Polarized fluorescence of the dinucleotides
by Robert Wilfred Wilson

A thesis submitted in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in Chemistry
Montana State University

Abstract:
The polarization of the fluorescence of twelve dinucleotides in a mixture of 7 parts ethyleneglycol to 3 parts water was studied at -125° C, where this mixture forms a rigid glass, using the method of photoselection.

The dinucleotides ApA, ApC, and ApU were found to fluoresce as excimers and had polarization ratios which were much different from the polarization ratios of the monomers.

The fluorescence of dpApT was attributed to a mixture of monomer and excimer fluorescence. The dinucleotides dpTpT, GpG, ApG, GpA, CpG, UpG, GpU, and dpGpT were shown to not exhibit excimer fluorescence. However, even in these cases, the polarization ratios indicated some interactions between the monomers, as these polarization ratios were different from those observed for the monomers. In all cases studied, the polarization ratios for excitation and fluorescence in the region of the O-O transition were found to be very high, approaching the maximum limit indicating that the absorbing and fluorescing moments were approximately parallel. The polarization of the dinucleotides were compared with the results of both a vibronic exciton theory, such as that of Fulton and Gouterman, and Forster's theory of very weak coupling energy transfer. In all cases, the exciton resonance theory could not satisfactorily explain the observed polarization ratios. It was found necessary in most cases to include a transition moment directed out of the plane determined by the monomer moments to explain the decrease in polarization ratios with the decrease in the energy of fluorescence which was observed for all of the dinucleotides studied. This out-ofplane moment is likely due to a contribution of charge resonance character to the fluorescence state. Extended Huckel calculations were done to determine the amount of out-of-plane character in the fluorescence of CpC which had been shown to have the largest drop in polarization ratios across the fluorescence band. It was found that the amount of out-of-plane character was extremely sensitive to the orientation assumed for the two chromophores.

Additionally studied were the fluorescence excitation spectra of several bases, nucleosides, nucleotides, and dinucleotides. These excitation spectra were found to be unusual in that they were not coincident with the absorption spectra. This non-coincidence is discussed in light of two explanations. One assumes the fluorescence to be due to a minor tautomeric form which accounts for a small fraction of the absorption. The other explanation involves radiationless transitions from vibrationally excited states. It is thought unlikely that tautomerization is responsible for this non-coincidence in all cases.

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Approved:

Patrick R. Callis
Chairman, Examining Committee

E. W. Gnacker
Head, Major Department

K. Goering
Graduate Dean

MONTANA STATE UNIVERSITY
Bozeman, Montana

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ABSTRACT

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I. INTRODUCTION

Since the discovery of the excimer fluorescence of pyrene in 1954 (1), the phenomenon of excimer formation and fluorescence has been the subject of considerable investigation [for recent review, see (2)]. The term excimer, which is short for excited dimer, has been applied to molecular associates that exist only in excited electronic states. These molecular associates owe the knowledge of their existence to their characteristic fluorescence spectra, which are shifted to lower energies and broadened relative to the spectra of the monomer components. The forces responsible for excimer formation are thought to result from a combination of exciton resonance (3) and charge resonance (4,5) effects.

Polarized fluorescence experiments yield information regarding the relative directions of the transition moment that governs the absorption of light by a molecule, and that which governs its fluorescence. From such information, insight can be gained into the states that these moments connect. It was thought that measurements of the fluorescence polarization from excimers would lead to a better understanding of the nature of the excimer state and the interactions which are responsible for its creation.

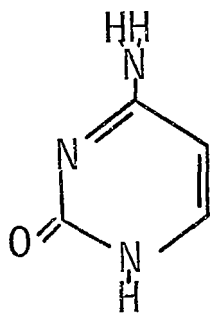
Excimer formation occurs when an excited molecule combines with an unexcited molecule to form a complex. In the case of inter-molecular excimers, this formation can be seen to be concentration dependent. That is, high concentrations promote excimer formation. Likewise, the molecules must be mobile, so that the excited and unexcited molecules may come together before the excitation energy is lost. These conditions, which promote excimer formation, are those which lead to the loss of polarization information.

The problem discussed above can be avoided by studying intra-molecular excimers, that is, by studying excimers which are formed from chromophores which are held in close proximity in the ground state. The dinucleotides have been shown to form excimers under conditions which are ideal for fluorescence polarization studies. They are known to fluoresce as excimers at low concentrations in a highly viscous glass. Furthermore, these compounds and their interactions are of obvious biological interest.

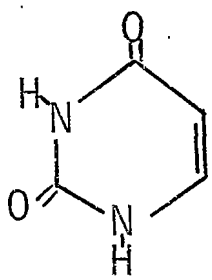
Several members of the class of compounds known as the dinucleotides have been shown to exhibit excimer fluorescence at liquid nitrogen temperatures in an ethyleneglycol-water glass (6). In general, these

compounds have absorption spectra which are little different from those observed for an equal-molar mixture of their component mononucleotides. The major difference between the absorption spectra of the component monomers and those of the dinucleotides is a reduction in the area under the absorption curves of the dinucleotides which is referred to as hypochromism.

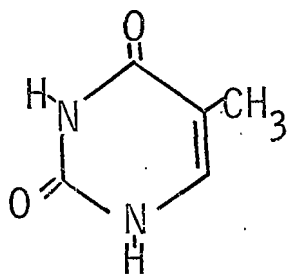
The component chromophores of a dinucleotide may include the DNA bases: adenine, guanine, cytosine, and thymine, or, in the case of a dinucleotide derived from RNA, uracil in place of thymine. These five molecules will hereafter be referred to as "the bases". The structures of these five bases, in what are thought to be their principle tautomeric forms are shown below.



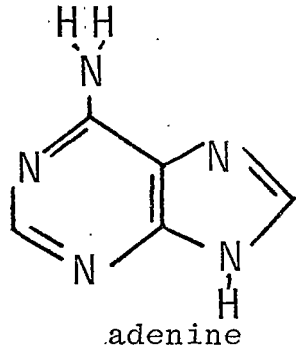
cytosine



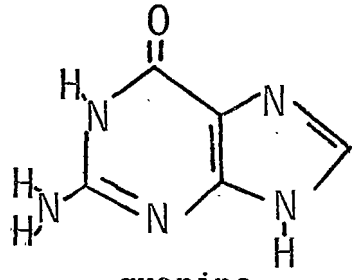
uracil



thymine



adenine



guanine

A nucleotide is composed of a base linked to either a ribose sugar (in the case of RNA) or a 2-deoxyribose sugar (in the case of DNA). This linkage is either from the 1 position (for pyrimidines) or the 9 position (for purines) to the 1′position of the sugar.

A nucleotide is the sugar-O-phosphate ester of a nucleoside. The phosphate linkage may be at either the 2′, 3′, or 5′ position for ribonucleotides or at the 3′ or 5′ position for deoxyribonucleotides.

A dinucleotide is obtained by linking two nucleosides through a phosphate bridge. Unless otherwise indicated, all of the dinucleotides discussed will be bridged between the 3′ and 5′ positions and abbreviated such that the base with the sugar bonded to the phosphate at the 3′ position is indicated first. For example, the abbreviation, ApC, stands for adenylyl(3′-5′)cytidine.

A knowledge of the nature and directions of the electronic transitions in the individual bases is required for any understanding of the interactions between coupled bases. The spectral properties of the bases have been extensively studied, and the nature of the transition moments responsible for the absorption in the region from 180 nm to 300 nm is fairly well established [for a review, see (7)]. In all cases, it appears that the strong transitions in the absorption are π-π^* transitions, and that their fluorescence is also due to π-π^* transitions. It is important to note that π-π^* transitions will have transition moments which are oriented parallel to the plane of the aromatic molecule. The absolute directions of the transition moments of the bases are not as well known. These directions, where known, will be discussed as needed in subsequent sections.

Besides their ability to form excimers, these compounds have some other interesting fluorescent properties. At room temperature and neutral pH, none of the bases, nucleotides, or dinucleotides fluoresce strongly enough for their fluorescence to be studied by ordinary means. Just recently, Daniels (8) has been able to study these compounds at room temperature using signal accumulation

techniques. In addition, it has been found that all of these compounds have fluorescence excitation spectra which are different from their absorption spectra. These observations point to the need to study the radiationless pathways in these compounds.

The purpose of this dissertation is to report the results of fluorescence polarization experiments performed on several dinucleotides, and to compare these results with theories regarding the interactions between paired chromophores. In addition, a discussion of fluorescence excitation experiments performed on several bases, nucleosides, nucleotides, and dinucleotides will be presented along with a discussion of possible explanations for the non-coincidence of these excitation spectra with the absorption spectra of these compounds.

II. EXPERIMENTAL

A. Instruments

1. Emission apparatus. The apparatus used for studying emission spectra and fluorescence polarization is a composite of a number of components arranged by Dr. P. R. Callis. Referring to Figure II-1, each component will be described as follows.

a. Source (A): An Osram XBO 150 W/1 high pressure zenon lamp was used to supply light for emission studies. This lamp is well suited to these studies for it is a stable, high intensity source with a nearly continuous output in the accessible ultraviolet. The lamp is operated at 18 volts, while drawing 7.7 amps of current.

b. Lamp power supply (B): An Oriel Optics Corporation universal power supply model C-72-20 was used to supply the proper voltage and current to the lamp.

c. Condensing lens (C): A lens assemblage consisting of 3 quartz lenses is supplied with the monochromator and was used to focus the

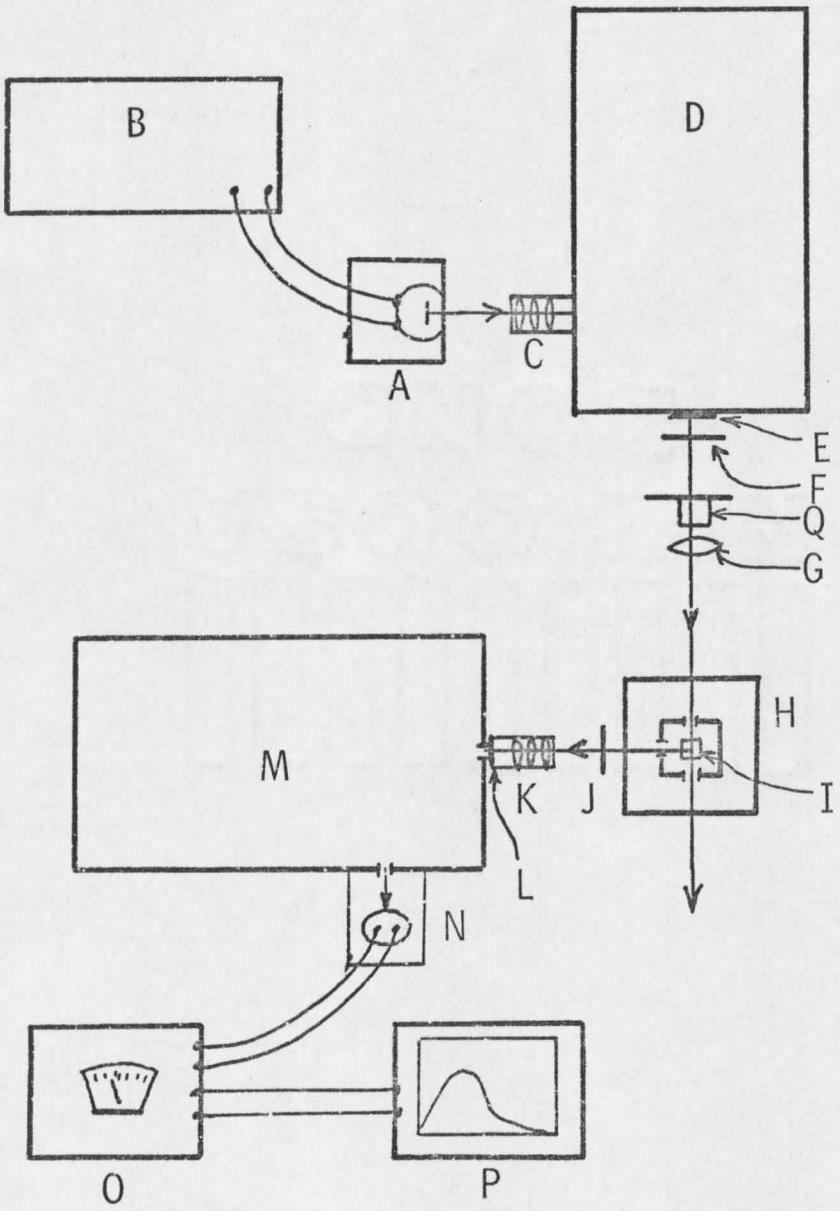


Figure II-1 Emission Apparatus

exciting light on the entrance slit of the source monochromator.

d. Source monochromator (D): This is a 500 mm focal length Bausch and Lomb grating monochromator with a reciprocal linear dispersion of 3.3 nm per mm slit width.

e. Quartz wedge (E): A 3/4° quartz wedge is positioned on the exit slit of the source monochromator to depolarize the light from this monochromator.

f. Source polarizer (F): A Polacoat ultraviolet polarizing filter supplied by Polacoat Incorporated, 9750 Conklin Road, Cincinnati, Ohio, was used to polarize the exciting light. This filter has good transmission properties and polarizing abilities to below 240 nm as indicated by Polacoat and confirmed by Dr. Callis in our lab. It was found that only a small fraction of light polarized perpendicular to the direction of polarization was passed in the spectral region of interest.

g. Condensing lens (G): A 2 inch diameter quartz lens with a 7.5 cm focal length was used to focus the slit image of the source monochromator on the sample cell.

h. Cell holder (H): An adjustable table is connected to the analyzing monochromator with an optical rail. This table can be altered to accept either a cell holder for room temperature studies or a stainless steel dewar for low temperature studies. In addition, the table can be moved to position the cell in the beam of the source monochromator, and to position the cell relative to the analyzing monochromator.

