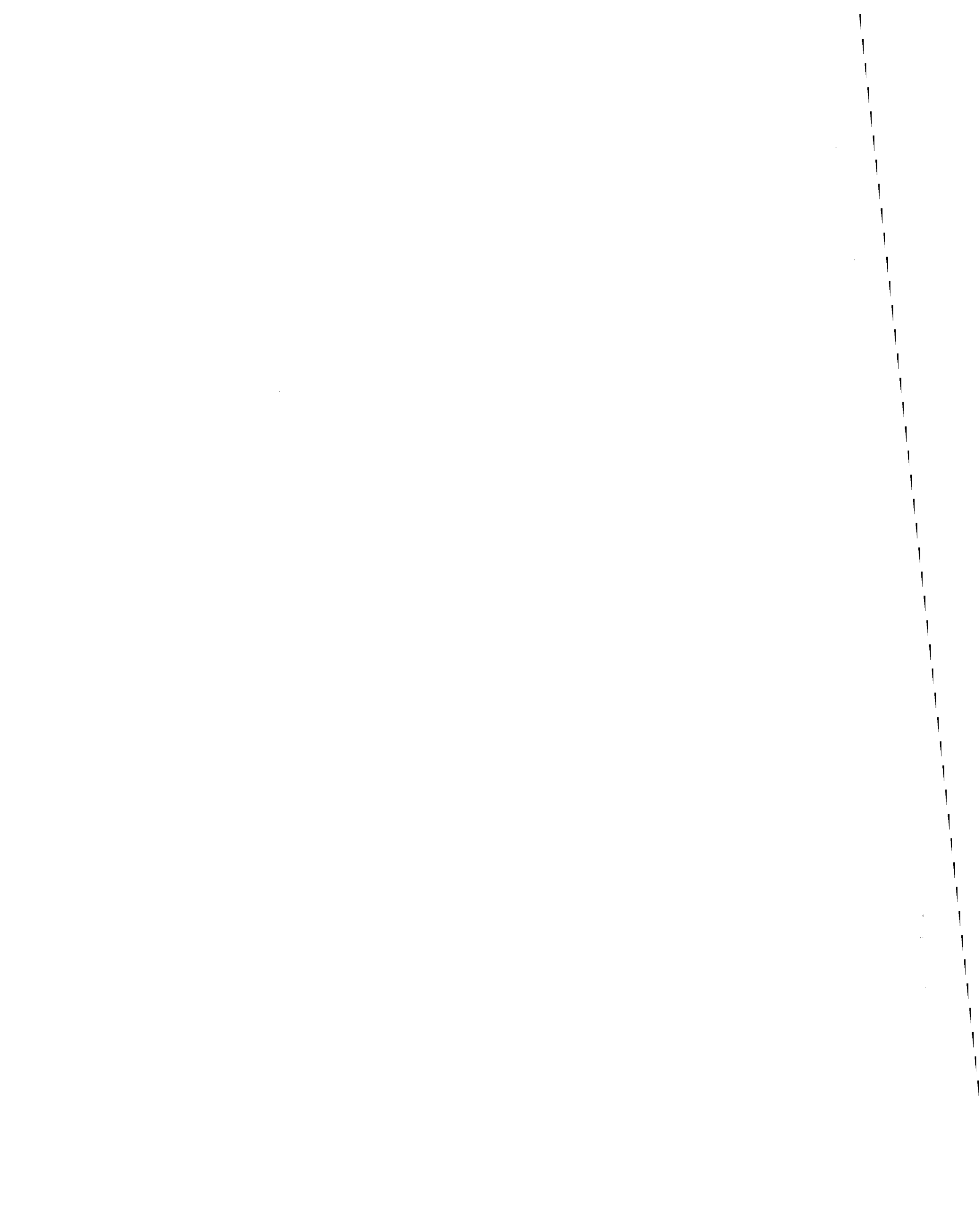
More careful studies of the polarization and of the efficiency of the sensitization as a function of wave length (with narrow band width) in the region of DNA absorption are needed to analyze the efficiency of transfer as a function of the optical properties of individual bases and dyes and to gain eventual information on preferential intercalation between given base pairs. It will simultaneously help to solve the problem of efficiency of base to base transfer to which a first approach can be made on the basis of the preceding results.

The ratio of quantum yield in the U.V. to quantum yield in the visible has been plotted in Fig. 9 as a function of P:D for the case of acridine orange. The higher values obtained (approximately 5) in comparison with proflavine (approximately 3, see Fig. 6) are likely to be due to the better overlap of the fluorescence spectrum of the bases with the absorption spectrum of the dye. For the lowest experimental value of P:D = 6 the ratio of sensitization is 2. Taking into account the respective coefficients of absorption of DNA and the bound acridine orange (approximately 7000 and 30,000, respectively) this means that nearly all the energy absorbed by the bases is transferred to a dye molecule, i.e. the ratio absorbance (complex)/absorbance (bound dye) is 2.4. If the "free path length" of excitation energy ^{in the polymer} was infinite, which means a totally efficient transfer with no loss of energy, the ratio of sensitization should then increase indefinitely with the ratio P:D. The experiment shows that in fact this ratio increases only slightly to level off for values of P:D of the order of magnitude of 40.

