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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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.

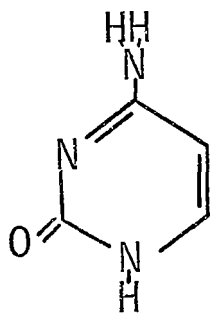
Excimer formation occurs when an excited molecule combines with an unexcited molecule to form a complex. In the case of inter-molecular excimers, this formation can be seen to be concentration dependent. That is, high concentrations promote excimer formation. Likewise, the molecules must be mobile, so 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.

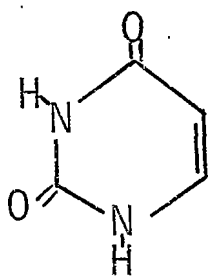
Several members of the class of compounds known as the dinucleotides have been shown to exhibit excimer fluorescence at liquid nitrogen temperatures in an ethyleneglycol-water glass (6). In general, these

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.

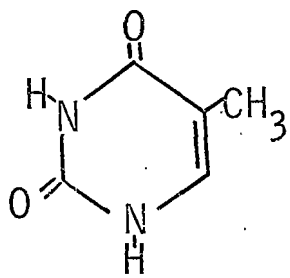
The component chromophores of a dinucleotide may include the DNA bases: adenine, guanine, cytosine, and thymine, or, in the case of a dinucleotide derived from RNA, uracil in place of thymine. These five molecules will hereafter be referred to as "the bases". The structures of these five bases, in what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



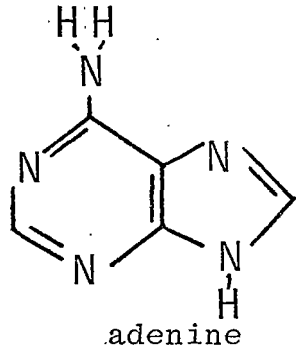
cytosine



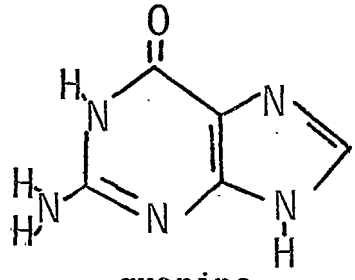
uracil



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

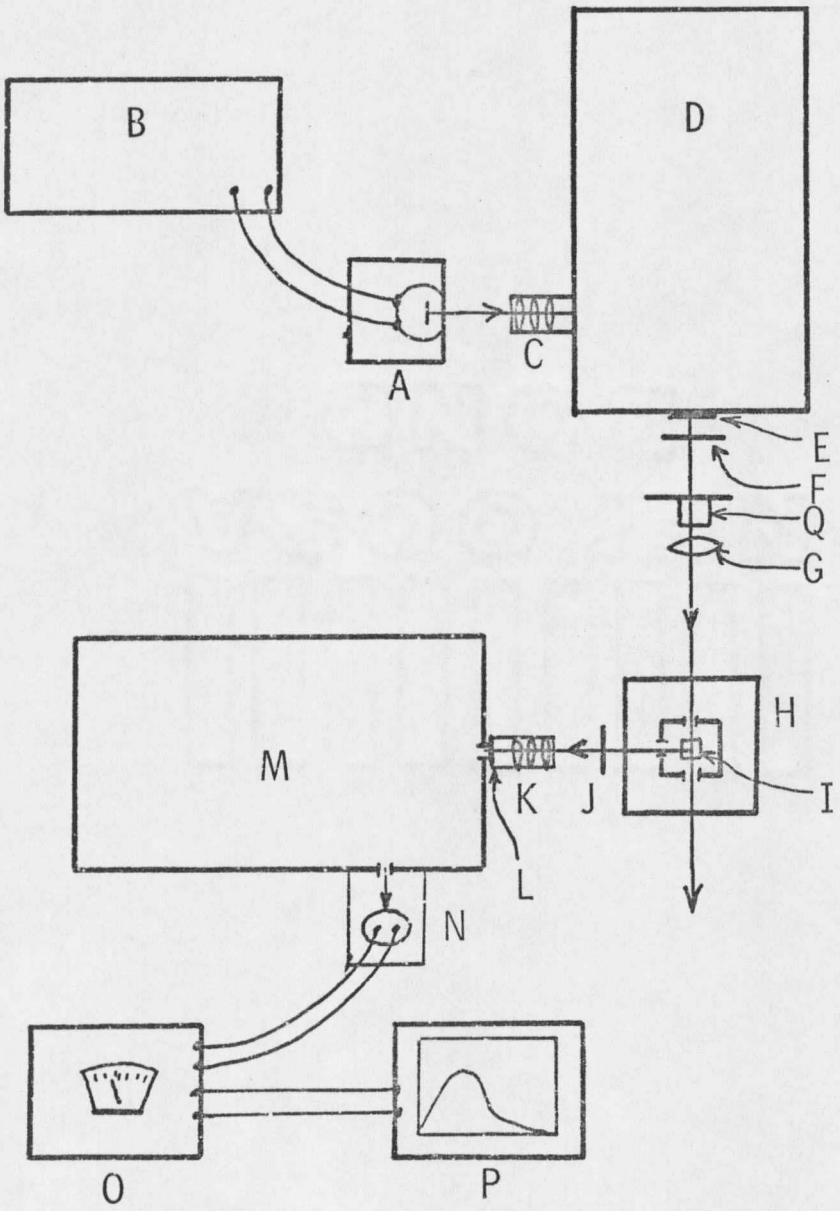


Figure II-1 Emission Apparatus

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.