For low temperature studies, the cell is positioned in a stainless steel dewar which has three quartz windows which allow its use for either absorption or right angle fluorescence studies. When in use, a stream of nitrogen gas is bubbled through liquid nitrogen, and then passed onto the sample cell contained in the dewar; thus cooling the sample. A copper-constantine

thermocouple referenced to ice water is used to monitor the temperature at the sample cell. A signal from the thermocouple is amplified by the X-Y recorder and displayed in millivolts. The temperature at the sample cell can be varied by altering the flow rate of the nitrogen gas to the liquid nitrogen. A plot of temperature versus mv potential was prepared by Dr. Callis and was used regularly. The thermocouple set-up was checked periodically by plunging the detector end of the thermocouple in both ice water, which gives a reading of 0 mv, and liquid nitrogen, which gives a reading of 5.5 mv. The outside windows of the dewar were kept from fogging by passing a stream of air over them. This dewar was fabricated by Sulfrian Cryogenics.

i. Cell (I): Two types of rectangular fluorescence cells were used in this study. Both cells were purchased from Opticell Optical Cell Company and were made of high purity fused silica. One cell has

dimensions: O.D. = 12×12×23 mm, I.D. = 6×6×20 mm, and the other: O.D. = 12×12×23 mm, I.D. = 3×3×20 mm.

j. Analyzing polarizer (J): A commercial ultraviolet Polaroid sheet sandwiched between quartz plates was used to polarize the emitted light before entrance into the analyzing monochromator. This polarizer was found by Dr. Callis to have good transmittance and polarizing properties to 275 nm with the light passed perpendicular to the direction of polarization only a few percent of the total light transmitted.

k. Condensing lens (K): A three lens assemblage identical to c. is used to focus the light emitted from the sample cell on the entrance slit of the analyzing monochromator.

l. Quartz wedge (L): Another quartz wedge identical to e. was used to depolarize the emitted light prior to its entrance into the analyzing monochromator.

m. Analyzing monochromator (M): This monochromator is identical to the source monochromator. It is positioned with its entrance slit at approximately 90° to the direction of the exciting light.

n. Detector (N): The detector is an EMI 9558QC phototube which has fused silica windows. This phototube has a spectral range from 165 nm to 850 nm.

o. Photometer (O): A Pacific Photometric Laboratory Photometer Model 11 was used to measure the photocurrent from the detector. The photometer will give full meter deflection on its most sensitive setting with a signal of 10^{-9} amperes.

p. Recorder (P): A Hewlett Packard Model 7030A X-Y recorder was used to display the signal from the photometer in a recording made on a piece of graph paper such that a graph of signal intensity versus wavelength is obtained.

q. Chopper and lock-in amplifier (Q): In addition to the above components, for some

experiments a light chopper and a Princeton Applied Research Model 122 lock-in amplifier were used. The chopper is a cardboard disc having equal areas of light passage and blockage driven by an A.C. motor. It was used to supply a D.C. signal and reference signal to the lock-in amplifier. In this mode the emission apparatus may be used to study fluorescence in the presence of phosphorescence. Phosphorescence, being much slower than fluorescence, represents a D.C. signal which is removed by the lock-in amplifier.

2. Low temperature ultraviolet absorption apparatus. The same dewar that was used in the emission apparatus was fitted with a collar which replaces the cell compartment lid on a Cary 14 recording spectrophotometer. The collar can be adjusted for height and translation so that the sample cell may be centered in the light beam of the spectrophotometer. As before, a stream of air is blown on the outside windows of the dewar, and cooled nitrogen gas is used to cool the sample.

B. Origin and Purity of Materials

1. Nucleotides, nucleosides, dinucleotides, and bases. These compounds were obtained in pure cyrstalline form from several sources. They were used for absorption, emission, and polarized fluorescence experiments without further purification, as their spectral properties, except for a few dinucleotides which will be discussed later, showed no hint of impurities. In order to investigate purity, the emission spectra of the compounds were recorded upon excitation at several different wavelengths, and invariance in the shapes of the spectra was taken to indicate purity. Also, their absorption spectra were compared with published spectra.

The compounds used and their sources are as follows:

Name	Company
Guanine	Calbiochem A Grade
Guanosine	Calbiochem A Grade
7 Me-Guamine	Sigma
GMP	Sigma
GMP	Calbiochem A Grade
GpG	Sigma
ApG	Sigma
GpA	Sigma
CpG	Sigma
GpC	Sigma

Name	Company
UpG	Sigma
GpU	Sigma
dpGpT	Collaborative Research
Adenine	Calbiochem A Grade
AMP	Calbiochem A Grade
7 Me-Adenine	Sigma
ApA	Sigma
ApC	Sigma
CpA	Sigma
2′-5′ApC	Sigma
ApU	Sigma
UpA	Sigma
dpApT	Collaborative Research
Polyadenylic acid (5′)	Sigma
Cytosine	Calbiochem A Grade
CMP	Sigma
5 Me-Cytosine	Calbiochem A Grade
5 Me-Deoxycytidine	Calbiochem
CpC	Sigma
UpC	Sigma
CpU	Sigma
Polycytidylic acid (5′)	Sigma
dpCpC	Collaborative Research
Thymine	Calbiochem A Grade
TMP	Calbiochem A Grade
dpTpT	Collaborative Research

2. Miscellaneous compounds.

Name	Company
Tryptophan	
Tyrosine	
Salicylic Acid	Mallinchrodt
Rhodamine B	J. T. Baker
Quinine Bisulfate	Eastman
Activated Carbon	Nuchar

3. Solvents. The solvents used for emission experiments were treated so as to have virtually no emission as compared with distilled water when excited from 240 nm to 310 nm.

a. Ethylene glycol was purchased from Eastman, and Matheson, Coleman, and Bell. As ethylene glycol from both of these sources displayed considerable fluorescence in the spectral region studied, it was necessary to purify it. This was done by introducing activated charcoal into the solvent and then gravity filtering the mixture through a millipore filter to remove the charcoal.

b. Methanol was obtained from J. T. Baker, and, depending on the batch, was used without

further purification or treated with activated charcoal as was the ethylene glycol.

C. Experimental Procedures

1. Preparation of glasses. Two basic types of glasses with slight modification of the concentration of components were used in this work.

a. Glasses consisting of 12 parts methanol and from 1 to 3 parts water by volume were used at temperatures of around -135° C.

b. Glasses consisting of 5 parts ethylene glycol and 5 parts water to 7 parts ethylene glycol and 3 parts water by volume were used at temperatures from around -110° C to -135° C where the glasses may become too rigid and crack. The aqueous phase used in each of these glasses contained 1×10^{-2} M phosphate to buffer the pH of the solutions near 7. In the rest of this thesis, these glasses will be abbreviated as G-50 to G-70.

2. Alignment of the monochromators. In order to insure that the wavelength of light being passed by the monochromators used corresponded to the reading on the

wavelength drum on each, a preliminary alignment was performed. This was done by monitoring the output of the analyzing monochromator which was irradiated by a low pressure Hg lamp. By comparing the maximum signal responses as the violet and ultraviolet spectral regions were scanned with the known emission spectra of Hg, corrections were made by turning the drum on the wavelength drive so as to make its reading correspond to the correct wavelength. After this was done, light from the source monochromator was reflected into the analyzing monochromator and both monochromators were set to the same wavelength. Then the source monochromator was made to scan on both sides of the wavelength setting. The output of the detector was monitored during this time, and the wavelength drum of the source monochromator was turned so that its reading was identical to that of the analyzing monochromator when the maximum signal was observed.

3. Determination of relative source intensities. In order to determine quantum yields and correct observed emission action spectra, it is necessary to know the intensity of the excitation source as a function of wavelength. In practice, it is much easier to obtain precise

relative intensity measurements than to determine precise absolute intensities. The method used to obtain the relative intensities of the light at the sample was the rhodamine B quantum counting technique as described by Melhuish (9). A solution containing 3 g/l of rhodamine B in ethylene glycol was put in the sample cell and positioned in the cell holder. Due to the high optical density of the rhodamine B solution, nearly all of the light incident on the cell is absorbed in the first few millimeters of solution. Therefore, the intensity of the fluorescence from this part of the solution is proportional to the intensity of the incident light. The fluorescence of the solution at its fluorescence maximum of 620 nm was therefore monitored as the wavelength of the incident light was varied from 220 nm to 320 nm. Table II-1 shows the results of experiments done with a 6 cm I.D. fused silica cell position in the dewar, which is the arrangement used in all low temperature work reported, and of experiments done with a 1 cm fluorescence cell outside the dewar. The 1 cm cell was stoppered and used as a fluorescence standard prior to subsequent fluorescence determinations.

Table II-1. Relative Incident Light Intensities

Wavelength (nm)	I_0 [a]	I_0 [b]
220	0.024	0.023
225	0.038	0.037
230	0.066	0.064
235	0.12	0.11
240	0.17	0.17
245	0.25	0.25
250	0.33	0.32
255	0.41	0.41
260	0.50	0.50
265	0.60	0.61
270	0.70	0.74
275	0.84	0.87
280	1.00 [c]	1.00
285	1.15	1.19
290	1.35	1.39
295	1.52	1.60
300	1.72	1.80
305	1.93	2.02
310	2.11	2.25
315	2.32	2.50
320	2.46	2.75

a - Measurements taken in a 1 cm fluorescence cell.

b - Measurements taken in a 6 mm fluorescence cell inside the low temperature dewar.

c - All measurements were taken at the front edge (maximum signal) of a solution of rhodamine B with the slits of the source monochromator set at 1 mm and 1 mm and the relative intensities normalized to 1.00 at 280 nm.

In addition to the above determination, similar experiments were performed using fluorescein and quinine bisulfate. The fluorescein gave similar results, but the quinine bisulfate was found to give poor results due to a deep minimum in its absorption spectra near 270 nm.

To further verify our relative intensity measurements, the fluorescence excitation spectra of four compounds, which are known to have quantum yields that are independent of the wavelength of excitation, were corrected for variations in the intensity of the exciting light using our relative intensity data. The absorption spectra and corrected excitation spectra of salicylic acid, tyrosine, and quinine bisulfate are shown in Figure II-2. Equally good results were obtained for salicylic acid at low temperature in G-70, thus allowing these corrections to be applied to our experimental conditions.

4. Calibration of the emission detector. The spectra obtained from the emission instrument used are plots of phototube signal versus wavelength. Since the analyzing monochromator/phototube combination has a sensitivity which depends on the wavelength of light measured, true

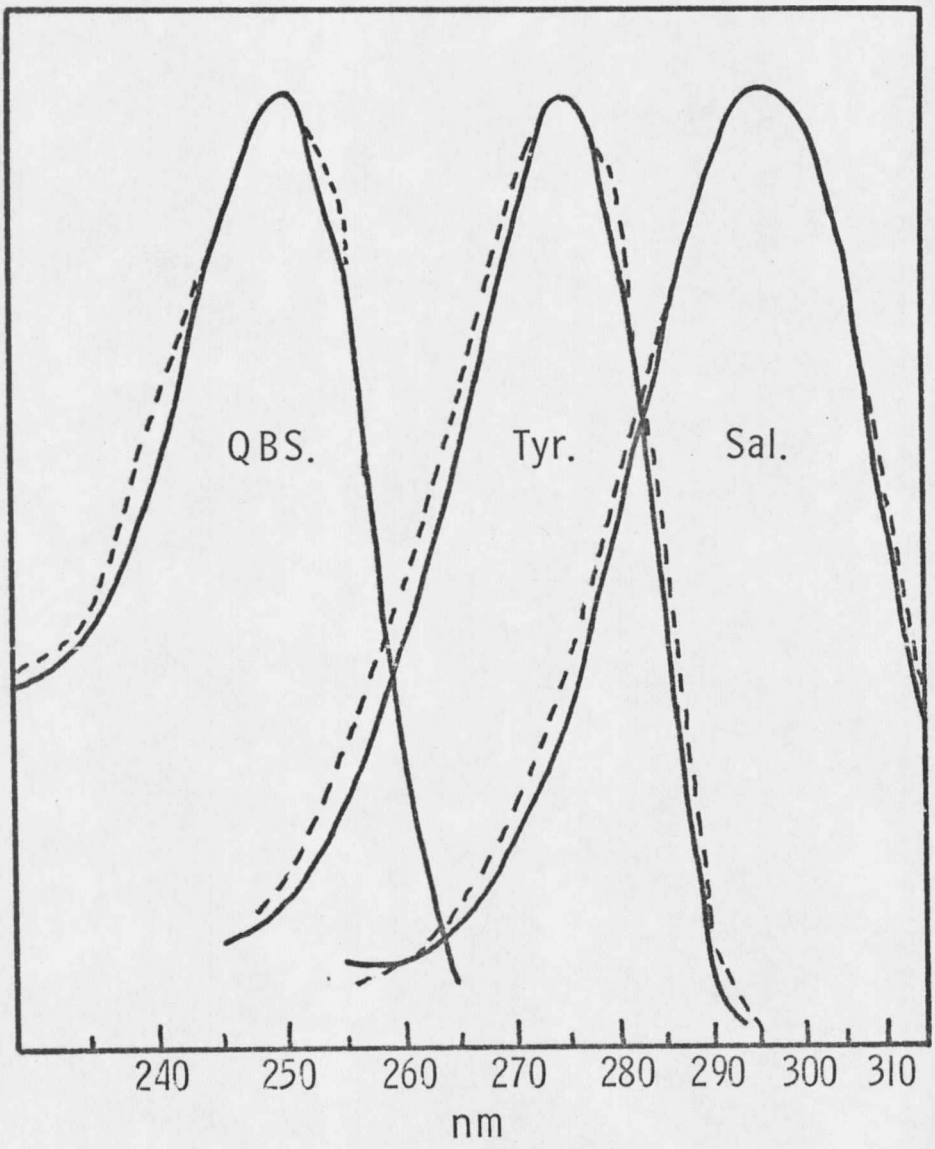


Figure II-2 Fluorescence Excitation Spectra --- and Absorption Spectra —— of Quinine Bisulfate, Tyrosine, and Salicylic Acid

emission spectra can be obtained by taking this variation in sensitivity into account.