TABLE 4

Polarization of Fluorescence of the Complexes in the U.V. Region
(Emission between 5200 and 5500 Å)

Excitation wave length	2600	2700	2800	2900	3000	3100	3200	3400	3600
A.O.-DNA									
P:D = 25		.175	.160	.150	.130				
P:D = 50	.20		.17		.16		.10	.05	.13
Pro-DNA									
P:D = 30	.15	.18	.17	.17					
P:D = 60	.19	.17	.19	.18	.18		.07	.11	.27



This provides a value for the path length of excitation from base to base equal to ten or twenty base pairs, after which the excitation energy has been released into vibrational quanta. This result is to be expected from the heterogeneity of the base pairs, and could account for the absence of fluorescence of DNA as such. It gives an experimental basis to theoretical discussions of the concept of exciton migration in the DNA molecule.

Similar experiments with homopolynucleotides in the helix and coil shape, and the use of dyes with different gross structure and electronic properties will be valuable to investigate the geometrical requirements for energy transfer and the possibility of longer path length of excitation (or exciton) migration.

Quantum yield of the isolated bound dye: So far we have neglected the large difference in the change of quantum yield occurring upon binding of the dye to a large excess of polymer for acridine orange and proflavine. The simple effect of viscosity should be an increase actually observed in the case of acridine orange, while in the case of proflavine binding results in quenching. Slight changes of absorption spectrum in the presence of large quantities of purine nucleotides have been reported⁷ and we have measured the change in fluorescence of the dyes under the following circumstances: a) When bound to excess of polyadenylic acid, both in the helix and in the coil configuration; b) in presence of 10^{-2} M concentration of different nucleotides. The results are given in Table 5.

These preliminary results would lead to the notion that the binding constant can be influenced by the nature of the base and the difference between the two dyes may be due to different coupling with different bases. The enhancement of fluorescence of acridine orange by AMP should be compared with the proposed formation of a complex on the basis of phosphorescence measurements²⁰.

TABLE 5

Variation of the Intensity of Fluorescence of the Dyes in the Presence of Excess of Mono and Polynucleotides.

Nucleotide	P:D	Acridine orange		Proflavine	
		% change	polarization	% change	polarization
polyA coil	200	+ 100	.28	0	.16
PolyA helix	200	+ 100	.23	0	.25
AMP	104	+ 66	0	-15	0
TMP	104	0	0	-25	0
CMP	104	0	0	0	0

SYNOPSIS

Dye-macromolecule complexes provide good models for the study of the effects of coupling between chromophores. In addition to modifications of the visible and U.V. absorption spectra of the dyes at small interchromophore distances, very efficient energy transfer has been demonstrated at longer distances. The probability of non-radiative transition increases with the number of excitation transfers so that an array of oscillators close to one another becomes non-fluorescent.

The insertion of a dye molecule, acting as a trap for the excitation energy, in the highly ordered system of chromophores constituted by the purine and pyrimidine bases of native DNA has given results supporting the intercalation model of Lerman and providing an experimental approach to the problem of the path length of energy migration in the DNA molecule. The average excitation path length seems to be of the order of only ten base pairs, a result which can explain the lack of fluorescence of the DNA.

APPENDIX

Calculation of the correction factors due to the variation in transmission through the monochromators with the polarization of the light.

The transmission of the horizontally polarized component does not vary in the narrow range of an absorption or of a fluorescence band. The unpolarized light produced by the source would normally give four fluorescent components related by the relations:

$$V_h = H_v = H_h; \quad V_v = H_h (1 + p)/(1 - p)$$

Due to the instrumental factors t_1 and t_2 the instrument gives:

$$H_h' = H_h; \quad V_h' = t_2 H_h; \quad H_v' = t_1 H_h \text{ and } V_v' = t_1 t_2 (1 + p)/(1 - p)$$

The correct intensity of fluorescence in unpolarized incident light is:

$$I = I' \frac{4 - 2p}{(1 - p) [1 + t_1 + t_2 + t_1 t_2 \frac{1 + p}{1 - p}]} \quad (1)$$

where I' is the observed value without polarizers and the true polarization of fluorescence.

