



Polarized one- and two-photon fluorescence excitation spectroscopy on selected nucleic acid bases
by Scott Allan Williams

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Chemistry

Montana State University

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Abstract:

The first two-photon fluorescence excitation spectra of thymine, cytosine and the four nucleotides of deoxyribonucleic acid (DNA) are reported. These were obtained using neutral aqueous solutions at room temperature in the spectral range 400-600nm delivered by a pulsed (8nsec) ND-YAG pumped dye laser. The low fluorescence quantum yields (10^{-4}) established a new detection domain for the two-photon fluorescence excitation technique, for molecules in solution, made possible by strong two-photon absorptions and short lifetimes. The two-photon polarization ratio ($\delta_{\text{cir}}/\delta_{\text{lin}}$) was low and constant for the 260nm region of thymine and TMP, but exhibited a maximum at 220nm, therefore, confirming the singular nature of the lowest $\pi\pi^*$ band and the composite nature of the 205nm band. The data for CMP is consistent with either three or four $\pi\pi^*$ transitions in the 192-300nm region. In no case did the two-photon peaks exactly coincide with their one-photon counterparts. This result was puzzling for the thymine and TMP 260nm band which is strongly allowed under both one- and two-photon absorption. Extensive testing procedures are reported to confirm the intrinsic origin of the data obtained. The experimental two-photon data for the nucleotides and bases are compared to the predicted values calculated using the current semiempirical molecular orbital (INDO/S) methods.

Polarized one-photon fluorescence excitation and emission spectra of thymine in room temperature aqueous solution as a function of pH are reported. In contrast to the high and constant fluorescence anisotropy across the first absorption envelope found for thymine dissolved in a pH 5.0 aqueous buffer, a loss in fluorescence anisotropy ($\Delta R \approx 0.2$) is observed for thymine at neutral pH when excited at wavelengths greater than 280nm. This loss in fluorescence anisotropy for thymine at neutral pH is explained by the presence of a small equilibrium concentration of the highly fluorescent thymine anion ($\text{pK}_a=9.9$). No evidence for the presence of a weakly absorbing low-lying state under the first absorption band is found for thymine in room temperature aqueous solution. The fluorescence anisotropies of uracil and UMP are also reported for the first time.

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APPROVAL

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Scott Allan Williams

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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In memory of my college roommate and chemistry buddy,
Phelps Langtry.

VITA

Scott Allan Williams was born January 13, 1962, in Huntington, Indiana, to Inge S. and A. Ernest Williams. After living in Indiana a majority of his youth, Scott received his diploma from Clarkstown High School in New City, New York. The following year he attended Purdue University, receiving his B. S. degree in biochemistry in 1984. In May 1984 he married Carol Rairdon, a fellow Purdue alumnus. In June 1988 he became the father of Amanda Kay Williams.

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ABSTRACT

The first two-photon fluorescence excitation spectra of thymine, cytosine and the four nucleotides of deoxyribonucleic acid (DNA) are reported. These were obtained using neutral aqueous solutions at room temperature in the spectral range 400-600nm delivered by a pulsed (8nsec) ND-YAG pumped dye laser. The low fluorescence quantum yields (10^{-4}) established a new detection domain for the two-photon fluorescence excitation technique, for molecules in solution, made possible by strong two-photon absorptions and short lifetimes. The two-photon polarization ratio ($\delta_{\text{cir}}/\delta_{\text{lin}}$) was low and constant for the 260nm region of thymine and TMP, but exhibited a maximum at 220nm, therefore, confirming the singular nature of the lowest $\pi\pi^*$ band and the composite nature of the 205nm band. The data for CMP is consistent with either three or four $\pi\pi^*$ transitions in the 192-300nm region. In no case did the two-photon peaks exactly coincide with their one-photon counterparts. This result was puzzling for the thymine and TMP 260nm band which is strongly allowed under both one- and two-photon absorption. Extensive testing procedures are reported to confirm the intrinsic origin of the data obtained. The experimental two-photon data for the nucleotides and bases are compared to the predicted values calculated using the current semiempirical molecular orbital (INDO/S) methods.

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INTRODUCTION

Molecules and light interact. For light incident on biologically important molecules, the effect can be dramatic. Light is required to initiate the process of photosynthesis for organisms containing chlorophyll, and therefore, light supports life. The absorption of visible light by 11-cis-retinal induces a conformation and structural change to all-trans-retinal and the result is sight. The photochemical mechanisms displayed by nature are the subject of extensive spectroscopic research as the methods of isolation of these systems improve.