In order to determine the detector sensitivity of the instrument as a function of wavelength, a procedure similar to that described by Parker (10) was used. In this procedure, light of a known intensity is reflected from a MgO screen into the analyzing monochromator, and the phototube signal is noted. Since the reflectivity of MgO is nearly constant for the wavelength of interest, the relative response of the monochromator/phototube combination can be obtained by dividing the signal from the phototube by the intensity of the incident light. The incident light intensity was determined by the rhodamine B quantum counting technique described before. Figure II-3 shows the relative response of the detector system as a function of wavelength from 250 nm to 575 nm.

This relative response data were used in integrating the fluorescence envelopes of the compounds for which fluorescence quantum yields were determined. It can also be used to obtain the true emission spectra from the observed spectra which will be reported in this work.

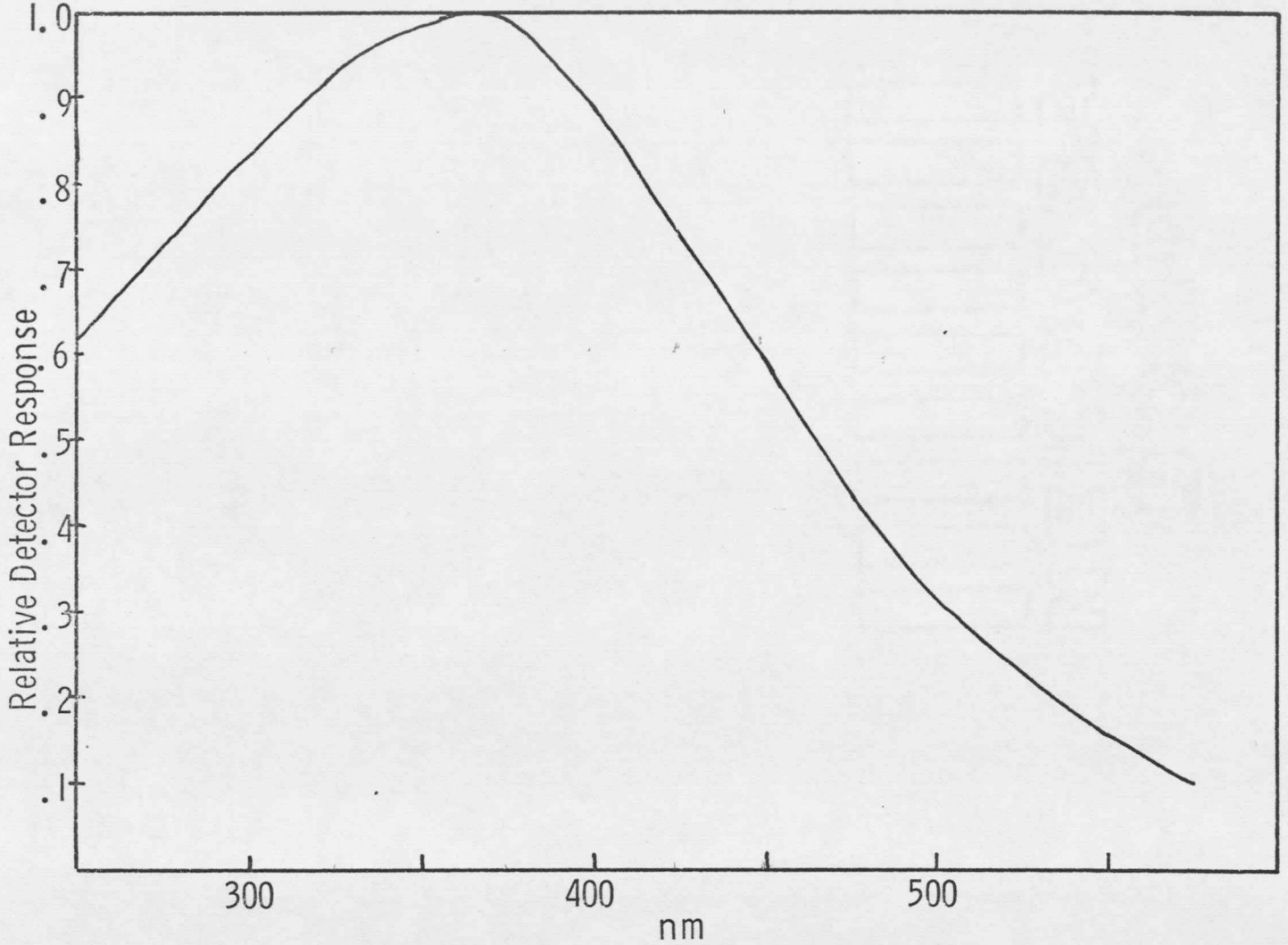


Figure II-3 Relative Response of the Emission Detector

5. Low temperature absorption spectra. All absorption spectra reported were taken on a Cary 14 dual beam absorption spectrophotometer versus air in the reference compartment and corrected for absorption due to the solvent and sample container used. This was done by first inserting the cell, containing the pure solvent, or the cell and the dewar for low temperature work, into the instrument. The wavelength drive was set to a wavelength at which the sample was known not to absorb. This wavelength was 340 nm for the dinucleotides. The pen was then adjusted to zero absorption, and the spectral region of interest was scanned from low energy to high, thus recording the absorption spectrum of the solvent. Then a solution of the compound of interest is added to the cell and the absorption outside the absorption band is again monitored to see that it is the same as the pure solvent. If this criterion is met, the spectrum is scanned from low to high energy. The absorption spectra reported here are the difference between these two spectra.

If low temperature spectra are desired, at this point the sample is cooled to the temperature desired and allowed to sit at this temperature for about ten minutes.

If no change in the base line is noted, the spectrum is taken and reported as above.

All solutions used for absorption work were prepared by adding a small amount of the compound of interest to the solvent contained in a small flask for batch work, or in the sample cell for single determinations. Their absorptions were adjusted by addition of sample or solvent to around 0.5 to 0.7. The solutions were then allowed to sit for a period of time and their absorption was checked to make sure all the sample was in solution. This period of time varied from several minutes for most compounds up to a few days for guanine.

6. Emission spectra. Since none of the DNA bases, nucleosides, nucleotides, or dinucleotides have any emission detectable by our apparatus at room temperature and neutral pH, all emission spectra reported were taken at low temperatures in rigid glasses. In all cases, the sample cell containing the sample was placed in the stainless steel dewar and cooled to the desired temperature, as described before. A short time was allowed for the sample to cool, as there seemed to be a lag between the cooling of the sample and the cooling of the cell holder which

contained the thermocouple end. An emission spectra was than taken by choosing a wavelength of excitation within the absorption band of the chromophore and monitoring the emission as a function of wavelength. After waiting a short time, the emission was again scanned to see that there was no change in the emission spectra, indicating that the temperature of the sample had stabilized. Then the emission spectrum of the sample was taken at a number of different wavelengths to see that the shape of the emission spectra was indpendent of the wavelength of excitation within the absorption band of the sample, and that there was no significant emission when exciting outside of its absorption band. This independence of shape on varying excitation wavelength was a criterion for purity of the sample. In some cases, the shape of the emission band was not independent of wavelength, and these dependences are not thought to be due to impurities. These cases will be dealt with at length later.

If the emission spectrum contained both fluorescence and phosphorescence, and only the fluorescence spectrum was desired, some electrical connections on the apparatus were interchanged, and the light chopper was inserted in the path of the exciting light. In this mode,

the signal from the detector is amplified by the lock-in amplifier rather than the photometer. The photometer is used to amplify a reference signal from another phototube which is positioned to sense the chopped light of a small light bulb. The output of the lock-in amplifier can then be displayed on the X-Y recorder and represents the emission spectra in the absence of slow phosphorescence.

7. Polarized fluorescence. In all polarized fluorescence experiments performed, the low temperature fluorescence of the sample was first recorded as above. The polarizers were then mounted in their respective positions and the analyzing polarizer was turned against a stop, thus positioning it such that light polarized perpendicular to the plane of the table will be passed. The source polarizer was then turned so that it would pass light polarized perpendicular to the plane of the table, that is, both polarizers are "parallel". The analyzing monochromator was then set to a wavelength near the center of the fluorescence spectrum and this wavelength was recorded. The source monochromator was set to wavelength near the low energy edge of the absorption spectra, and the signal from the detector was read from the X-Y recorder, thus giving

the fluorescence intensity when the polarizers are parallel ($I_{\parallel}$). The source polarizer was then rotated 90° and the intensity was again read giving $I_{\perp}$. This procedure was repeated as the wavelength of excitation was decreased in 5 nm intervals. The analyzing monochromator was then set to a wavelength near the high energy side of the fluorescence spectrum, and the process repeated. The process was again repeated with the analyzing monochromator set on the low energy side of the fluorescence. Then the source monochromator was set to three wavelengths: one near the low energy side, one in the middle, and one near the high energy side of the absorption band. The intensities $I_{\parallel}$ and $I_{\perp}$ were recorded as the analyzing monochromator was varied from the high energy to the low energy side of the fluorescence band in increments of 10 nm.

In earlier experiments, the orientation of the source polarizer was kept constant, and the analyzing polarizer was rotated. It was found that this method gave the same results as the method described above. Since it was more convenient, it was decided to rotate the source polarizer rather than the analyzing polarizer.

8. Fluorescence excitation spectra. All of the emission spectra discussed up to this point were the result of using exciting light of a fixed wavelength and noting the response of the phototube as the wavelength of light passed to the phototube is varied. There is another type of emission spectrum known as the emission excitation, or emission action spectrum. These spectra are generated by viewing the emission at a fixed wavelength and monitoring the phototube output as the wavelength of excitation is varied. If Vavilov's law and Kasha's rule (11) hold, then the intensity of the emitted light should be proportional to the amount of light absorbed. This can be written as

$$E(\lambda) = KI_0(\lambda)\cdot(1-10^{-A(\lambda)}) \qquad (2.1)$$

where $E(\lambda)$ is the emission intensity for excitation at wavelength λ, $I_0(\lambda)$ is the incident intensity of the excitating light, and $A(\lambda)$ is the absorbance of the solution at wavelength λ. For low absorbances, the emission intensity becomes approximately proportional to the absorbance.

$$E(\lambda) \simeq KI_0(\lambda)A(\lambda) \qquad (2.2)$$

Therefore, the emission excitation spectrum of a solution with a sufficiently low absorbance, and after correcting for variations of I_0, should have exactly the same shape as the absorption spectra.

All of the fluorescence excitation spectra reported here were obtained in the same manner. Solutions were prepared and their room temperature and low temperature absorption spectra were taken as described in the previous section. The samples were then diluted so as to give an absorbance at the maximum of 0.2 when taken in a 6 mm cell at low temperatures. Prior to making any emission measurements, the variation of I_0 with wavelength was checked using the rhodamine B solution described earlier. The sample was then placed in the sample cell and was cooled to the desired temperature. The emission spectrum of the sample was then determined as in Section 5. The analyzing monochromator was then set to the desired wavelength, usually the wavelength of the maximum fluorescence signal, and the wavelength of the exciting light was varied from 220 to 320 nm. In the case of weakly fluorescing samples, the excitation spectrum of the solvent was determined so as to provide a solvent base line. This was done by immediately replacing the sample with the pure solvent and

recording the excitation spectrum of the solvent under the same conditions as those used for the sample.

All excitation spectra were taken with the sample cell arranged so that the detector system was viewing the front part of the cell. Since the detector only views approximately 3 mm of the cell, this provides an effective absorbance maximum of about 0.1. In addition, all spectra were obtained with the slits of the excitation monochromator set at 1 mm.

The uncorrected excitation spectra as obtained above were corrected for variations in the lamp intensity and plotted along with the absorption spectra using a Fortran computer program, the M.S.U. Sigma 7 computer, and a Calcomp Drum Plotter.

III. POLARIZED FLUORESCENCE OF THE DINUCLEOTIDES

A. Application of the Technique of Photoselection

The application of photoselection to polarized fluorescence has been reviewed by Albrecht (12). In this technique, polarized light is used to excite molecules held in a rigid media. A rigid media is necessary because it is required that the molecules do not rotate from the time of excitation until they fluoresce. The light emitted by these molecules is then passed through another polarizer which is oriented first parallel, and then perpendicular to the direction of the source polarizer. The ratio of the intensity of the emitted light when both polarizers are parallel to that when they are perpendicular is observed. This ratio can be expressed as

$$I_{\parallel} / I_{\perp} = (2S+1)/(2-S) \qquad (3.1)$$

where

$$S = \frac{\sum_{k} \sum_{\ell} c_{k\ell} m_k^2 m_\ell^2 (\vec{\ell} \cdot \vec{k})^2}{\sum_{k} \sum_{\ell} c_{k\ell} m_k^2 m_\ell^2} \qquad (3.2)$$

where m_k and $\vec{k}$ are the magnitude and direction of the transition moment responsible for the kth transition in the

absorption, m_ℓ and $\vec{\ell}$ are the magnitude and direction of the transition moment responsible for the ℓth transition in the emission, and $c_{k\ell}$ is the probability that light absorbed by moment k will be emitted by moment ℓ. If all the $c_{k\ell}$'s are equal, which is thought the usual case for molecules, then

$$S = \sum_k \sum_\ell r_k q_\ell (\vec{k} \cdot \vec{\ell})^2 \qquad (3.3)$$

where r_k and q_ℓ are the fractions of light absorbed by the kth transition moment and the fraction emitted by the ℓth transition moment.