$$p = \frac{V_v' - t_2 H_v'}{V_v' + t_2 H_v'} \quad (2)$$

If starting from a solution of free dye at a given concentration, emitting an intensity I_0' , the addition of the polymer to a given ratio lets a fraction q of the molecules remain unbound, with an unpolarized fluorescence, and binds a fraction $(1 - q)$ of the molecules which now give rise to a polarized emission, the polarization of fluorescence of the bound dye p_1 can be calculated from the actual values of the intensities of fluorescence of the two solutions, I' and I_0' , the value of q , and the corrected polarization of fluorescence p of the solution containing the polymer. If the subscript 1 stands for the bound dye the four components of the emission are:

$$V_V' = t_1 t_2 H_{ho} q + t_1 t_2 H_{hl} (1 + p_1)/(1 - p_1)$$

$$H_V' = t_1 (H_{ho} q + H_{hl})$$

$$V_h' + t_2 (H_{ho} q + H_{hl})$$

$$H_h' = H_{ho} q + H_{hl}$$

$$\text{The polarization } p = \frac{H_{hl} (2 p_1/1 - p_1)}{2 H_{ho} + H_{hl} (2 p_1/1 - p_1)}$$

$$(3) \quad = \frac{(I' - I'_0 q) \frac{4 - 2 p_1}{4 (1 - p_1)} (1 + t_1 + t_2 + t_1 t_2 \frac{1 + p_1}{1 - p_1})^{-1} \frac{2 p_1}{1 - p_1}}{2 I'_0 q (1 + t_1)^{-1} (1 + t_2)^{-1} + (I' - I'_0 q) \frac{4 - 2 p_1}{4 (1 - p_1)} (1 + t_1 + t_2 + t_1 t_2 \frac{1 + p_1}{1 - p_1})^{-1} \frac{1}{1 - p_1}}$$

In most cases, a negligible error is introduced by the replacement of $I'_0 q [(1 + t_1) (1 + t_2)]^{-1}$ by $I'_0 q [(1 + t_1 + t_2 + t_1 t_2 \frac{1 + p_1}{1 - p_1}) (1 - p_1)]^{-1}$

p_1 is then the root of the equation

$$(4) \quad 2 p_1^2 (I' - I'_0 q) - p_1 [2 p (I' + I'_0 q) + 4 (I' - I'_0 q)] + 4 p I' = 0$$

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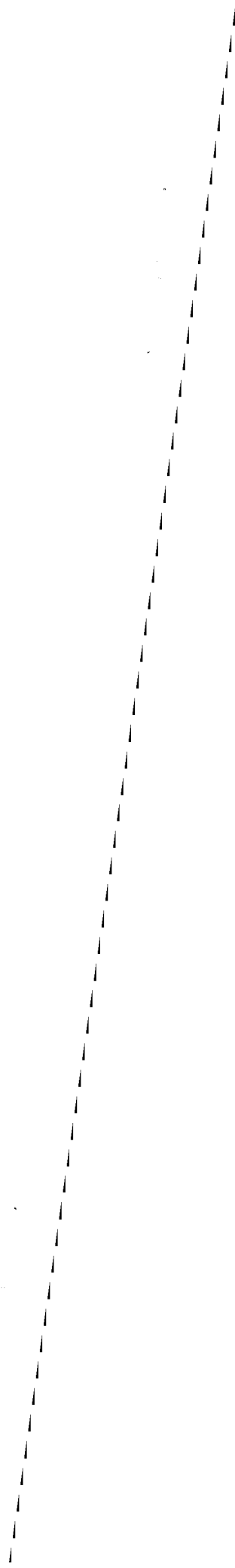


FIGURE LEGENDS

Fig. 1. Optical properties of proflavin⁺ in 10⁻¹ M acetate buffer, pH 5.5.

_____ Absorption spectrum, 5 x 10⁻⁶; _____ Absorption spectrum, 10⁻³;
- - - - Fluorescence spectrum, 2 x 10⁻⁶; _____ Polarization of
fluorescence.

Fig. 2. Optical properties of acridine orange monocation in 10⁻¹ M

acetate buffer, pH 5.5. _____ Absorption spectrum, 5 x 10⁻⁶;
_____ Absorption spectrum, 5 x 10⁻⁴; - - - - Fluorescence,
2 x 10⁻⁶; _____ Polarization of fluorescence.

Fig. 3. Titration of proflavine and acridine orange cations with polyphosphates.

Top: Proflavine, 2 x 10⁻⁵ M. _____ Pure dye; - - - - P:D = 0.25;
_____ P:D = 0.75; P:D = 1.25; _____ P:D between 5 and 50.
Bottom: Acridine orange, 2 x 10⁻⁵ M. _____ Pure dye; - - - - P:D = 1;
.... P:D = 2; _____ P:D between 5 and 50.

Fig. 4. Changes in absorption by fixation on DNA.

Top: Proflavine total concentration, 2 x 10⁻⁶ M in 10⁻³ M acetate buffer.
_____ Free dye; _____ P:D = 4; - - - - P:D > 20.
Bottom: Acridine orange total concentration, 2 x 10⁻⁶ M in 10⁻³ M
acetate buffer. _____ Free dye; - - - - P:D > 20.
p = polarization of fluorescence of the complex with P:D = 50.