Optical spectroscopic techniques are based on the same classical relationship between molecules and light. The mechanism of interaction provides detail with respect to molecular structure and environment. As outlined by the selection rules of quantum mechanics, the information gained depends on the specific properties of both the molecule and the light. So, for a single molecule with intrinsically defined structure, complete characterization may be ideally achieved by varying the properties of the light. The

variation in wavelength, for example, may dictate whether the observed data reveals electronic, vibronic or rotational states (or all three). Polarized light may also be required to take advantage of unique structural features.

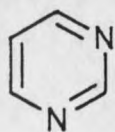
For the first time, two-photon fluorescence excitation spectroscopy has been applied to the monomeric bases and nucleotides of deoxyribonucleic acid (DNA). A new and detailed one-photon photoselection study of thymine and uracil is also part of this work. With the complete elucidation of the electronic structure may surface the reasons for photochemically induced mutation pathways or changes in tertiary structure with environment. The selection rules inherent in each of these methods allow additional and complementary information to be gained. Before detailing the structural and spectroscopic features of the nucleic acid bases, a brief chronological perspective is offered with regard to the advancement of DNA research.

Historical Background

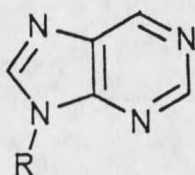
The isolation of the first DNA material, a complex of DNA and protein, was achieved by Friedrich Miescher in 1868 from discarded surgical bandages (1). Although the material was then identified as being a constituent of the cell

nucleus, no explanation of function was pursued. Twenty years after the initial isolation, protein-free DNA had been successfully purified. By the 1920's, the scope of knowledge only reached to the understanding that all cells contained this material. The structural arrangement and composition of DNA was still uncertain nearly sixty years after the initial discovery.

From 1920 to the late forties, researchers could not agree on the type of sugar present in DNA. Consequently, the exact overall structure of the monomeric units was not known. Eventually, by 1949, the sugar was identified. DNA was now known to be composed of four nucleotides based on a pyrimidine (I) and purine (II) template.

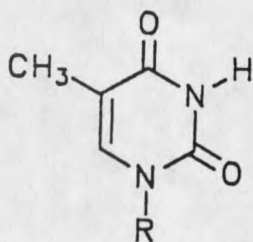


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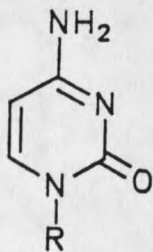


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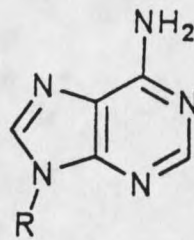
R= H, DEOXYRIBOSE



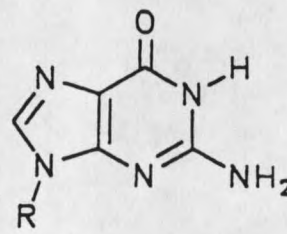
III



IV



V



VI

Thymidine (III) and cytidine (IV) comprise the pyrimidine class of molecules. The glycosidic linkage between deoxyribose and these bases is located on the N1 position. Members of the purine class, adenine (V) and guanine (VI) are derivatized with deoxyribose at the N9 position. Several more years would pass before the ratio of nucleotides would be determined and the structure resolved. Finally, in 1953, Drs. J. Watson and F.H.C. Crick published the double helix as the first complete structural arrangement for DNA (2). Although nearly 100 years had elapsed between discovery and complete structural elucidation, functional aspects were instantaneously realized. In fact, by 1960 (seven years after the structure was published), most of the framework for the genetic expression of DNA was known.

Through a cooperative ensemble of enzymes and protein structures, the DNA code can be replicated into an identical copy for the purpose of cell division or transposition. Alternatively, the DNA can be transcribed into ribonucleic acid (RNA) for translation into protein or ribosomal construction. Considering the vastness of the code, for example a human genome, this matter of cellular business is nearly error free. Problems arise when the code is

mishandled or changed in a way that modifies the message being copied. Such errors or mutations can arise from several mechanisms.

The severity of a mutation depends upon the location of the code and the type. Some mutations can be silent, such as point mutations, provided that the location is not critical. Sickle cell anemia is an example of a devastating point mutation (3). Shift mutations are generally more severe to the normal functions of the cell. In many cases, these mutation mechanisms are naturally mediated through base pair mismatching or improper enzyme recognition. Mutations can arise, however, from external stimuli. Two examples of the introduction of external "monkey wrenches" would be a chemical or radiation (4,5).

Chemical mechanisms predominately involve the intercalation of compounds, usually mono- and polycyclic aromatic rings, into the DNA covalently or electrostatically locking the DNA for further use. Interaction of wavelengths shorter than 315nm with DNA poses the second natural hazard (6,7). To understand the molecular photochemistry, a complete knowledge of the electronic characteristics of the base chromophores is paramount. Structural chemists using circular dichroism (CD) techniques to resolve tertiary

structure also require detailed electronic information (8-10). Particularly important to the CD spectroscopist are those wavelengths shorter than 200nm.