Electronic transitions are generally composite in nature so r_k is a function of the excitation frequency ν_a and q_ℓ is a function of the frequency of the emitted light, ν_e. Therefore, S and $I_\parallel / I_\perp$ are functions of both ν_a and ν_e, and it is the variation of the polarization ration with frequency with which this section deals.

By using equations (3.1) and (3.3), it can be seen that if all the light absorbed at a given wavelength is absorbed by moment k and all the light emitted is by a moment ℓ, and ℓ and k are parallel, then S = 1 and $I_\parallel / I_\perp$ = 3. This represents the maximum theoretical value of S

which is obtained when the absorbing moment is parallel to the emitting moment. If k and ℓ are perpendicular, then $S = 0$ and $I_{\parallel} / I_{\perp} = 0.5$, this being the minimum theoretical value. In practice, these limits are never attained, and a value of $I_{\parallel} / I_{\perp} \simeq 2.5$ for parallel transitions is actually observed. This deviation from theory is handled by introducing a factor, $\varepsilon = 3/2 \sin^2\theta$, which randomizes the direction of ℓ around a cone with angle θ to the axis. With this randomization factor

$$\frac{I_{\parallel}}{I_{\perp}} = \frac{2S(1-\varepsilon) + 2/3\varepsilon + 1}{2 - \frac{\varepsilon}{3} - S(1-\varepsilon)} \qquad (3.4)$$

where S is the same as before.

B. Spectral Features and Fluorescence Polarization of dpTpT

The absorption and fluorescence spectra and the fluorescence polarization ratios for TMP at -125° C in a G-70 glass are shown in Figure III-1. It can be seen that the polarization ratios are very high for excitation at any wavelength within the first absorption band when the fluorescence is obtained at any wavelength within the fluorescence band. This uniformly high polarization

Figure III-1

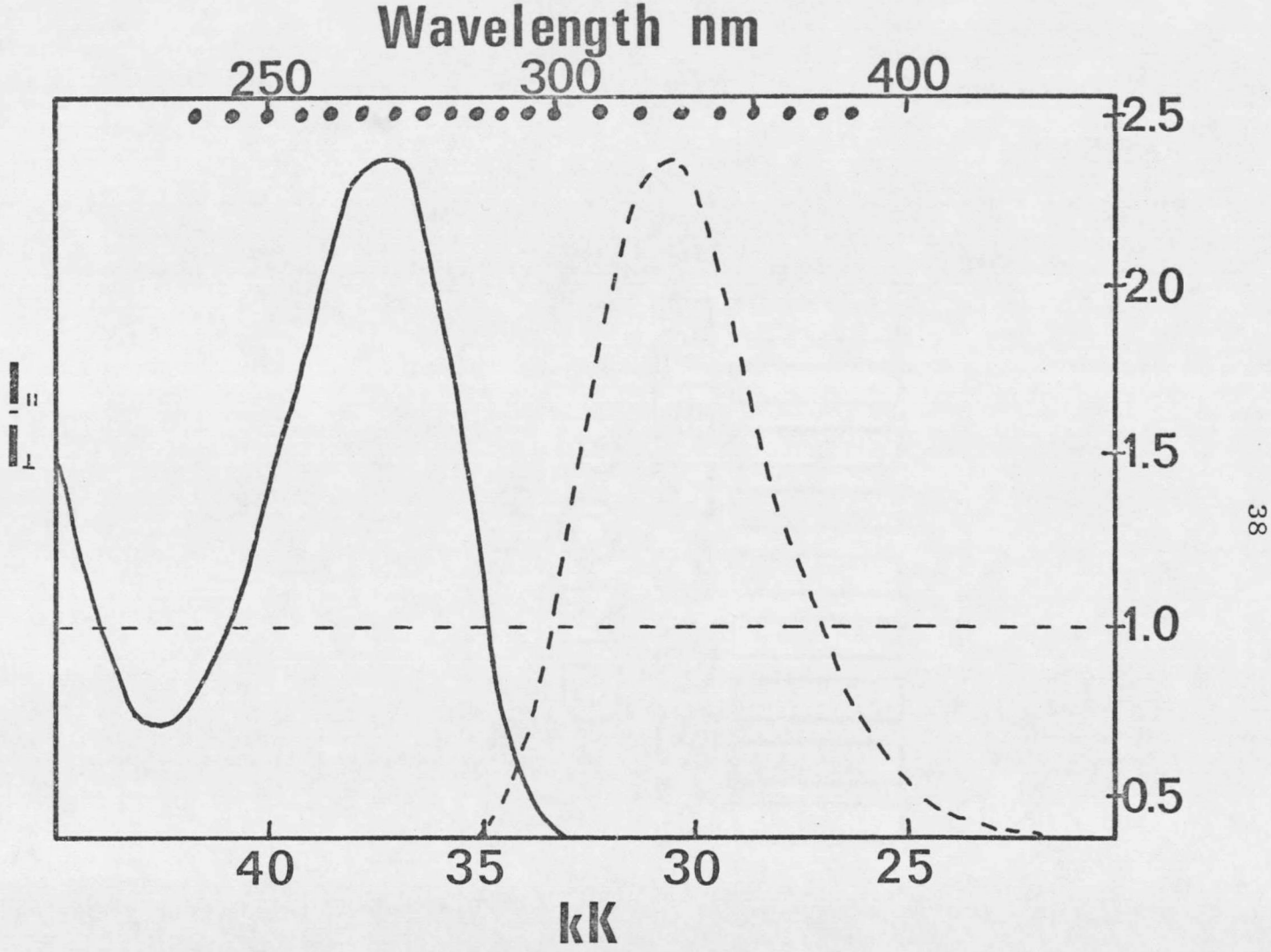
TMP in G-70 at -125° C

—— Absorption

--- Fluorescence

The Points on the Left Show the Polarization as a Function of the Excitation Wavelength While Observing the Fluorescence at Any Wavelength from 305 nm to 360 nm (●)

The Points on the Right Show the Polarization as a Function of the Fluorescence Wavelength While Exciting at Any Wavelength from 240 nm to 290 nm


Wavelength nm
250
300
400
2.5
2.0
1.5
1.0
0.5
40
35
30
25
kK

indicates that only one transition moment is responsible for all of the absorbance fluorescence of this molecule in the wavelength region studied.

The absorption and fluorescence spectra of dpTpT, as shown in Figure III-2, are nearly identical to those of TMP. However, in contrast to the uniformly high fluorescence polarization ratios of TMP, those of dpTpT can be seen to drop across both the absorption and fluorescence bands. This change in polarization ratios is evidence for an interaction between the molecules forming the dimer.

As TMP would appear to have only one transition moment in the wavelength region studied, the dpTpT molecule represents a good system for comparison between experimentally determined values, and values calculated using the exciton resonance model for dimers. For a discussion of this model, see Appendix I.

In order for a model to be considered satisfactory, it must be capable of correctly predicting not only the general shapes and maxima of the absorption and fluorescence spectra of the dimer, but must also correctly predict the observed polarization ratios. Callis (13) has discussed the possibility of correctly predicting the polarization ratios of CpC using an exciton resonance

Figure III-2

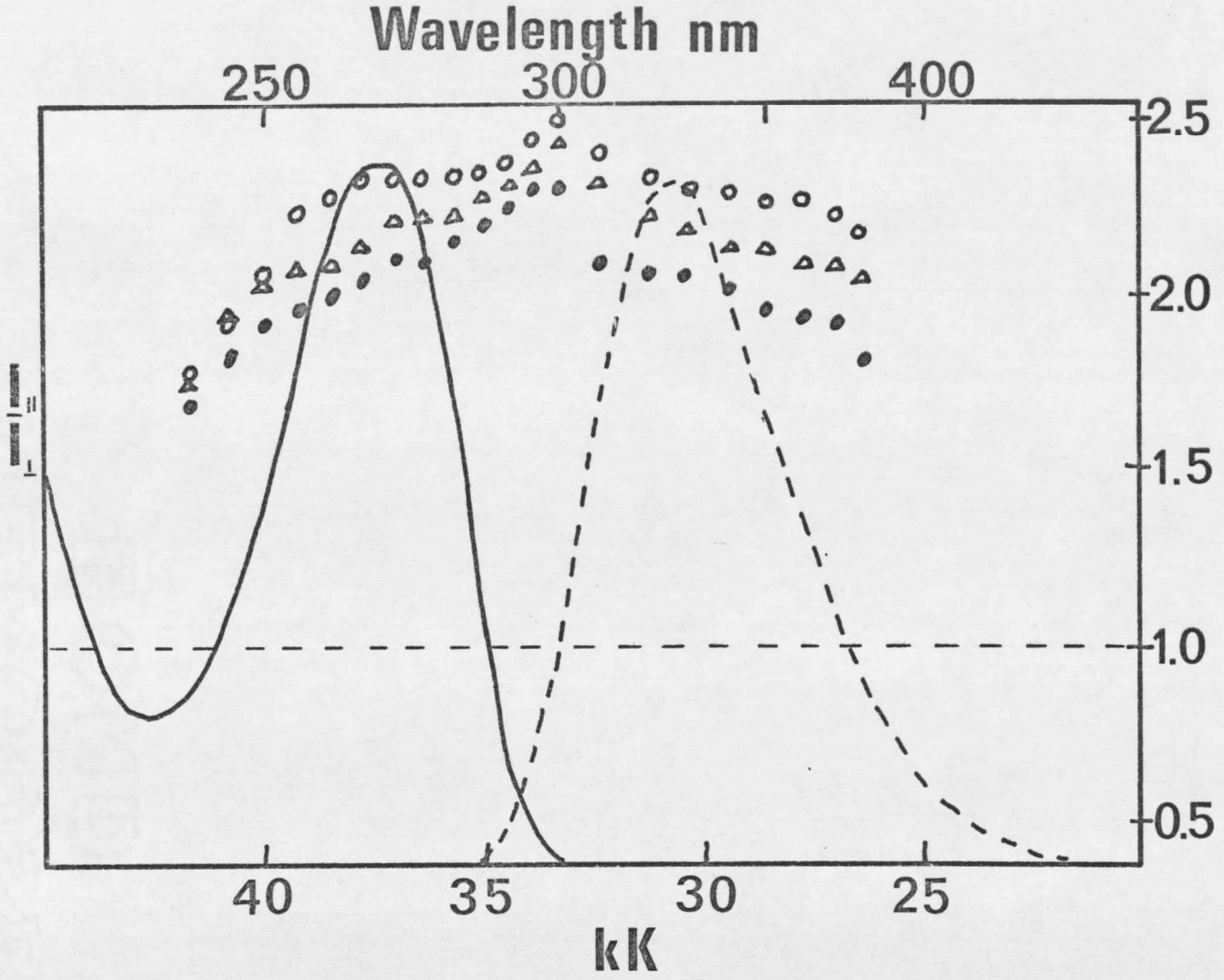
dpTpT in G-70 at -125° C

—— Absorption

--- Fluorescence

The Points on the Left Show the Polarization as a Function of the Excitation Wavelength While Observing the Fluorescence at 305 nm (○), 330 nm (△), and 370 nm (◎)

The Points on the Right Show the Polarization as a Function of the Fluorescence Wavelength While Exciting at 290 nm (○), 270 nm (△), and 250 nm (◎)


Wavelength nm
250
300
400
2.5
2.0
1.5
1.0
0.5
40
35
30
25
kK

approach. The behavior of CpC with regards to its fluorescence polarization is similar to dpTpT. That is, the polarization ratios for CpC also drop across both the absorption and fluorescence bands. However, this drop is considerably larger in the case of CpC, and furthermore, CpC exhibits excimer fluorescence, meaning that is fluorescence spectrum is red shifted relative to that of CMP. Despite these differences, some of the arguments presented in this article concerning the applicability of the exciton resonance model can be used in discussing dpTpT.

For a dimer composed of identical monomers, a particular monomer absorption band will be split into two bands in the dimer. These two bands will have transition moments which are perpendicular to one another given by

$$\vec{m}_- = \frac{1}{\sqrt{2}} (\vec{m}_A - \vec{m}_B)$$

and

$$\vec{m}_+ = \frac{1}{\sqrt{2}} (\vec{m}_A + \vec{m}_B)$$

where $\vec{m}_A$ and $\vec{m}_B$ are the transition moments of monomers A and B.

Since the absorption and fluorescence spectra of dpTpT are little different from those of TMP, the coupling in this dimer is thought to be weak. In a weak coupling situation, the plus and minus absorption bands will overlap so that transitions having plus and minus polarization occur throughout the band. The distribution of the plus and minus intensity throughout the band will depend on the sign of the coupling constant. In dimers with the component monomers oriented face to face, as is thought to be the case for the dinucleotides (14,15), the dipole-dipole approximation predicts a positive coupling constant. This results in having the minus intensity distributed more to the low energy side and the plus intensity distributed more to the low energy side and the plus intensity distributed more to the high energy side of the absorption band. As this theory has the result of allowing absorption or fluorescence, which is governed only by two perpendicular transition moments, expression (3.3) can be written as

$$S = r_+ q_+ + r_- q_- \qquad (3.5)$$

The fluorescence polarization of dpTpT will therefore be discussed with respect to this expression.