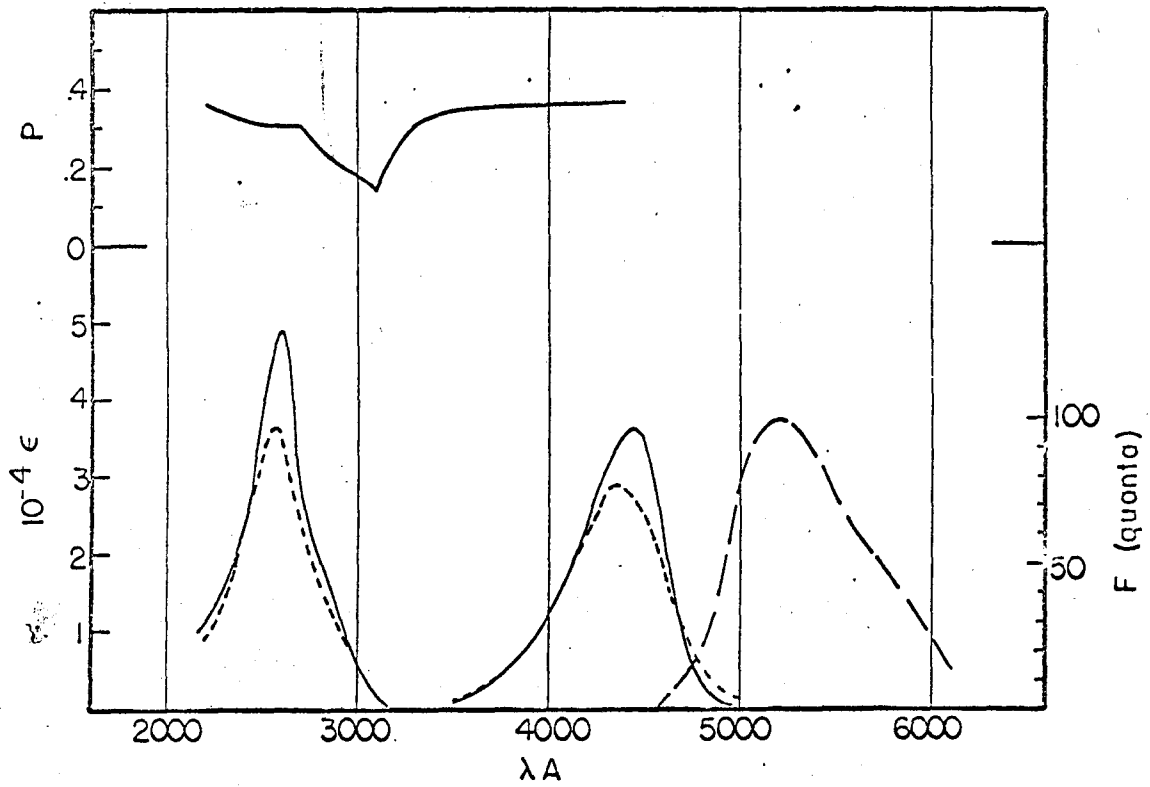
Fig. 5. Shift of the absorption and fluorescence spectra of the free and
bound dye (P:D = 100). (Relative values obtained by fitting the
maximum values arbitrarily to 1). _____ Free dye; - - - - bound dye.

Fig. 6. Relative quantum yield of bound proflavine as a function of P:D.
Open circles, excitation in the visible; closed circles, excitation
at 2600 Å.

Fig. 7. Relative quantum yield of bound acridine orange as a function of P:D.
Closed circles, excitation in the visible; open circles, excitation
at 2600 Å.

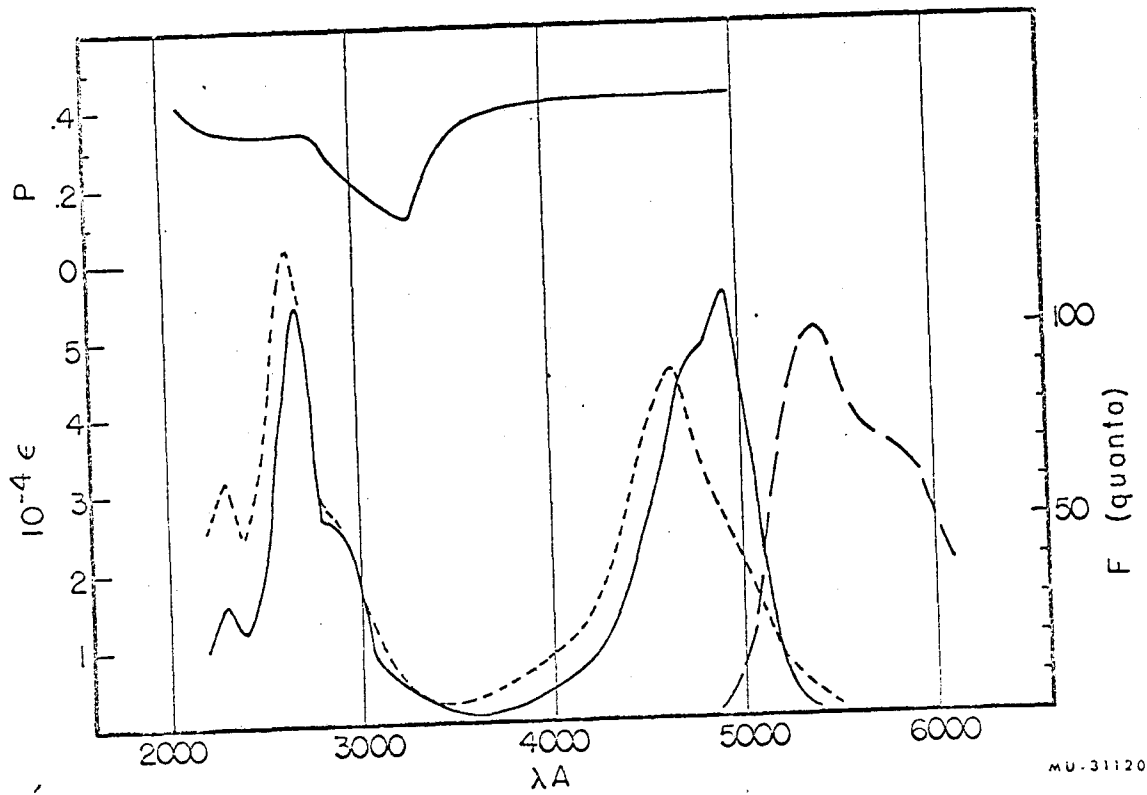
Fig. 8. Change of the polarization of fluorescence of the complex excited in the visible with the loading of the DNA.