Although the first applications of a one-photon spectroscopic technique were conducted in the early 1940's, the bulk of the spectroscopic effort did not begin until after the structure and questions concerning function were forwarded post 1953 (1). From 1953 to the present, a large body of information detailing aspects of electronic structure have been compiled. A recent review by Dr. P. Callis summarizes some of these results (11).

The electronic properties reported have been useful in determining the mechanisms of several well known DNA modification events. Most noteworthy is the photochemically induced pyrimidine-pyrimidine cyclization reaction (12-14). However, with regard to all of the bases, the conclusions as to the identity and number of electronic states within the near and far- ultraviolet (UV) region are diverse and contradictory. The theoretical and experimental reasons will be addressed in the next section, but the relatively young age of DNA excited state research should be evident. In fact, this research is only slightly older than the author.

The following section will address properties common to all of the nucleic acid bases from both a structural and excited state view. The final section in this introduction will expose the problems and techniques used to address structural questions of these biomolecules.

General Electronic and Structural Characteristics

Because of the common structural and electronic properties inherent in the DNA bases, the experimental and theoretical difficulties and advantages of one are often applicable to all (11). The bases and purine are members of a point group which is void of any symmetry beyond a single reflection plane, C_s . This classification makes the analysis of spectroscopic data challenging since all dipole (and quadrupole) transitions between states are symmetry allowed. Pyrimidine contains higher order symmetry, C_{2v} . The excited states for molecules of C_s symmetry transform into two separate types with no other possible subdivision. Since the nucleic acid bases are nitrogen heterocycles containing amine and carbonyl functional groups, the types of transitions are limited to predominately in-plane polarized $\pi\pi^*$ and out-of-plane $\sigma\pi^*$ in the UV region of 200 to 350nm. The $\sigma\pi^*$ transitions are normally labeled $n\pi^*$ due

to the non-bonding origin of the excited electron. A Rydberg series beginning at 182nm was observed for pyrimidine in a crystal matrix, and therefore, must not be overlooked when interpreting data in the far-UV and near-VUV (15). Theoretical classification of these excited states is made difficult by electronic configurations with more π electrons than centers. Uracil and cytosine, for example, are isoelectronic with m-xylylene dianion (11). Purine, on the other hand, is isoelectronic with the annulenide ($C_9H_9^-$) system. To correlate these relatively unknown systems with theoretically well established molecules such as benzene (six π electrons on six centers), is not appropriate but has been attempted (8-11,16).

The radiative properties of the nucleic acid bases for excited state relaxation also display common characteristics. The fluorescence quantum yield (Φ_f) for all the neutral DNA bases is on the order of 10^{-4} in room temperature aqueous solution (17-21). This yield increases by as much as three orders of magnitude under low temperature (77K) conditions (21-23). One-photon molar absorptivity maxima for the neutral bases at room temperature are on the order of $10^4 \text{ M}^{-1}\text{cm}^{-1}$ (24). For all of these bases in aqueous solution, the UV absorption spectra

are broad with a full width at half maximum (FWHM) of roughly 5000 cm^{-1} for the first absorption envelope. From the integration of the first absorption band, a radiative lifetime (τ_r) of about seven nanoseconds (nsec) can be calculated for each base (19,25). By definition, the radiative lifetime is equivalent to the inverse of the fluorescence rate constant in the absence of any non-radiative mechanism. So, the fluorescence lifetime (τ_f) may be calculated from the product of the Φ_f and τ_r . The general experimental result, $\tau_f \approx 1$ to 10 picoseconds (psec) for the DNA bases, has been indirectly verified (19,21,26).

With a short τ_f compared to the rotational correlation time, the relative transition moment direction can be experimentally derived from the observed fluorescence emission anisotropy. If a molecule, with an isolated lowest energy transition, has a rotational diffusion time significantly greater than the lifetime of the excited state, then the emission moment observed will be oriented along the same direction as the absorption moment resulting in a high anisotropic emission. Conversely, for those molecules with a long τ_f in relation to rotational diffusion, the polarization information will be lost to sample orientational averaging (27). That is, the emission

will be isotropic. For the bases, all with τ_f less than the time for rotational motion, the relative polarization of each transition moment is ideally resolvable by experiment in room temperature aqueous solution.

In conjunction with the polarized fluorescence data, the τ_f for these biomolecules also reveals the transition type. The life of the excited state is related to the allowedness of the relaxation event. A short radiative lifetime is characteristic of an allowed transition. In contrast, a less allowed transition would have a comparatively longer radiative lifetime. Notice that the word "forbidden" is not used because such a term implies "not at all". An example of long radiative lifetime would be that associated with triplet transition in which a spin inversion is required. For thymine, the constant emission anisotropy across the first absorption band is high (nearly equivalent to the theoretical maximum) indicative of a collinear absorbing and emitting transition moment (19,28,29). Therefore, with a strong absorption moment, short τ_f and high anisotropy, the lowest excited singlet state is $\pi\pi^*$ in neutral aqueous solution. From similar analysis, the lowest excited singlet states for all of the DNA bases is of $\pi\pi^*$ character in polar solvents (19).