The polarization ratio for excitation at 295 nm, while observing the fluorescence at 300 nm, can be seen to be about 2.5. This would indicate that the absorbing and emitting moments are parallel. Therefore, S(295, 305) equals 1. Using the assumption of a positive coupling constant and equation (3.5), then $r_-(295) = q_-(305) = 1$. If the excitation wavelength is changed to 250 nm and the fluorescence is still viewed at 305 nm, the polarization ratio drops at a value of about 2.1. From this it is found that $r_-(250) = 0.86$ and $r_+(250) = 0.14$.

The fluorescence polarization can be seen to drop as the wavelength at which it is viewed is increased. For excitation at 295 nm, while the fluorescence is viewed at 380 nm, a polarization ratio of about 2.2 is obtained. This results in $q_-(380) = 0.90$ and $q_+(380) = 0.10$. Finally, for excitation at 250 nm and fluorescence at 380 nm, the observed polarization ratio is 1.8. This value of 1.8 corresponds to an S value of 0.75. The S value determined using the equation $S = r_-(250)q_-(380) + r_+(250)q_+(380)$ is .79 giving a polarization ratio of 1.9. The difference between the 1.8 and 1.9 values obtained is within experimental error. Therefore, it would seem possible to explain the polarization of dpTpT in terms of only these

plus and minus moments. It should be pointed out that this result would have been obtained even if the coupling constant had been assumed to have a negative sign. In fact, any two mutually perpendicular vectors can be shown to give the same result.

Even though it has been shown that is possible to explain the observed polarization ratios using only these two perpendicular plus and minus transition moments, the problem of theoretically generating the correct percentage of plus and minus absorption and emission, as a function of wavelength, remains. If the coupling constant has a positive sign, as it would for a stacked dimer, exciton theory predicts that the highest energy, 0-0, fluorescence will have a minus transition moment. Referring to the upper curve on the left of Figure III-2, which is for 305 nm fluorescence, the high polarization for excitation at 295 nm indicates that absorption at that wavelength is also due to a minus transition moment. As the wavelength of excitation decreases, the polarization ratios are also seen to decrease. This indicates that the ratio of plus to minus absorption is increasing as the wavelength of excitation decreases. Since the ratio of plus to minus absorption goes from 0 to 0.16, everywhere in the absorption band the

minus intensity is greater than the plus intensity. This behavior is not consistent with that predicted by the exciton theory for a positive coupling constant. That is, the absorption should be largely due to a plus transition moment in the high energy regions of the absorption band. The ratio of plus intensity to minus intensity for integration across the entire absorption band is given by

$$\frac{\langle m_+^2 \rangle}{\langle m_-^2 \rangle} = \cot^2\left(\frac{\theta}{2}\right) \qquad (3.6)$$

where θ is the angle between $\vec{m}_A$ and $\vec{m}_B$. If θ is 90°, then this ratio can be seen to equal 1.0. This is the minimum ratio of plus to minus intensity possible. Therefore, it can be seen that it is impossible for the minus intensity to be greater than the plus intensity everywhere in the absorption band, as was required to fit the polarization data if a positive coupling constant is assumed.

A similar analysis can be performed for the assumption of a negative coupling constant. In this case, the fluorescence from the 0-0 transition will be due to a plus transition moment. Using the same reasoning as before, the low energy absorption must also be due to a plus transition moment. As the wavelength of excitation is decreased, the

exciton theory predicts that more and more of the absorption will be due to a minus transition moment. In this situation, everywhere in the absorption band the plus intensity is greater than the minus intensity. This is in line with the predictions of the exciton theory which, as can be seen from equation (3.6), predicts that the ratio of the total absorption due to the plus and minus moments is greater than or equal to 1.0.

Up to this point, the effect of the sign of interaction on the variation of polarization ratios across the fluorescence band has been ignored. It is possible that the magnitude, and even the sign of this coupling constant, may change after excitation. It is thought that an increase in the magnitude of this constant after excitation is in large part responsible for excimer formation. Although dpTpT does not exhibit excimer fluorescence, it is entirely possible that the coupling constant changes after excitation even in this case. As pointed out in Appendix I, the effect of the coupling constant in lowering the energy of the fluorescent state is severely moderated by the displacement of the nuclear positions at which the potential energy minimum of the excited state of the monomers occur. Therefore, rather large changes in the

coupling constant will have little effect on the shape of the fluorescence spectrum. It should be obvious from the preceding discussion that in order to have high polarization when exciting and viewing the fluorescence in the 0-0 region, the absorbing moment and emitting moment must be parallel. If the coupling for the absorption is negative, this absorbing moment will be a plus moment. In order for the fluorescing moment also to be a plus moment, the coupling in the fluorescence must also be negative.

To a first approximation, the coupling constant is given by the transition-dipole, transition-dipole interaction. Using this approximation

$$\beta = \frac{\vec{m}_A \cdot \vec{m}_B}{r^3} - \frac{3(\vec{m}_A \cdot \vec{r})(\vec{m}_B \cdot \vec{r})}{r^5}$$

where $\vec{r}$ is a vector between the centers of charge of the component monomers. For face to face dimers $\vec{m}_A \cdot \vec{r} = \vec{m}_B \cdot \vec{r} = 0$ and so β must be positive. The geometry of dpTpT in solution is not known. In fact, crystal structures have only recently been obtained for three dinucleotides: GpC (16), ApU (17), and UpA (18). Both GpC and ApU were shown to crystallize in the form of a right handed, antiparallel

double helix with Watson-Crick hydrogen bonding between either the uracil and adenine, or the cytosine and quanine bases. This is the structure which had been deduced for double-stranded RNA. The structure of UpA was very different from that of ApU or GpC in that it showed no resemblance to that proposed for double-stranded RNA. Using the structure reported for ApU and GpC, and substituting thymines in the place of guanine and cytosine, or adenine and uracil, a calculation for the value of the coupling constant for dpTpT can be made. Assuming the centers of charge to be at the centers of the respective pyrimidine rings, and using the transition moment directions for 1 - Me thymine (19) results in a positive value of β.

The fact that a positive β results from this calculation should be considered of very little consequence. The basic approximation that dpTpT should exist in solution in a form similar to that of crystalline GpC or ApU is very crude at best. Both GpC and ApU are composed of Watson-Crick pairs, the hydrogen bonding between base pairs on separate dinucleotides may be very important in determining the relative geometries of bases on the same molecule. Furthermore, one of the three dinucleotides studied did not show a double-stranded RNA type geometry. UpA

instead had a structure with two different geometries for the dinucleotide, and if a calculation similar to that discussed above is done for the structure designated in the article UpA[2], a negative β results.

It is therefore felt that no constraint is placed on the sign or magnitude of β by the results of crystal structures reported to date.

The results of a vibronic exciton calculation for a symmetric dimer, assuming negative coupling constants for both the ground and excited states, are shown in Figure III-3. The major trends in the polarization ratios are very much like that observed for dpTpT. In addition to the calculation presented, many other calculations were also done to illustrate the effects of changing the coupling constants for both the absorption and fluorescence, and changing the angle which the two monomer moments make with each other. As long as both coupling constants are assumed negative, the result of increasing the absolute magnitude of the coupling constant for absorption is to increase the polarization ratios at the low energy side of the absorption band, and decrease those at the high energy side. The effect of increasing the absolute magnitude of the fluorescence coupling constant is to increase the

Figure III-3

Results of a Vibronic Exciton Calculation

Vertical Lines Represent the Energy and Intensity of Individual Vibronic Transitions

Solid Curves Represent the Absorption Spectrum, Left, and Fluorescence Spectrum, Right, if Each Vibronic Transition is Spread Over a Gaussian Curve with a Half Width of 1.0 $h\nu_0$

The Symbols on the Left (×, +, and ◁) Represent the Polarization Ratios as a Function of Absorption Frequency for Fluorescence Viewed at the Frequency Indicated by the Small Corresponding Symbols on the Top of the Figure

The Symbols on the Right (◇, ◁, and ⋉) represent the Polarization Ratios as a Function of Fluorescence Frequency for Excitation at the Frequency Indicated by the Small Corresponding Symbols on the Top of the Figure

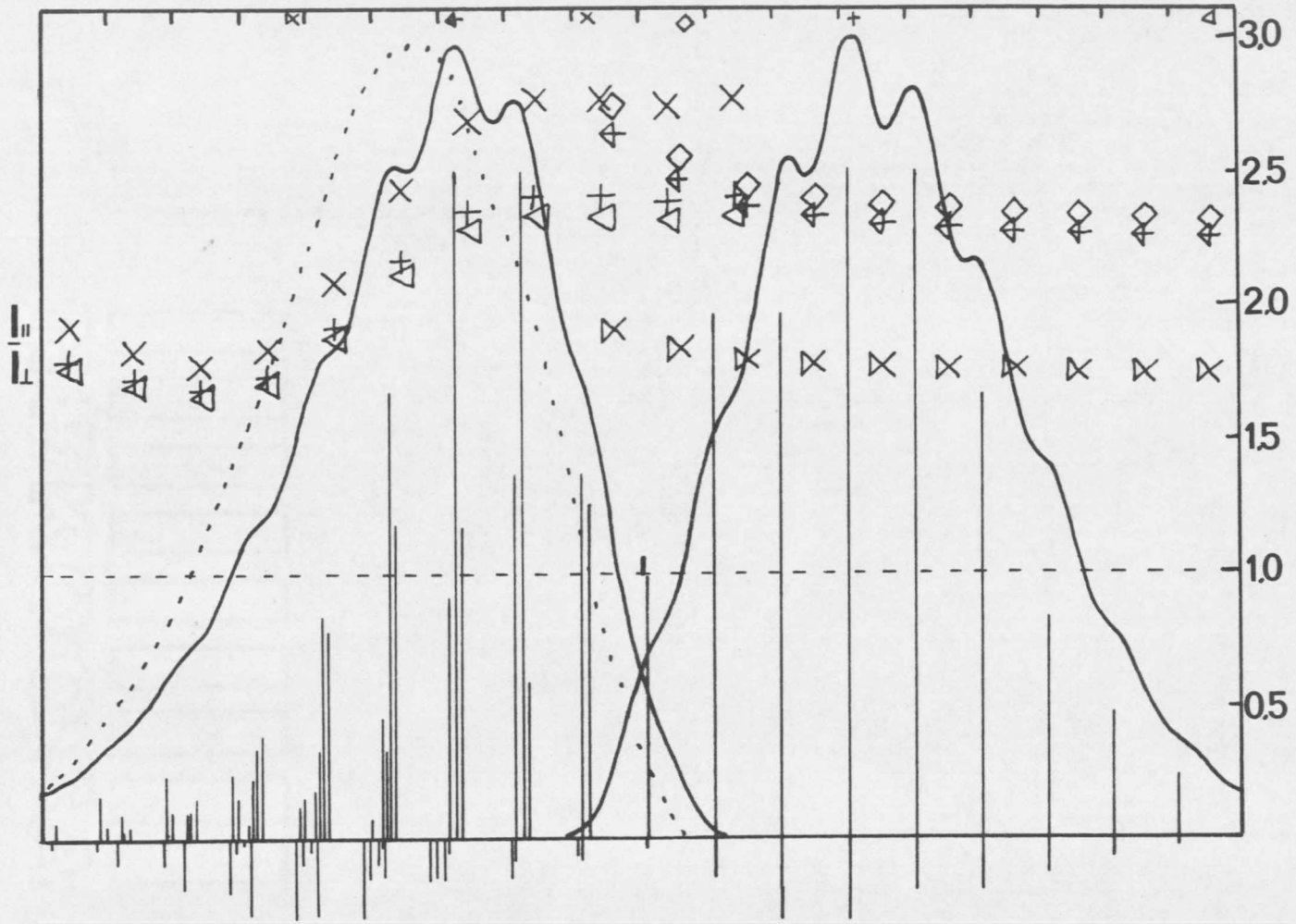
Dotted Curve is the Absorption Spectrum of the Monomer Components

Absorption $\beta = -0.75$

Fluorescence $\beta = -0.75$

$\lambda = 2.00$

The Angle Between the Monomer Transition Moments = 45°


3.0
2.5
2.0
1.5
1.0
0.5

polarization ratios at the low energy side of the fluorescence band relative to those at the high energy side. The effect of increasing the angle between the monomer moments is to decrease the polarization throughout the absorption band with the biggest decrease occurring on the high energy side of the band.

Although the results of the exciton resonance calculation shown in Figure III-3 represent the best fit to the observed polarizations of dpTpT obtained, the fit is far from perfect. A very serious failure in the model is its inability to predict the correct maximum and shape of the absorption spectrum. That is, if the coupling constant for the absorption is assumed to have a large enough absolute value to give the observed drop in polarization ratios across the absorption band, then the maximum absorption will be shifted about 1kK to lower energy relative to that of the monomer. In addition, the general shape of the absorption will be skewed towards lower energies. As the shape and maximum of the absorption of dpTpT are nearly identical to those of TMP, this large effect cannot be neglected.

Another spectral feature that should not be overlooked in considering if an exciton resonance approach can

explain the observed polarization ratios is the similarity of the fluorescence spectrum of dpTpT to that of TMP. This similarity would allow the interaction between the monomers in the excited state to be considered small. If this is true, then the lowest two exciton resonance states will be very close in energy. In fact, the separation between these states may be less than kT. The Boltzman distribution set-up between these two close-lying states should allow fluorescence from both of these states. If the sign of the coupling constant is positive, the higher of these two states will have a plus transition moment, while the lower will have a minus moment as discussed before. If the coupling constant is negative, the lowest state will have a plus moment while the higher state will have a minus moment. In either case, the fluorescence will be a mixture of plus and minus intensity. This would have the effect of lowering the polarization ratio for excitation in the front of the absorption band. This would also have the effect of increasing the polarization ratios for excitation at higher energies relative to that observed before consideration of the Boltzman distribution. This added consideration will not help to explain the polarization ratios observed for dpTpT.