Fig. 9. Change of the sensitization ratio (ratio of quantum yield for excitation at 2600 Å and in the visible with the loading of the DNA.



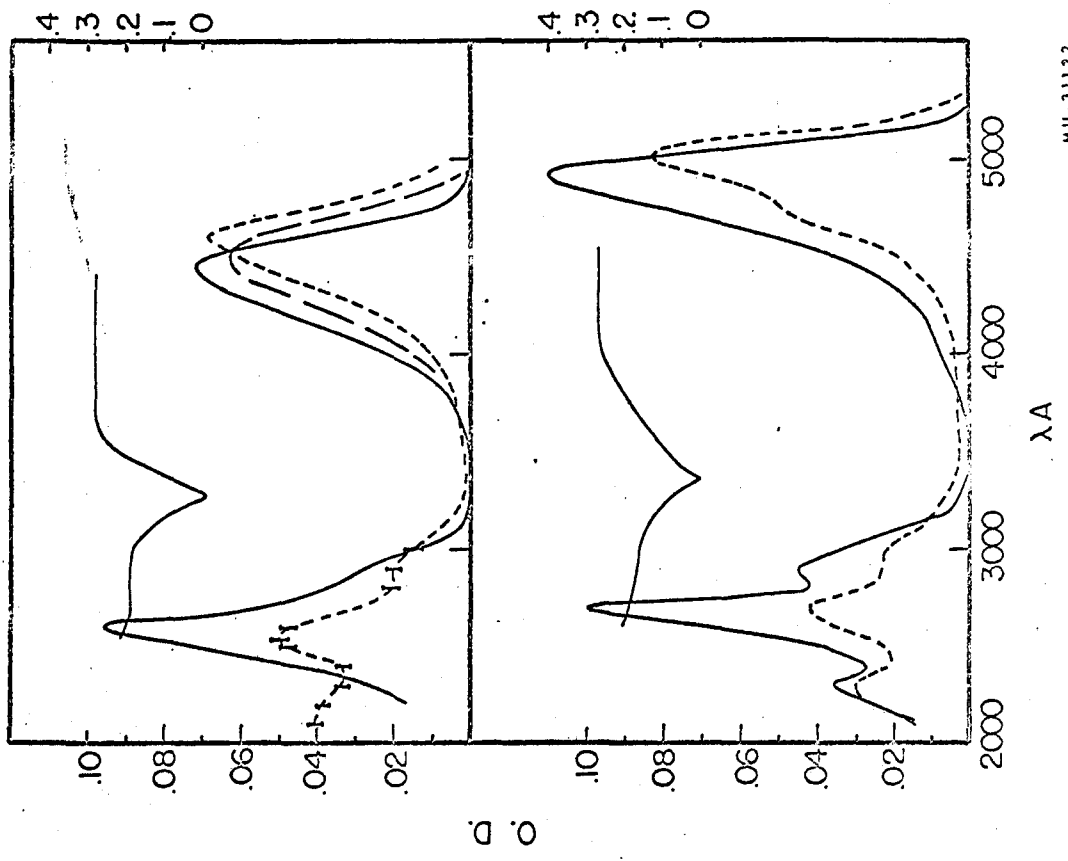
MU-31119

Fig. 1



MU-31120

Fig. 2

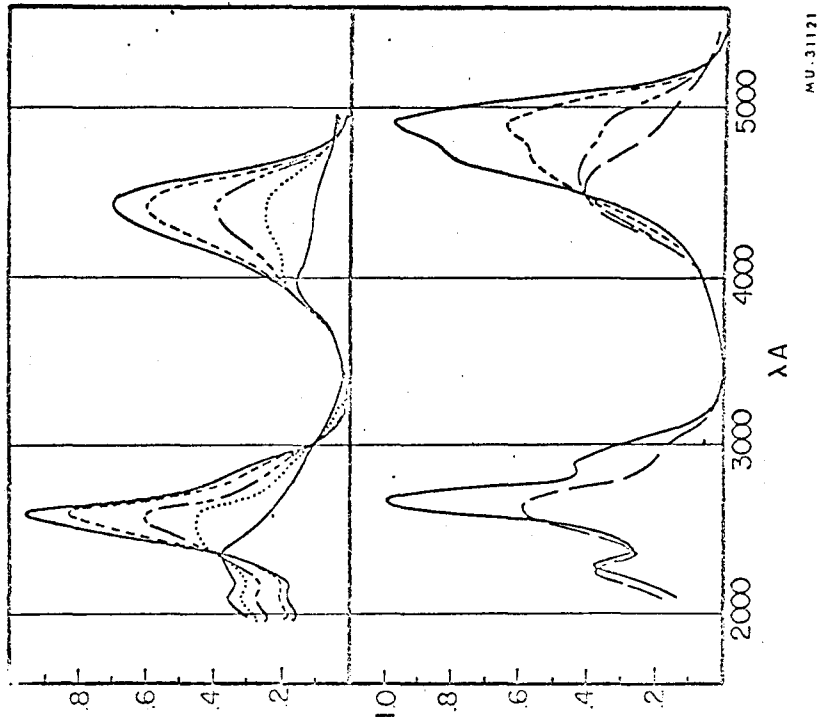


0 0

Fig. 3

MU.31122

3
4



0 0

Fig. 4

MU.31121