Supporting experimental and theoretical methods will be discussed in subsequent sections. Interpretation of fluorescence data of molecules dissolved in various solvents depend on the identification of the lowest excited state. Because of the presence of both $n\pi^*$ and $\pi\pi^*$ states, another common property to all bases, the polarity of the solvent must be specified.

Since these molecules are nitrogen heterocycles with substituent amine and carbonyl groups, two types of $n\pi^*$ states can occur. The carbonyl $n\pi^*$ event represents the weakest of the $n\pi^*$ transitions due to the small overlap between the non-bonding p-orbital on the oxygen and the π network. The integrated absorption strength is roughly 100 times weaker than a $\pi\pi^*$ transition. The second and slightly stronger $n\pi^*$ transition is centered on the hybridized ring nitrogen. With regard to experimental observations of the DNA bases, the relative energies of the $\pi\pi^*$ and these $n\pi^*$ states vary depending on the molecular environment (19,28,30,31). Theoretical predictions with all-valence electron parameterization support the close proximity of these transition types (32,33). This is not the case, however, with the parent molecules of pyrimidine and purine where the $n\pi^*$ state is the lowest excited singlet state

under a variety of conditions (34-39).

For the nucleic acids the separation in energy for the $n\pi^*$ and $\pi\pi^*$ states must be either sufficiently small or extremely sensitive to the type of solvent. With uracil and adenine as examples, the lowest excited state is determined by the solvent. In a polar environment and under supersonic jet expansion, the lowest excited singlet state unquestionably displays $\pi\pi^*$ character for all the DNA bases (8-10, 29, 40-48). The presence of the $n\pi^*$ state appears in polarized absorption, reflectance or fluorescence spectra only under three circumstances: (1) when there is a large separation in states; (2) when the $n\pi^*$ state resides in energy corresponding to a minimum in the $\pi\pi^*$ profile; and (3) when these molecules are dissolved in non-polar aprotic solvents resulting in a state reversal. The second and third experimental observations have been reported for adenine, thymine and uracil (8-10, 30, 40, 41, 46). The $n\pi^*$ transition type may also be observable using CD spectroscopic methods (8-10). Since, in regard to this dissertation, the fluorescence spectroscopic methods applied to these bases were conducted using polar aqueous solvents, the origin of the fluorescence, in all cases, will be characteristic of a $\pi\pi^*$ transition.

The common structural characteristics of the nucleic acid bases does not end with their radiative similarities. Because of the composition of these bases, the possibility of prototropic tautomerism must be adequately addressed when studies are conducted in aqueous solvents. For pyrimidine and its associated bases, the problem of spectral interference from significant concentrations of tautomer impurities is theoretically and experimentally found to be nonexistent. Both the diketo form of uracil (thymine by implication) and amino-keto form of cytosine are dominant in room temperature aqueous solution (19,29,50-53). The latter, cytosine in the amino-keto form, is found to be $10^5:1$ more concentrated than the imine-keto form which is the second most abundant species (54,55). Only in non-polar or high temperature environments does the imine-keto form of cytosine become appreciable (55). The nucleotide of each base was chosen for study to limit the influence of tautomer contributions. The result of tautomeric stability for the pyrimidines is very comforting with respect to the structural integrity of DNA. If the base pair hydrogen bonding scheme were energetically unstable, then there may be a higher probability for chance mutations or constant DNA melting. For the spectroscopist studying the purine bases,

however, the concentrations of interfering tautomers is of concern.

For purine and its derivatives, the optical spectra are influenced by equilibrium mixtures of tautomer impurities. Of particular concern is the relative concentration of the 7-H and 9-H tautomers of purine and adenine. In the case of adenine, the equilibrium constant for the relative concentrations of 7-H verses 9-H is 0.28 (56,57). The absorption maxima for these two forms are also distinguishable since they differ by about 10nm (57). Even though the 9-H tautomer has a 3:1 concentration advantage, there is nearly a 100:1 domination of the fluorescence by the 7-H tautomer in low temperature studies (29). Fluorescence from adenine solutions at room temperature also originates primarily from the 7-H tautomer (19). As in the case of the pyrimidines and with the exception of purine, the purines of adenine and guanine used in this dissertation are derivatized at the nine position, and therefore, the spectra observed for these systems will be characteristic of the 9-H tautomer.