Up to this point, it has been assumed that the monomer energy states are degenerate so that the transition moments of both monomers contribute equally to absorption or fluorescence from any dimer state, thus giving the plus and minus directions for dimer transition. This assumption is valid as long as β is large as compared with the energy difference of the two monomers. If β is very small, it is not inconceivable that small energy differences caused by slightly different environments for each of the component monomers could invalidate the assumption of degenerate states. Furthermore, the permanent dipole-transition dipole-interactions, V_{01}, have also been ignored. In the absence of a plane of symmetry for the dimer, V_{01} may not equal V_{10}. This results in the configuration $A^{\circ}B'$ having a different energy than $A'B^{\circ}$. This also causes the assumption of degenerate states to be invalid. As this energy difference, ΔE, becomes large as compared with β, the resultant dimer wavefunctions will become more and more like monomer wavefunctions [see Appendix I]. These wavefunctions will, however, be connected by radiationless transitions which will result in the appearance of energy transfer. That is, if absorption occurs to a state that has the excitation largely located on molecule B,

there is a probability that fluorescence will be observed from a state that has the excitation largely located on molecule A. A formal discussion of energy transfer has been presented by Förster (20). He has pointed out that for molecules with unresolved vibrational levels, transfer may be due to two types of interaction; either strong or very weak coupling. The dinucleotides have unstructured absorption spectra and exhibit no major change, relative to the component monomers, of their absorption. Therefore, the coupling in these compounds must be considered using Förster's very weak coupling scheme.

Gueron et al. (21) have discussed energy transfer in the dinucleotides, considering both post- and pre-relaxation energy transfer using Förster's model for very weak coupling. Post-relaxation energy transfer will occur if vibrational relaxation occurs faster than transfer. In this model, the rate of transfer would be independent of the initially excited state and therefore independent of the energy of excitation. Pre-relaxation energy transfer occurs before any dissipation of the vibrational energy to the solvent. The rate of pre-relaxation energy transfer will depend on the energy of excitation. Either post- or pre-relaxation energy transfer can lead to depolarization

of the fluorescence. Because only pre-relaxation energy transfer is dependent on the initially excited state, only this type of transfer can lead to polarization ratios which vary with the wavelength of excitation. If no substantial increase in interaction occurs after transfer and before fluorescence, the fluorescence will originate from a monomer type state. Under these conditions, the fluorescence polarization ratios should exhibit the same dependence on fluorescence wavelength as that exhibited by the pure monomer. This is not the case for dpTpT which exhibits a decrease in polarization ratios as the fluorescence is viewed at lower energies, whereas TMP has no variation in polarization ratios with fluorescence wavelength.

Excimer formation has been discussed using both exciton resonance and charge resonance approaches. Although dpTpT does not exhibit excimer fluorescence, it is obvious that some sort of interaction must be responsible for the variation in the polarization across the fluorescence band. If pre-relaxation energy transfer is responsible for the variation in polarization across the absorption band, then the variation across the emission band cannot be due to exciton interaction. Exciton theory would require the fluorescence to be due to either a plus or minus transition

moment with both monomer moments making the same angle to either the plus or minus dimer moments. This would result in uniform depolarization of the fluorescence for excitation at any wavelength.

The observed polarization ratios can be predicted if it is assumed that pre-relaxation energy transfer is responsible for the polarization ratios across the absorption band, and that the fluorescence is due to a monomer moment and a moment which is perpendicular to the plane determined by the monomer moments. This out-of-plane component need only contribute a maximum of about 10% to the fluorescence at 380 nm, and less at shorter wavelengths. It must also be assumed that there is no post-relaxation energy transfer in order to account for the high polarization ratios observed for excitation in the low energy regions of the absorption band.

C. Spectral Features and Fluorescence Polarization of ApA

The absorption and fluorescence spectra along with the fluorescence polarization ratios of ApA in G-70 at -125° C are shown in Figure III-4. The fluorescence observed for ApA is approximately five times as intense as that predicted on the basis of the fluorescence intensity

Figure III-4

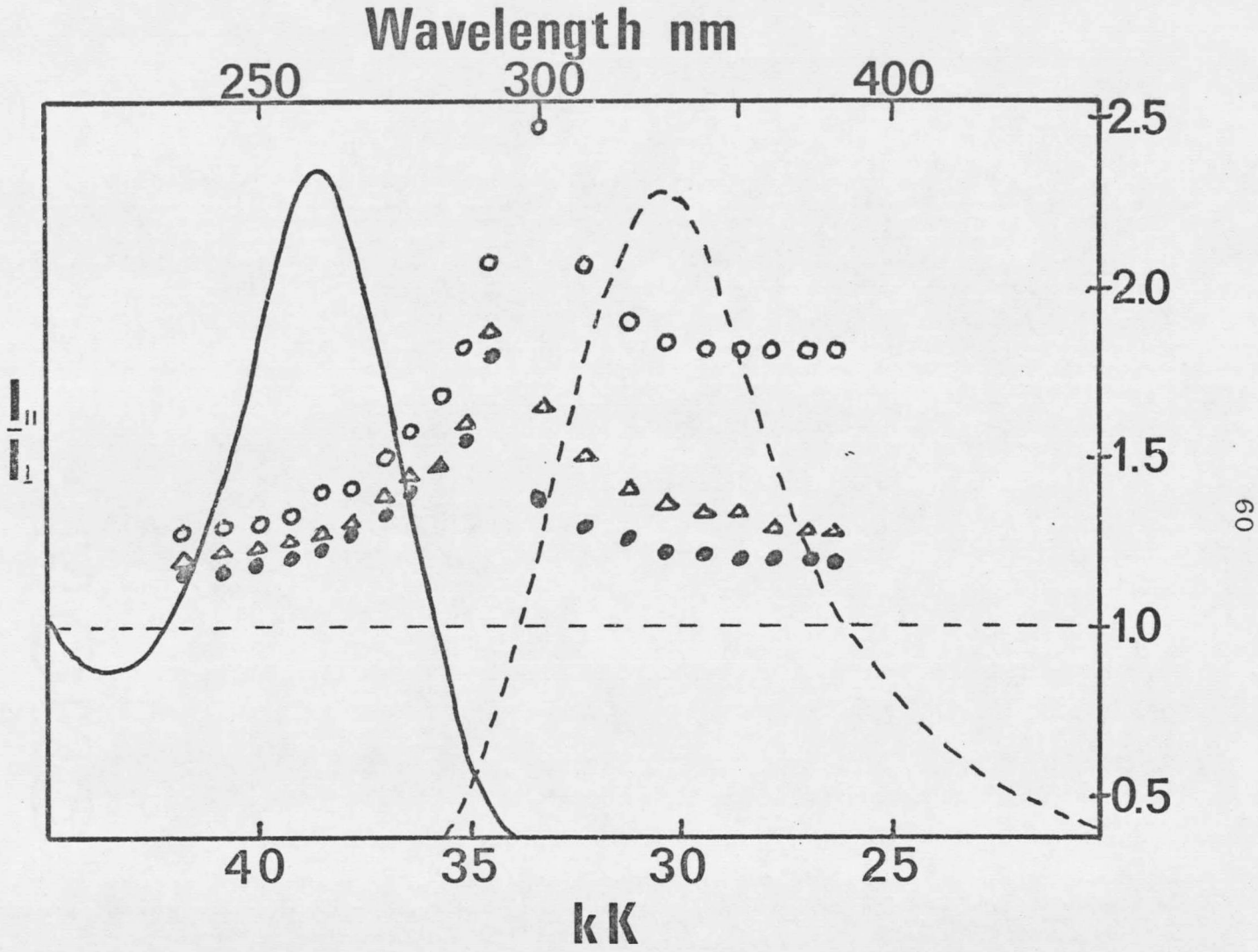
ApA in G-70 at -125° C

—— Absorption

--- Fluorescence

The Points on the Left Show the Polarization as a Function of the Excitation Wavelength While Observing the Fluorescence at 310 nm (O), 330 nm (Δ), and 360 nm (◎)

The Points on the Right Show the Polarization as a Function of the Fluorescence Wavelength While Exciting at 290 nm (O), 270 nm (Δ), and 250 nm (◎)


Wavelength nm
250
300
400
2.5
2.0
1.5
1.0
0.5
40
35
30
25
kK
∥
⊥

of AMP. Because AMP fluoresces very weakly, good fluorescence polarization ratios for this compound are not available. The fluorescence polarization of ApA will be compared with the fluorescence polarization results for adenine (22). The absorption of adenine is thought to be due to two different transition moments in the region from 240 nm to 300 nm, with the lower energy transition having an extinction coefficient about ten times that of the second transition. The higher energy transision has little effect on the observed polarization ratios for wavelengths longer than 250 nm. An analysis of the fluorescence polarization of ApA based on the assumption of only one transition moment for the monomer, AMP, should be possible. This analysis will be similar in many respects to that performed for dpTpT.

In determining if two mutually perpendicular transition moments may be responsible for a set of observed polarization ratios, heavy emphasis is placed on some point at which a very high polarization is noted. That is, a polarization ratio high enough to give a value of S equal to 1.0 is required to being the analysis. This is because only for S = 1.0 can the values of q and r be unambiguously assigned. Referring to Figure III-4, it can be seen that

excitation at 290 nm for fluorescence at 300 nm results in a polarization ratio of about 2.5. This polarization ratio results in the required value of S = 1.0. However, the uncertainty in this value must be considered rather high. Although the ratio reported represents the average of several separate determinations, in one instance a value of 2.2 was obtained. It is believed that the large uncertainty in this value is the result of scattered light contributing to the observed signals. The effect of scattered light would be to increase the observed polarization ratio, so that the reported value of 2.5 may be too high. For this reason, a rigorous mathematical approach, such as that used for dpTpt, was decided against.

Even in the absence of such an approach, a number of general comments can be made. In order for a vibronic exciton calculation, as that discussed in Appendix I, to predict a large decrease in the polarization ratios across the absorption band, the absolute value of the coupling constant would be large enough to result in a noticeable change in the absorption spectrum. The absorption spectrum of ApA is virtually identical to that of AMP. This indicates that a strictly vibronic exciton model of this type is not appropriate for a discussion of this dinucleotide.

The polarization ratios and spectral features of ApA can be accounted for using a model identical to that used for dpTpT. That is, the variation in polarization ratios across the absorption band is due to pre-relaxation energy transfer. Following relaxation to the lowest excited level, an increase in the interaction of the monomers leads to fluorescence due to a combination of a monomer transition moment and a moment that is directed out of the plane determined by the monomer moments. In order for this rationale to be applied, the angle between the component chromophores must be greater than 45°, for if there is complete transfer of energy, an angle of 45° is required to obtain the low polarizations observed for excitation at 250 nm. For any situation short of complete transfer, an angle larger than 45° is required to get this low polarization.

D. Spectral Features and Fluorescence Polarization of ApC

The absorption and fluorescence spectra, along with the fluorescence polarization ratios of ApC in G-70 at -125° C are shown in Figure III-5. Under these conditions, ApC exhibits excimer fluorescence. Its fluorescence maximum is shifted about 3.4 kK to lower energy relative to the

Figure III-5

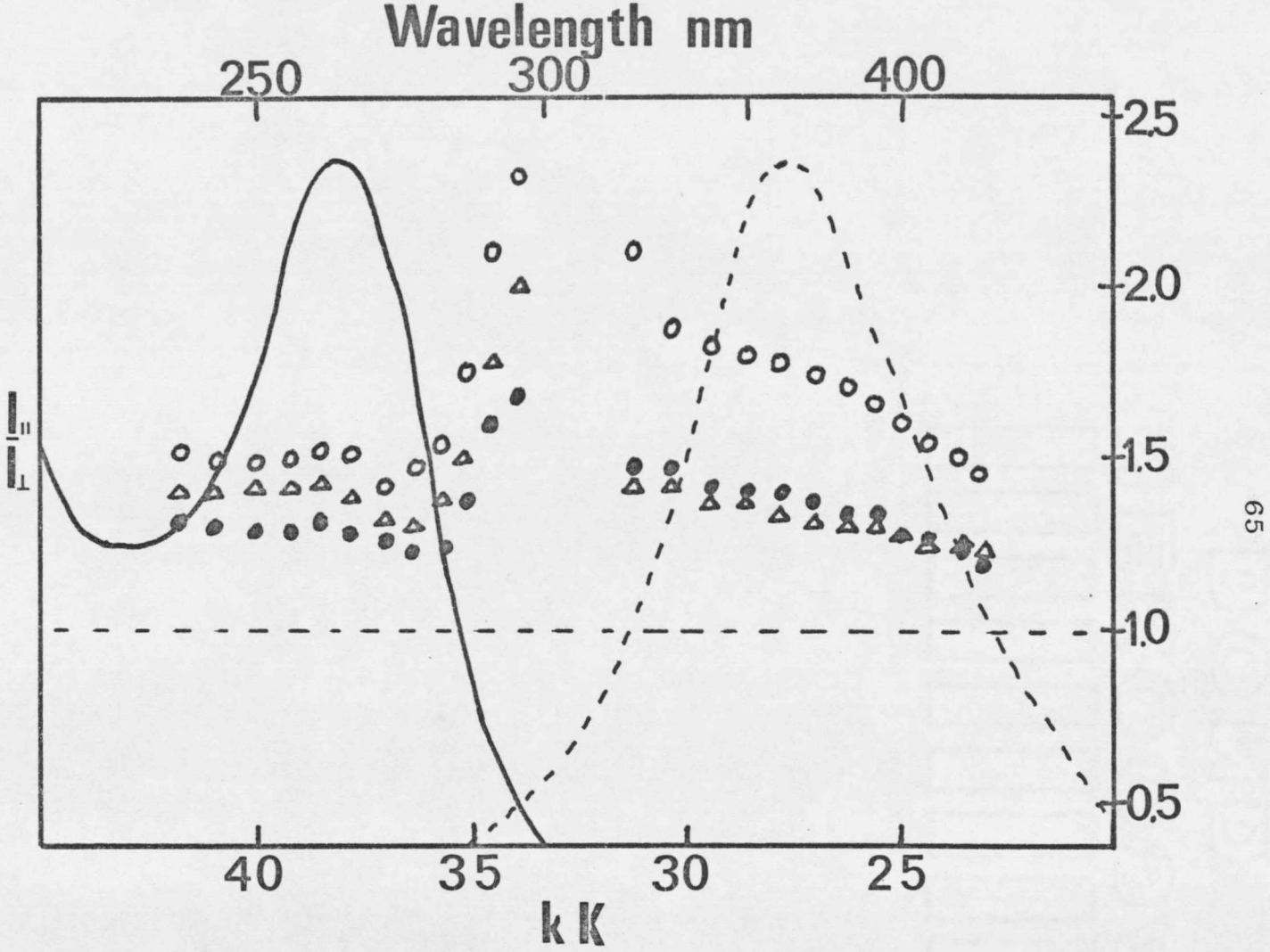
ApC in G-70 at -125° C

—— Absorption

--- Fluorescence

The Points on the Left Show the Polarization as a Function of the Excitation Wavelength While Observing the Fluorescence at 320 nm (O), 360 nm (△), and 400 nm (⊗)