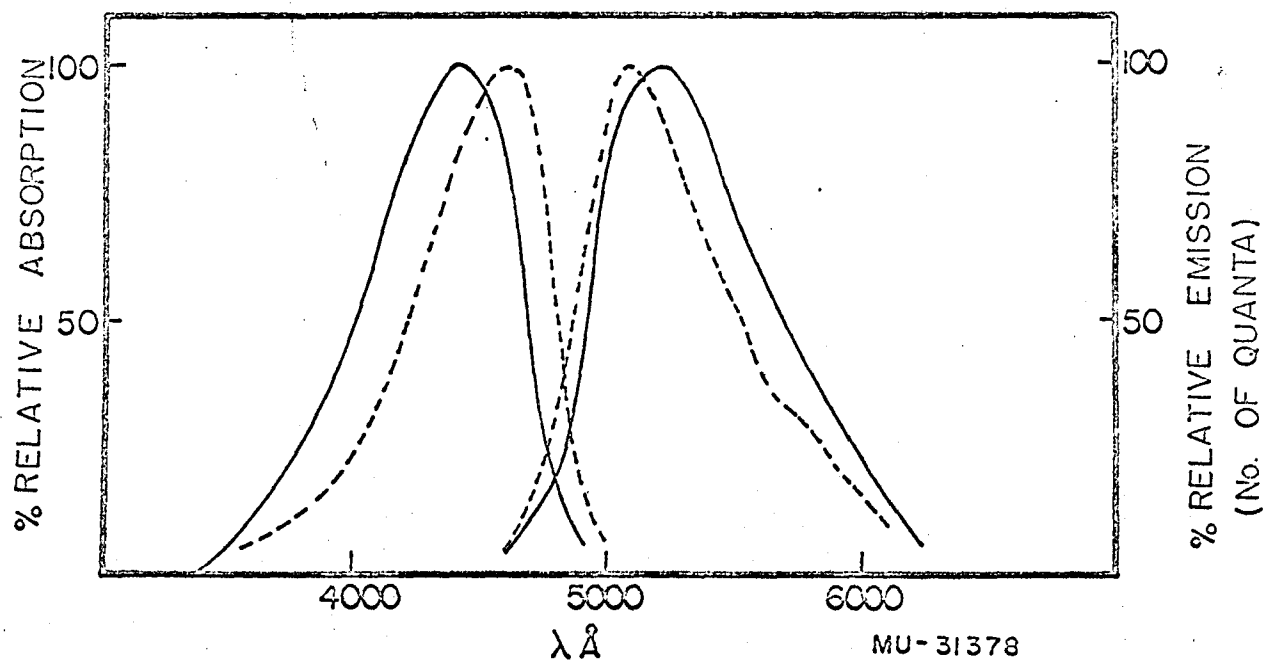


Fig. 5

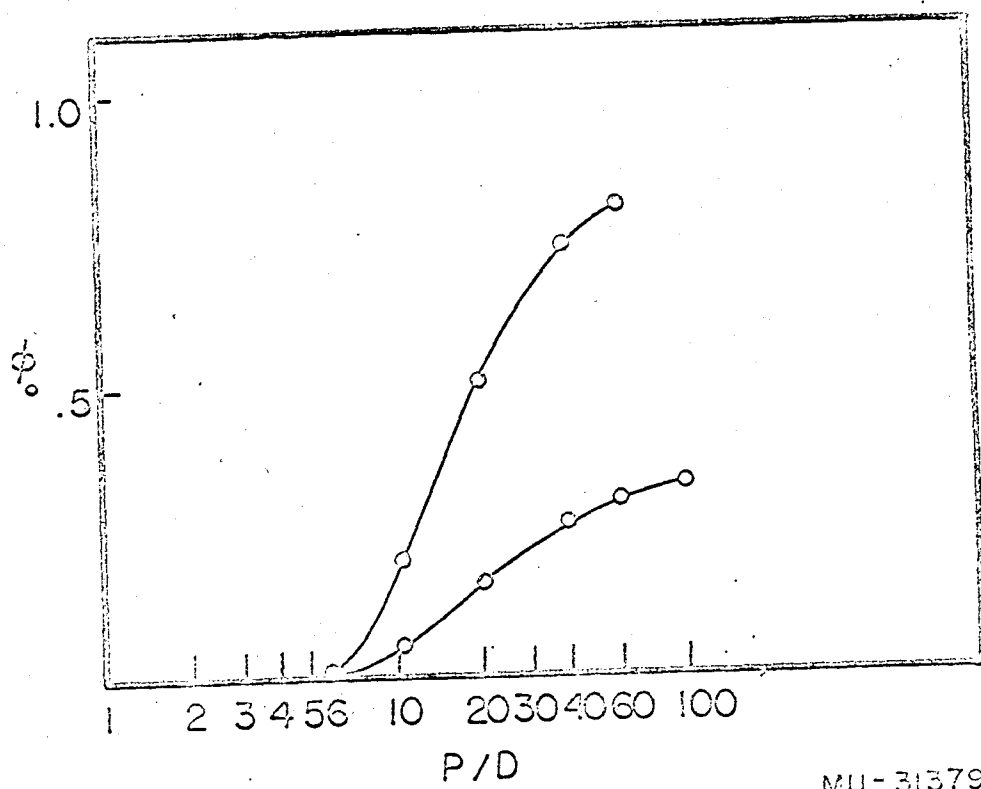


Fig. 6

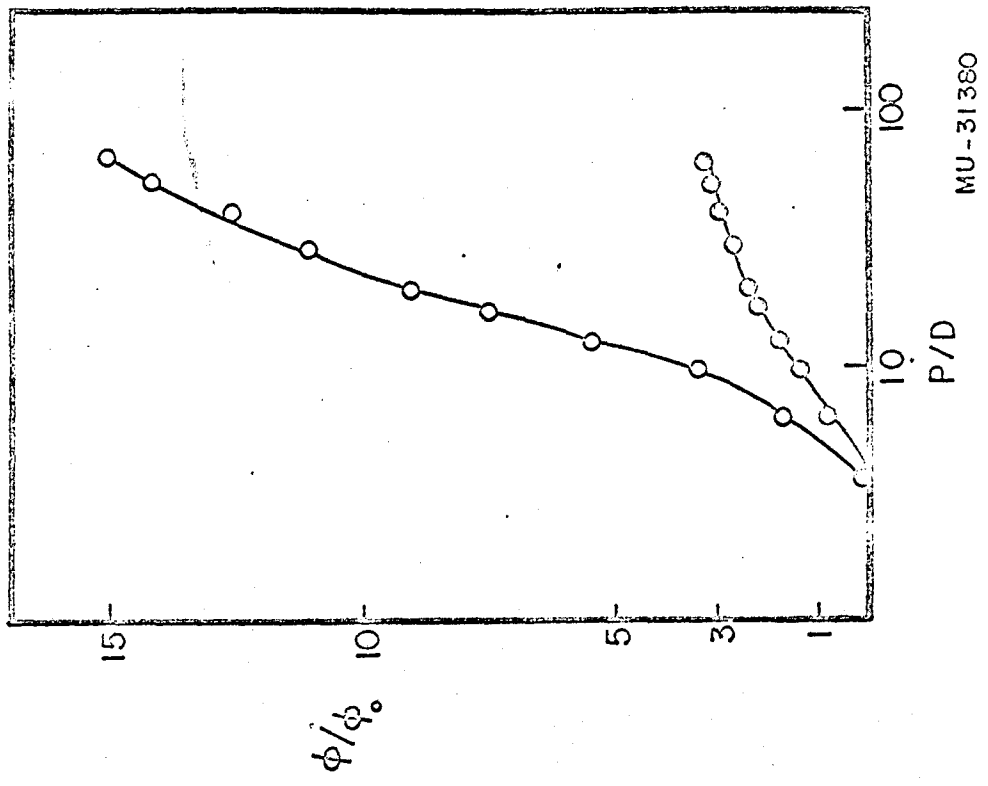


Fig. 7