A more serious issue of direct concern is the possibility of small equilibrium concentrations of various anionic and cationic species, which are seen in Table 1, to

often have comparatively high Φ_f relative to the neutral molecule. The presence of such interfering species is the result of a pKa in close proximity to neutral pH. Some of the bases have well documented Φ_f for their ionic forms and are listed in Table 1.

Table 1. Fluorescence Quantum Yields for Selected Neutral and Ionic Nucleic Acid Bases and Nucleotides at Room Temperature.

Nucleic Acid Base	pKa	pH	$\Phi_f (\times 10^4)^a$	ref.
Thymine	9.9	7.0	1.02	19
		12.4	17.0	17
TMP	9.9	7.0	1.14	60
CMP	12.3	7.0	0.84	60
5-methyl Cytosine	4.6	2.0	3.0	58
	12.4	7.0	5.0	58
		14.0	140.0	58
Adenosine	3.5	1.5	7.7	59
	12.4	7.0	0.49	60
Guanosine ^b	1.6	1.0	1600.0	22
	9.6	7.0	200.0	22
		11.0	900.0	22
GMP		7.0	0.8	20
AMP		7.0	0.5	20

a at the maximum absorption wavelength

b at low temperature (77K)

Because of the presence of these ionic species, the pH of the solution must be known in order to account for the relative concentrations of species present. For this reason, a buffer is often used as the aqueous solvent.

Considerable care must be taken with unbuffered media (such as water) which can change pH with time, and therefore, vary the relative composition of the solution. However, even when the solution pH is accurately known, the presence of a small amount of ionic contaminant can be overlooked.

Statement of the Problem

After the analysis of several decades of research dedicated to identifying the types and number of excited electronic states, there still remains some confusion. In part, this is a direct result of the intrinsic structural and radiative properties. Irrespective of the discrepancies found in the description of the $\pi\pi^*$ states resolved in the UV range, there seems to be particular importance placed on the identification of the first $n\pi^*$ state in aqueous solvent. The elucidation of such a weakly absorbing and emitting state in a "sea" of strongly dominant $\pi\pi^*$ transitions may be comparable to finding the proverbial needle in the haystack. Thymine and uracil, more than the other bases, are the subjects of a constant tug-of-war regarding the type and number of transitions under the first absorption envelope. This may not be so much because of their importance to the photochemistry of DNA, but because

they are structurally and spectroscopically less complex than their purine counterparts.

To review the literature concerning the electronic structure assignments prior to this work, the optical spectra of uracil (by example) could be explained by the presence of at most four transition manifolds in the 195 to 300nm range (10,11). There is considerable agreement on the location of the two strongest $\pi\pi^*$ transitions (9,10,30,31,40,46-49,61-64). These two transitions are partitioned into each of the two absorption envelopes and carry the bulk of the integrated oscillator strength for each band. The controversy arises in where to assign the location of the remaining bands provided that they exist at all.

Crystal absorption, magnetic circular and circular dichroism work offers a composite picture of at least two bands for the higher energy envelope (9,10,47). A second transition located at about 220nm may be a $\pi\pi^*$ transition with a calculated one-photon polarization direction nearly equal with the first transition at 265nm according to molecular orbital theory (65). Such polarization character would explain the difficulty in resolution using polarized one-photon techniques. Because of the weak strength, the

assignment of this state as an $n\pi^*$ cannot be precluded.

The first absorption envelope has also been reported to be composed of at least two transitions (10,30,49,61-63). In contrast, several similar studies have reported that the first absorption envelope is generated by a single $\pi\pi^*$ transition (19,28,29,43,44,47). Even when applying the same technique to this question, the results are mixed and contradictory. For those who agree on the composite nature of this band, there appears to be equal disagreement on the position and type of second transition influencing the spectral characteristics of the uracils. Taking advantage of the structural and radiative properties previously discussed, polarized fluorescence emission and excitation studies were conducted on thymine to probe the excited state characteristics of the first absorption band.

In 1979, P.R. Callis published polarized fluorescence excitation data for thymine dissolved (0.01M) in distilled water, $\text{pH} \approx 5$ (19). From a high and constant fluorescence anisotropy ($R \approx 0.33$) across the first absorption band, the observed integrated oscillator strength was postulated to result from a single $\pi\pi^*$ transition. This conclusion was further advanced by the mirror-like image of the fluorescence emission with the absorption profile. Soon

after these results were reported, a similar study was completed by Drs. Morgan and Daniels (63).

In the Morgan and Daniels study (MD), a dilute thymine solution (about 10^{-4} M) was prepared using a neutral phosphate buffer solvent. Except for the buffer and sample concentration, the type of experiment paralleled the Callis procedure. Consequently, their results were similar in every respect except that MD observed a large drop ($\Delta R=0.2$) in fluorescence anisotropy on the red edge of the absorption band (280-300nm). The conclusion forwarded by MD was that there existed a second state, $n\pi^*$, under the first absorption band. This discrepancy in results for identical procedures was troublesome until the difference in experimental condition was realized (28).