The Points on the Right Show the Polarization as a Function of the Fluorescence Wavelength While Exciting at 290 nm (O), 270 nm (△), and 250 nm (●)


Wavelength nm
250
300
400
2.5
2.0
1.5
1.0
0.5
40
35
30
25
k K

fluorescence maximum of CMP. The absorption spectrum of ApC is very little different from that of an equal-molar mixture of AMP and CMP.

The variation in polarization ratios across the absorption band is very indicative of energy transfer from the adenine to the cytosine component of this dinucleotide. AMP has a 0-0 transition energy about 1.5 kK higher than that of CMP (23). As the absorption spectrum of ApC indicates very little interaction between the monomers, at the low energy edge of the absorption band, all of the absorption is due to the cytosine component of the dinucleotide. The high polarization ratios observed for excitation in this region, and fluorescence at the high energy edge of the fluorescence band, indicate that the fluorescence is also largely from the cytosine component. As the energy of excitation is increased, the polarization ratios are seen to drop very sharply and then level off. If it is assumed that excitation of adenine is followed by transfer of this excitation energy to cytosine with fluorescence strictly due to cytosine, the equation

$$S = r_C + r_{Ad}\cos^2\theta$$

can be used to predict the polarization ratios. Here r_C and r_{Ad} are the fractions of the light absorbed by the cytosine and adenine components, respectively, and θ is the angle the transition moments of the monomers make with each other. It is easily seen that when $r_C = 1$, as it would in the front of the absorption band, then $S = 1$ giving high polarization. As r_{Ad} increases, if $\theta \neq 0$, the value of S, and therefore the polarization ratio, decreases. This is what is observed as the energy of excitation, and therefore the fraction of the absorption due adenine, increases. In the region of the absorption spectrum where the polarization ratio is not changing, r_C and r_{Ad} must be constant. An analysis of the fluorescence polarization ratios using this relationship and the absorption spectra of AMP and CMP gives results somewhat different from those actually observed. In general, this relationship predicts that the polarization ratios will be higher in the region where the drop in ratios is observed. This analysis also fails to predict the noticeable dip in the polarization ratios for excitation around 270 nm. There is some question as to whether it is appropriate to use the absorption spectra of the monomers in this analysis. As pointed out in Section IV, the fluorescence excitation

spectrum of CMP is not identical to its absorption spectrum. Neither is the fluorescence excitation spectrum of adenine the same as its absorption spectrum. If the same mechanism that is causing the differences in these spectra is operative in the dinucleotide, it would perhaps be more correct to use the excitation spectra of the monomers rather than their absorption spectra in this analysis. The excitation spectra of all of the nucleotides studied have been shifted about 1.5 kK to lower energies relative to the absorption spectra. If this shift is taken into account, the calculated polarization ratios would be closer to those actually observed. The excitation spectrum of ApC is more like its absorption spectrum in that the maxima appear at the same wavelength. However, the fluorescence excitation spectrum of this dinucleotide is still not coincident with its absorption spectrum, as there is an apparent doubling of the fluorescence quantum yield for excitation at 290 nm as compared with that at 260 nm. The failure on the part of the model may also be due to the assumption of the fluorescence being strictly due to cytosine.

The assumption of purely cytosine fluorescence is obviously not valid, as ApC exhibits excimer fluorescence.

This indicates that there is some type of excited state interaction which causes a lowering in energy of the fluorescence maximum. As before, if energy transfer is responsible for the observed variation in polarization ratios across the absorption band, then the fluorescence cannot be considered as originating from a purely exciton resonance state. The polarization ratios can be explained if it is assumed that the fluorescence is due to a combination of a transition moment due to the cytosine component, and a transition moment directed out of the plane determined by the monomer transitions moments.

E. Spectral Features and Fluorescence Polarization of ApU

The absorption and fluorescence spectra along with the fluorescence polarization ratios of ApU in G-70 at -125° C are shown in Figure III-6. Under these conditions, ApU exhibits excimer fluorescence. Its fluorescence maximum is shifted about 3.5 kK relative to the fluorescence maximum of UMP. The absorption spectrum of ApU is very little different from that of an equal-molar mixture of its component monomers.

The similarities between the polarization ratios determined for ApU and those of ApC are very striking. UMP

Figure III-6

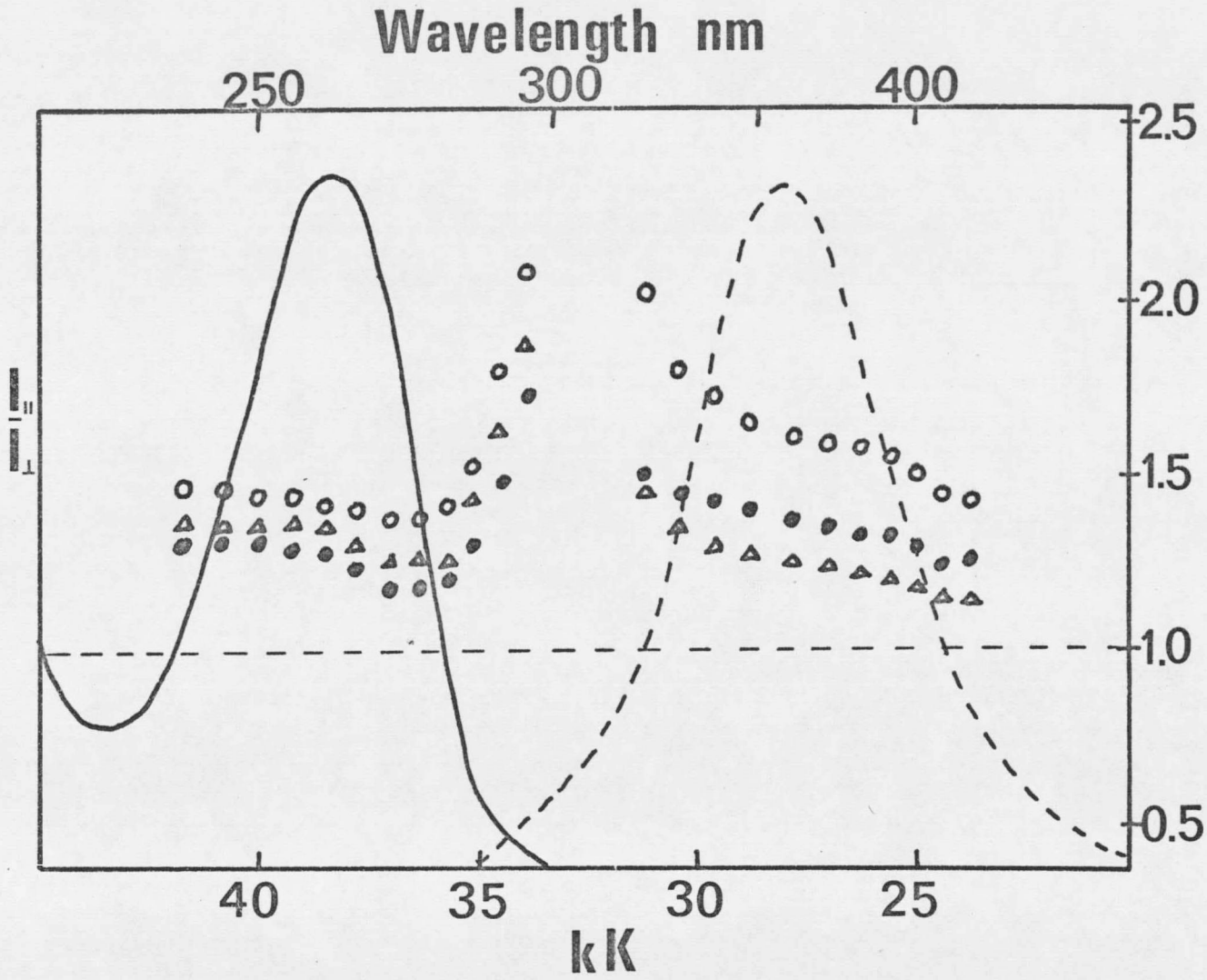
ApU in G-70 at -125° C

—— Absorption

--- Fluorescence

The Points on the Left Show the Polarization as a Function of the Excitation Wavelength While Observing the Fluorescence at 330 nm (○), 360 nm (△), and 400 nm (◎)

The Points on the Right Show the Polarization as a Function of the Fluorescence Wavelength While Exciting at 290 nm (○), 270 nm (△), and 250 nm (◎)


Wavelength nm
250
300
400
2.5
2.0
1.5
1.0
0.5
40
35
30
25
kK
II
T

has a 0-0 transition energy about 0.3 kK lower than that of AMP (23). Therefore, the arguments presented for ApC can easily be extended to ApU. That is, the spectral features and polarization ratios of ApU can be explained if it is assumed that energy transfer occurs from adenine to uracil, followed by fluorescence from a combination of a transition moment due to the uracil component, and a transition moment directed out of the plane determined by the monomer transition moments.

F. Spectral Features and Fluorescence Polarization of dpApT

The absorption and fluorescence spectra along with the fluorescence polarization ratios of dpApT in G-70 at -125° C are shown in Figure III-7. Its fluorescence maximum is shifted about 1 kK to lower energy relative to the fluorescence maximum of TMP. The absorption spectrum of dpApT is very little different from that of an equal-molar mixture of AMP and TMP.

Again, the polarization ratios determined for dpApT are similar in many respects to those of ApC and ApU. The major difference is the behavior of the polarization ratios with excitation wavelength when the fluorescence is viewed at the high energy side of the fluorescence band. If the

Figure III-7

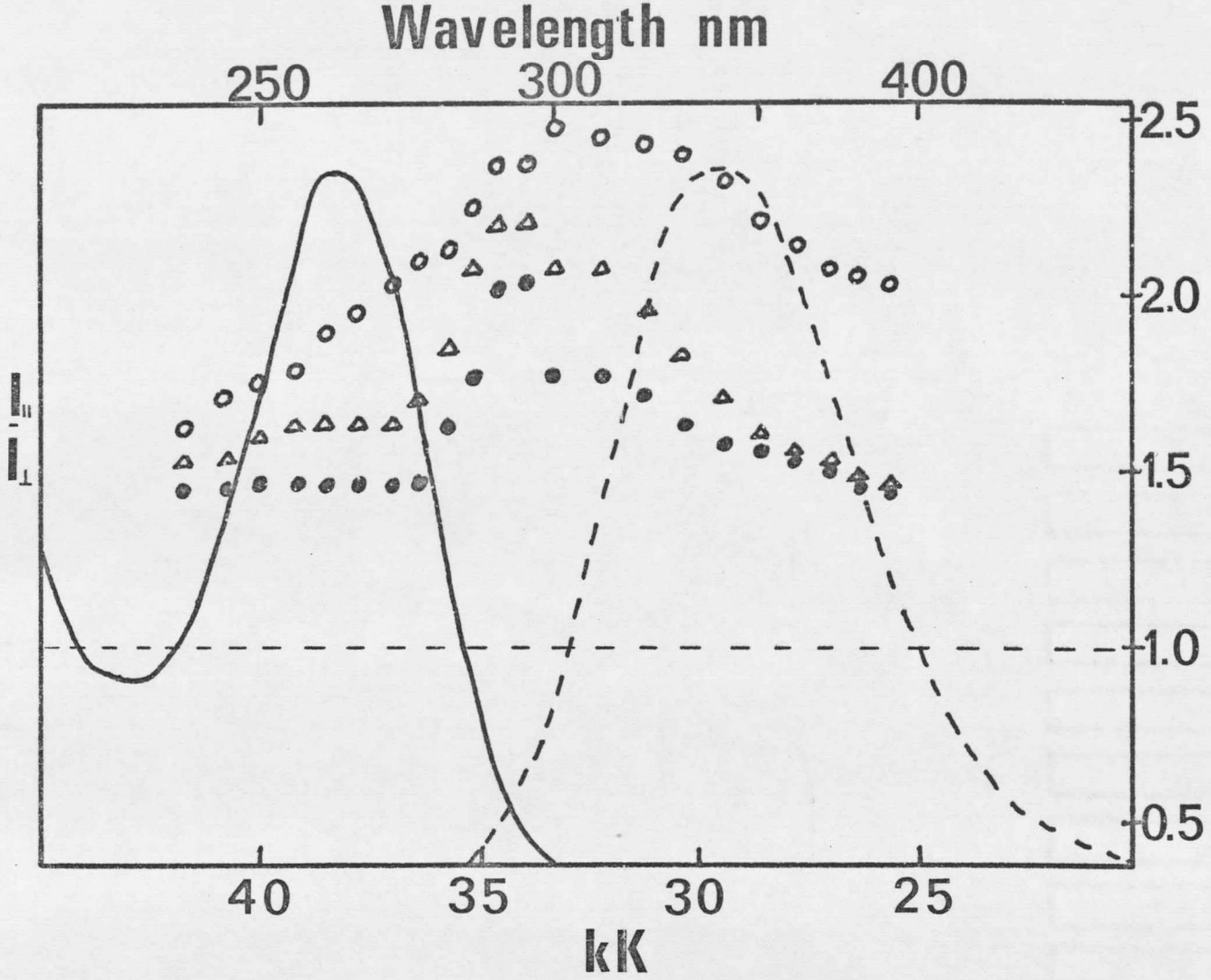
dpApT in G-70 at -125° C

—— Absorption

--- Fluorescence

The Points on the Left Show the Polarization as a Function of the Excitation Wavelength While Observing the Fluorescence at 315 nm (o), 345 nm (Δ), and 390 nm (o)