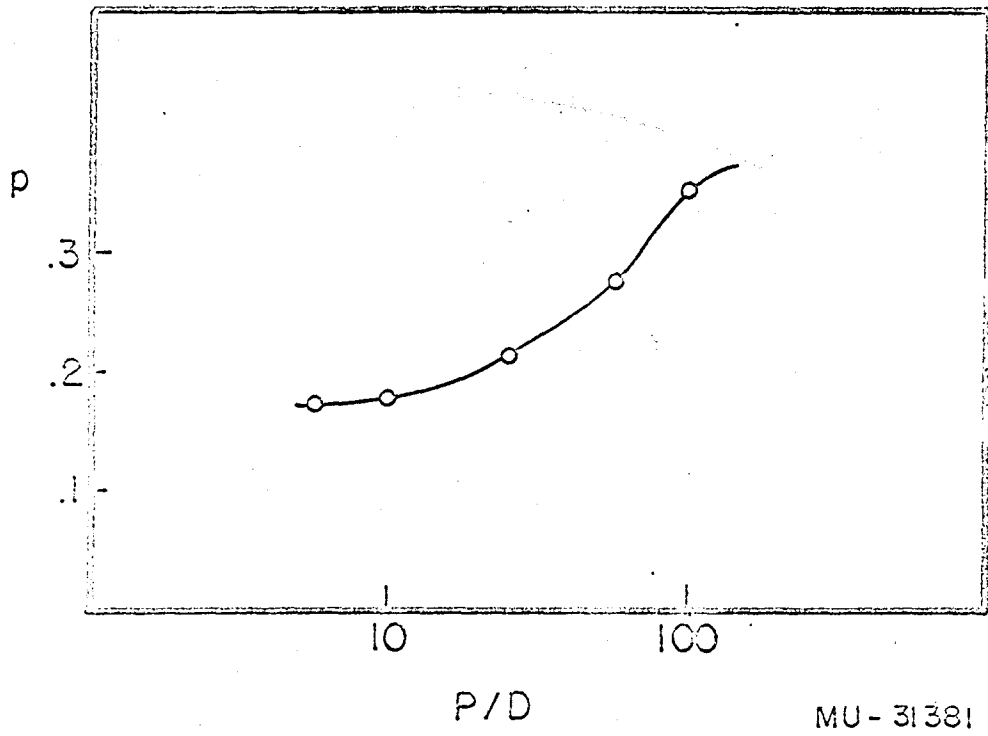
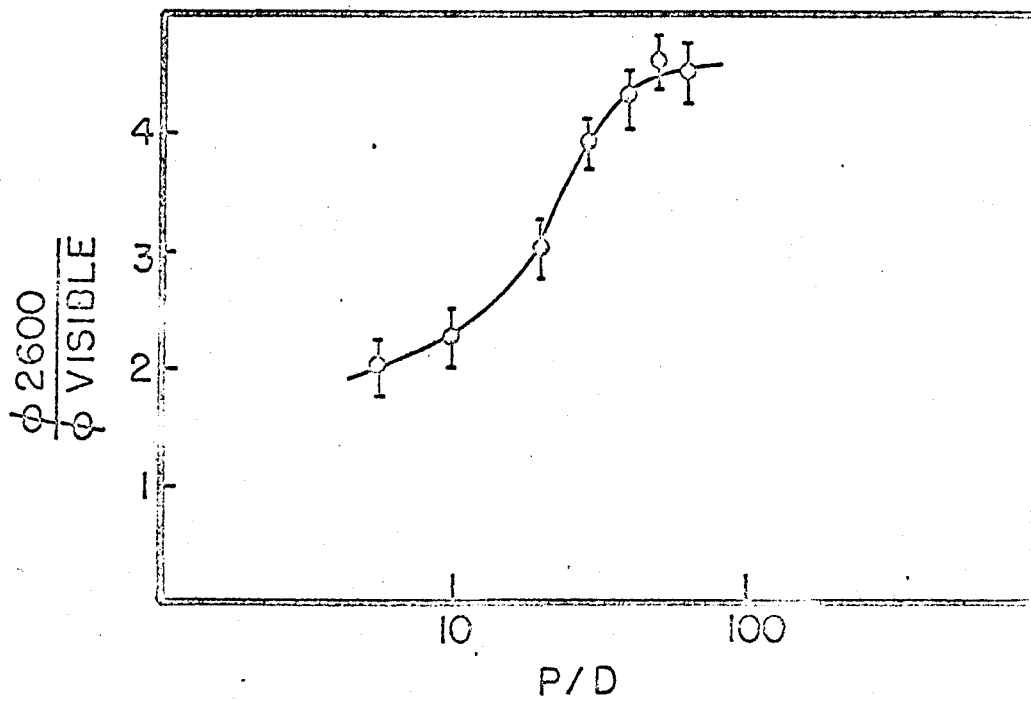


Fig. 8

7

8



MU-31377

Fig. 9

9

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