The critical divergence between the procedures was not in concentration, but that the solutions differed in pH. With a basic pK_a for thymine of only 9.9, the solution pH comparisons suggested the possibility that MD was not observing a new state, but interference from the highly fluorescent thymine anion. Structural and spectral characteristics of the thymine anion have been reported by several prior investigations (17,33,67). Therefore, fluorescence polarization spectra for thymine and several

derivatives will be re-evaluated with pH by experimentally observing and calculating the thymine anion contribution.

The theory describing the excitation from the ground state by simultaneous absorption of two photons was first proposed in 1931 by M. Goppert-Mayer (68). The theory of two-photon absorption proposed at that time could not be tested until the laser was developed to overcome the low transition probabilities. Because of this perception of low transition probabilities under two-photon excitation, only those molecules with large fluorescence quantum yields ($\Phi_f \approx 1.0$) were used when monitoring with a fluorescence detection scheme. In fact, as late as 1979, the two-photon absorption spectrum of benzene ($\Phi_f = 0.06$) was recorded using a thermal blooming detection method because "no fluorescence monitoring of benzene seems possible" (69,70). To answer this misconception, the two-photon fluorescence excitation spectra of both benzene and pyrimidine ($\Phi_f \approx 0.003$) were reported shortly after the thermal blooming studies (36,71).

With the development of advanced laser and detection methods, the idea of directing this two-photon fluorescence excitation method to the questions surrounding the bases of DNA was proposed. Perhaps, those states which were weak under one-photon excitation may be enhanced by two-photon

absorption. Also, the survival of two-photon polarization information for samples in solution may provide additional support to state location and order. In support of these possibilities, semiempirical molecular orbital theory predicted interesting properties which may be further calibrated with experimental results.

With these ideas also surfaces the realization that this would mean a new detection limit domain of $\Phi_f = 10^{-4}$! This represents a two to three order of magnitude lower yield than other molecules subjected to such a technique. In addition to quantum yield limitations, these molecules also have low room temperature aqueous solution solubilities. Therefore experiments on the DNA bases will also be concentration limited. The motivation to pursue the use of two-photon excitation further is that the two-photon cross section, which is related to absorptivity, is theoretically predicted to be about 100 GM (Goppert-Mayer) larger than several well-known substituted benzenes (27).

Since both techniques, polarized one- and two-photon fluorescence excitation spectroscopy, can provide essential excited state information regarding the bases of DNA, application of each will be presented in this dissertation. A particular emphasis will be placed on thymine and TMP for

reasons outlined in previous sections. The two-photon spectra of cytosine and the relevant DNA nucleotides will also be discussed in the context of polarized data obtained.

EXPERIMENTAL

One-Photon Studies

Instrumentation

Fluorescence excitation and emission measurements were made using a Spex Fluorolog2 (Model F211) spectrofluorometer equipped with a 150 W Xenon-lamp. A Datamate microprocessor was interfaced to the spectrofluorometer for the purpose of data acquisition and processing. Polarized fluorescence was differentiated via a single Polacoat film polarizer (3M) positioned before the emission monochromator. Depolarizing crystals were positioned in both the excitation and emission monochromators reducing instrumental corrections to a few percent. Sample concentrations and absorption determinations were conducted on a Cary 14 spectrophotometer. Measurements of solution pH were analyzed on a Sargent-Welch model NX meter with a S-30070-05 miniature combination electrode.

Measurements

For emission measurements, a 4.5nm bandpass was used on the excitation monochromators, and a 1.8nm bandpass for the emission monochromator. For excitation spectra, 1.8 and 18nm bandpasses were used on the excitation and emission monochromators, respectively. The time of signal integration was 30 seconds per point. In both the excitation and emission profiles, measurements were performed in one nanometer increments throughout the scanning range. Excitation scans were typically conducted from 240 to 300nm with the emission wavelength set at 360nm. Emission profiles were generated from 300 to 400nm with the excitation wavelength set at 295nm.

Sample Preparation

Thymine, uracil, and UMP (sigma) were dissolved directly into a 0.015M acetate (pH 5.0) or phosphate buffer (pH 7.6 or 12) at concentrations of 10^{-4} to 10^{-2} M. Thymine and uracil required gentle heating. The most accurate results were obtained with 0.01M solutions. Although these solutions were nearly saturated, the fluorescence was rigorously independent of excitation wavelength from 260-295nm and the polarization was very high, providing the pH was six or below. The high absorbance essentially

eliminated the need for corrections due to solvent emission and provided a higher signal. The solvent for the buffer, triply distilled water, was further purified over activated charcoal, which often increased the purity by ten fold. Also, purity determinations were made on the solvent and buffer before introduction of thymine. Purity was defined by the ratio of the water Raman peak to the buffer fluorescence. The pH of the sample was carefully determined after an experiment. Both the buffer and thymine solutions were made just before each run to assure purity.