The Points on the Right Show the Polarization as a Function of the Fluorescence Wavelength While Exciting at 290 nm (o), 270 nm (Δ), and 250 nm (o)


Wavelength nm
250
300
400
2.5
2.0
1.5
1.0
0.5
40
35
30
25
kK

fluorescence is viewed at 390 nm and the wavelength of excitation is varied, the polarization drops very sharply from 290 to 275 nm. From 275 to 240 nm, the polarization ratio is nearly constant. This would indicate that the fraction of light absorbed by the fluorescing moment is constant in this region. In the case of a strong coupling exciton model, this would mean that the ratio of plus to minus transition moments is constant. If the fluorescence is viewed at 315 nm, the observed polarization ratios are seen to continue to drop from 275 to 240 nm, indicating that the ratio of the two absorbing moments is not constant. From a strong coupling exciton resonance approach, this apparent discrepancy could be explained if the ratio of plus to minus fluorescence at 390 nm equals one. If this were the case,

$$S = r_+(.5) + r_-(.5) = 0.5$$

for any value of r_+ or r_-. This would result in an observed polarization ratio of 1.27 which is significantly different from the ratio of 1.45 actually observed. Also this mechanism would not allow the change in polarization ratios observed for excitation from 290 to 275 nm.

As before, the similarities between the absorption spectrum of dpApT and the absorption of an equal-molar mixture of AMP and TMP preclude the use of strong coupling exciton theory. Because the fluorescence of dpApT lies between the excimer fluorescence of ApC and ApU and the monomer fluorescence of TMP, it was thought that the fluorescence of dpApT may be composed of both excimer and monomer TMP fluorescence. Mixed excimer and monomer fluorescence has been observed for several dinucleotides (23). This dual nature of the fluorescence could account for the apparent discrepancy in the behavior of the polarization ratios for excitation across the absorption band. That is, the solution is composed of dinucleotides that are, or are not, capable of forming excimers depending on their ground state geometries. Therefore, at 315 nm, the fluorescence is largely due to a non-excimer component, while at longer wavelengths, the fluorescence is due to an excimer component.

As in the cases previously discussed, this model would require the fluorescence of the excimer to have an out-of-plane component to explain the decrease in polarization ratios across the fluorescence band.

G. Spectral Features and Fluorescence Polarization of GMP

The absorption spectrum of GMP in G-70 at -125° C as shown in Figure III-8 would seem to be composed of two electronic bands as indicated by the presence of a shoulder in the spectrum at about 285 nm. The composite nature of this spectrum and the relative directions of the two transition moments responsible for the absorption of GMP will be discussed and compared with published work on the directions of the transition moments of guanine, 9-ethylguanine, and guanosine. As the result of photoselection experiments performed on guanine and 9-ethylguanine, Callis et al. (22) came to the conclusion that the two electronic transition moments responsible for the composite absorption of these molecules in the region from 230 to 300 nm were directed approximately perpendicular to one another. In a later paper dealing with the polarized specular reflection of single crystals of 9-ethylguanine, Callis et al. (24) described two possible orientations for the transition moment directions in this molecule. Regardless of which orientation is considered, these moments are seen to be directed at about 70° from one another. Fucaloro and Forster (7), from data on the dichroic

Figure III-8

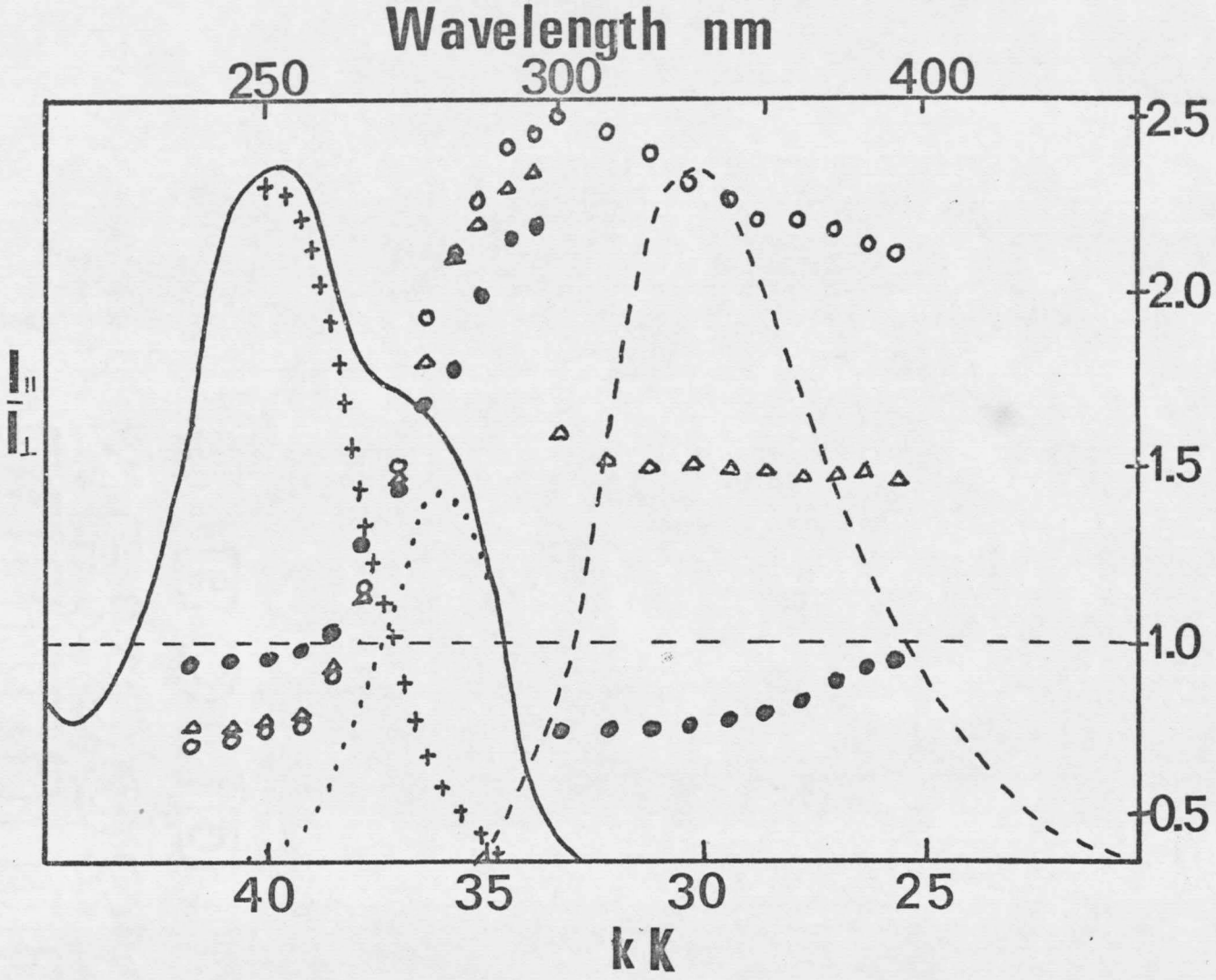
GMP in G-70 at -125° C

— Absorption

--- Fluorescence

The Points on the Left Show the Polarization as a Function of the Excitation Wavelength While Observing the Fluorescence at 310 nm (O), 330 nm (Δ), and 390 nm (⊙)

The Points on the Right Show the Polarization as a Function of the Fluorescence Wavelength While Exciting at 290 nm (O), 270 nm (Δ), and 250 nm (●)


Wavelength nm
250
300
400
2.5
2.0
1.5
1.0
0.5
40
35
30
25
kK

spectra of guanine and guanosine in stretched polyvinyl alcohol sheets, arrived at the conclusion that the two moments in these compounds are directed about perpendicular to one another.

Fluorescence polarization data obtained by the method of photoselection for GMP in G-70 at -125° C gives further evidence for the presence of two transition moments responsible for its absorption in the region from 240 to 300 nm. The open circles, extending across the absorption band in Figure III-8, represent the polarization ratios obtained when the fluorescence is observed at 310 nm and the wavelength of the exciting light is varied from 240 to 295 nm. A very high polarization ratio of 2.45 is observed when exciting at 295 nm. This indicates that the moments responsible for the absorption and fluorescence are very nearly parallel. As the wavelength of the exciting light is decreased to 240 nm, the polarization ratio can be seen to drop to a value of 0.70, indicating that the absorbing moment now is nearly perpendicular to the fluorescing moment, and therefore nearly perpendicular to the absorbing moment at 295 nm. Calculations were done to decide the angle which the high energy absorption moment makes with the low energy moment. The experimentally determined

polarization ratios were entered into the calculation as data, and the relative contributions of the two moments to the total absorption spectrum were computed as a function of the angle the two moments make with each other. The best fit, as determined by the shape and the absence of any low energy absorption at 240 nm, was obtained for an angle of 69°. This result compares well with the literature values cited earlier for compounds similar to GMP. The relative contributions of the two bands to the total absorbance of GMP are illustrated by crosses for the high energy band, and dots for the low energy band in Figure III-8.

When GMP was excited with light having a wavelength of 290 nm and the fluorescence was observed at wavelengths from 300 to 390 nm, the polarization was seen to drop. These polarization ratios, as given by the open circles extending across the fluorescence band in Figure III-8, can be seen to go from a value of 2.5 when viewing at 300 nm to a value of 2.1 at 390 nm. This drop in polarization across the fluorescence band is thought to be due to vibronic mixing between the two closely spaced electronic excited states of GMP. Assuming this to be true, the fluorescence band can be decomposed into contributions

from each of the two transition moments in a manner similar to that used for the absorption.

The triangles and darkened circles extending across the absorption band in Figure III-8 represent the fluorescence polarization ratios when the fluorescence is viewed at 330 and 390 nm, respectively. Using the information gained on the composition of the absorption spectrum from the polarization ratios obtained when viewing the fluorescence at 310 nm, and the composition of the fluoresence band determined for 295 nm excitation, the polarization ratios for viewing at 330 and 390 nm as a function of excitation wavelength were calculated. The calculated polarization ratios were found to be in good agreement with the experimental values. The only place that there was consistent disagreement, indicating some failure on the part of the calculation, was in the region of excitation below 260 nm while viewing the fluorescence at 390 nm. Even here, the disagreement was very small, and could have been caused by neglecting the mixing in of higher electronic states by vibronic borrowing, or by errors in the experimental values possibly due to depolarization caused by phosphorescence which was not totally removed by the lock-in-amplifier.

H. Spectral Features and Fluorescence Polarization of the Guanine-Containing Dinucleotides

Any discussion of the guanine-containing dinucleotides is very much complicated by the presence of the two transition moments in both the absorption and fluorescence spectra of GMP. In an exciton resonance approach, each of these two moments may be coupled with the transition moments of the other molecule forming the dimer by a different coupling constant. Furthermore, if the two molecules forming the dimer are not coplanar, the transition moments resulting from the exciton resonance calculation will also not all be in the same plane. The polarization ratios resulting from energy transfer considerations will also be complicated by the existence of these two moments. In view of these difficulties, the spectral features and polarization ratios of the guanine-containing dinucleotides will be discussed from a more qualitative point of view.

1. GpG. The absorption and fluorescence spectra of GpG in G-70 at -125° C, as shown in Figure III-9, are nearly identical to those of GMP. This would seem to indicate that the two component chromophores of GpG are acting entirely independently of one another under these conditions. If the component chromophores are acting

Figure III-9

GpG in G-70 at -125° C

—— Absorption

--- Fluorescence

The Points on the Left Show the Polarization as a Function of the Excitation Wavelength While Observing the Fluorescence at 310 nm (O), 330 nm (Δ), and 390 nm (⊗)

The Points on the Right Show the Polarization as a Function of the Fluorescence Wavelength While Exciting at 290 nm (O), 270 nm (Δ), and 250 nm (⊗)