Two-Photon Studies

Estimation of Signal Level

Using the specifications of the two-photon apparatus detailed in subsequent sections, the following calculation is offered to estimate the expected signal level of a 0.02M TMP solution in an one centimeter path length cell. Therefore, suppose that the two-photon cross section (δ) for TMP is on the order of 100 gm or 10^{-48} cm⁴ sec photon⁻¹ molecule⁻¹. The number of photons absorbed by such a sample can be calculated using the expression derived from a Beer's law relationship (72),

$$N_{\text{abs}} = N_0 \delta C I_0 l \quad (1)$$

where N_x is the number of photons, C is the concentration in units of molecules cm^3 , I_0 is the intensity (photons $\text{sec}^{-1} \text{cm}^{-2}$) which contains the beam focussing information and l is the length of the irradiated path.

For a laser pulse delivering 1MW peak power in 8nsec at 520nm, there are on the order of 10^{11} two-photon transitions per pulse. A beam area of 0.01cm^2 was used in a typical experiment. Given the Φ_f of TMP, there should be a subsequent emission of about 10^7 photons emitted across 4π steradians. In the current configuration, only about one percent of the total solid angle was collected. Consequently, the number of photons incident on the photomultiplier cathode, taking into account reflection losses on the optical surfaces and for the absorption of the UV pass filters, is about 10^5 photons.

Now that the photons are at the photocathode, the quantum efficiency and gain per dynode must be taken into account. For a typical photomultiplier, for example the Hamamatsu R955, the quantum efficiency is about eighteen percent at the TMP emission wavelength of 330nm. In the present dynode configuration, the expected gain is about 10^7 . Therefore, using the formula to convert number of incident photons into charge (in coulombs), the expected

value for 10^5 photons is 3×10^{-8} coulombs. If this charge is collected over 0.2 milliseconds with an integration time constant of $100 \mu\text{s}$, the expected voltage output would be approximately 1.5 volts per pulse. Since there is no stray light as the result of the use of both an emission monochromator and UV pass filters and that the photomultiplier housing is thermoelectrically cooled, this voltage is more than sufficient to encourage the use of the two-photon technique to the DNA bases.

Instrumentation

In order to overcome the low probability with which a two-photon transition is initiated, a laser capable of delivering a beam of sufficient power density was required in the place of conventional light sources. For the measurements discussed in this dissertation, a Lumonics laser system was used. The HY200 (oscillator only) pump laser, with neodymium:yttrium-aluminum garnet (YAG) as the amplifying material, typically delivered 70mj/pulse with the second harmonic (532nm) and 30mj/pulse with the third harmonic (355nm) of the 1064nm fundamental. The dye laser, a HyperDye 300 tunable system, was used to generate the visible wavelengths. Dyes used to access the entire profile region, listed in Table 2, were purchased through Exciton

and dissolved in reagent grade methanol (Baker). To minimize amplified spontaneous emission (ASE) to less than ten percent of the energy at maximum gain, the dye solutions were prepared in the concentration recommended by Lumonics for the coumarin dyes and half the recommended concentration for the rhodamine dyes. Estimation of ASE was obtained by blocking the rear mirror of the dye laser. In addition, diaminobicyclooctane (DABCO) was introduced to the coumarin dyes as a radical scavenger to extend the dye half-life (73,74). This reagent, purchased through Aldrich, was added at 1g/l concentration.

Table 2. Laser Dyes.

Dye	Typical Maximum Energy Per Pulse (mj)	Typical Wavelength Range (nm)
DPS	6	400-412
DPS/MSB	5	408-430
MSB	4	412-430
S420	7	415-445
C440	5	427-455
C460	6	450-481
C480	7	467-497
C500	8	492-537
C540A	6	525-575
R590+610	19	566-603
R610+640	10	597-623
DCM	17	607-680

All of the coumarin dyes, 400-575nm, were pumped with the 355nm harmonic while the Rhodamine set was generated through 532nm excitation. To achieve the pulse energies and ranges for the 400 to 455nm dyes, a 1800 grooves per millimeter grating was used in the dye laser and calibrated to second order. All power measurements were taken with a Scientech (model 380101) calorimeter. The configuration of these lasers with respect to the rest of the two-photon apparatus is diagrammed in Figure 1.

As shown in Figure 1, the dye laser beam (approximately 2mm cross sectional diameter) was expanded and recollimated by a 4X Galilean telescope (CL1) comprised of a 120mm focal length convex and -30mm focal length concave lens, provided by Oriel, in fixed mounts. The laser beam was then steered at right angles around the table by a pair of aluminum coated mirrors (M1,2) obtained from Newport. Between these mirrors, the beam was spatially filtered through an adjustable iris.

Polarization selection was achieved by a method previously outlined by B. Dick, et. al. (75). Briefly, the laser beam was sent through a fixed Glahn-Foucault polarizer (P1) for the purpose of further purifying the vertical polarization characteristics of the incident beam. The

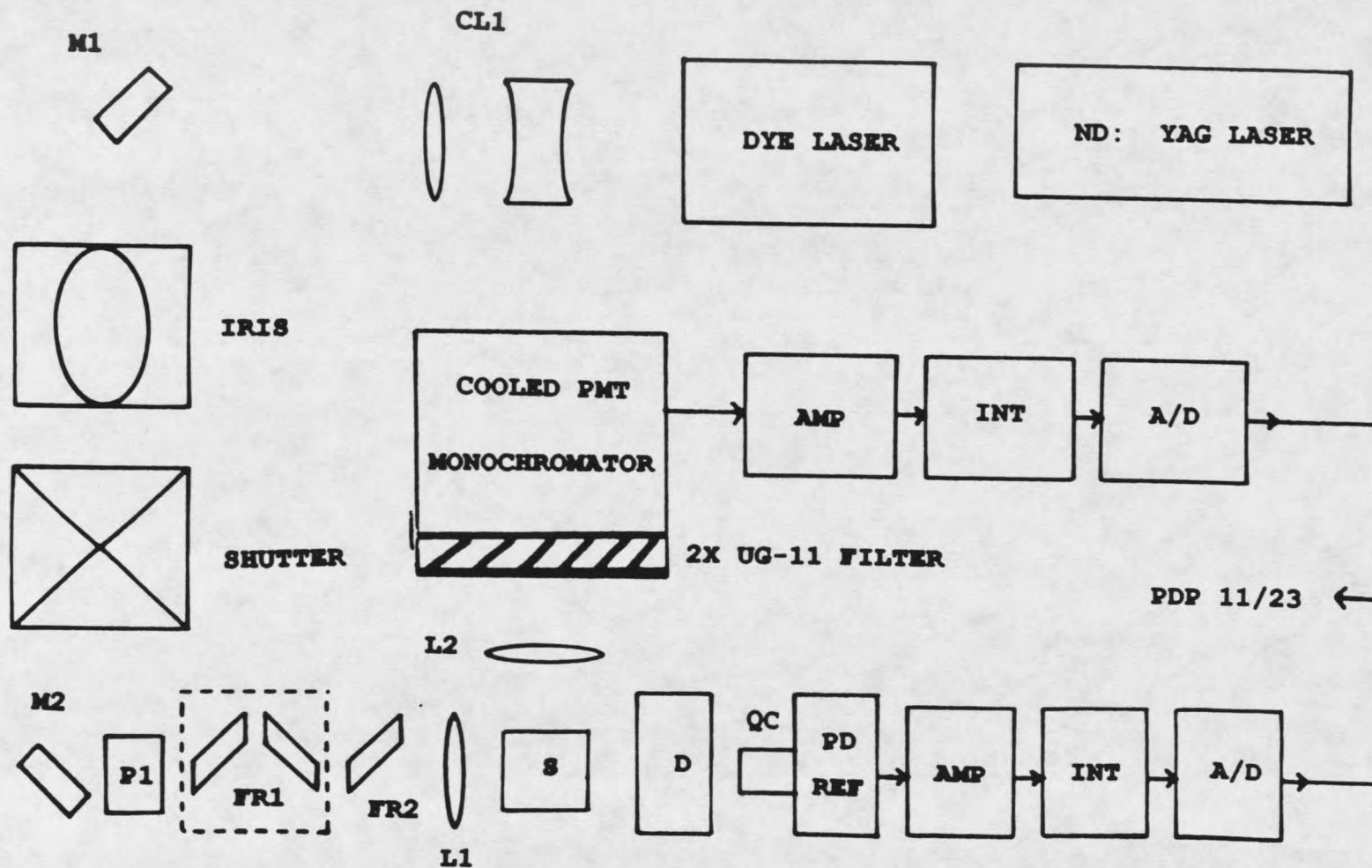


Figure 1. Polarized two photon fluorescence excitation apparatus

vertically polarized beam was then directed into a rotatable double Fresnel rhomb arrangement (FR1) which functions as an achromatic half wavelength ($\lambda/2$) device; that is, for every rotation through Θ of the FR1, the plane of polarization of the electric field becomes rotated through 2Θ (76). Down field from the FR1 was a third Fresnel rhomb (FR2) which acts as an achromatic quarter wave device. For the polarization measurements used for the two-photon experiment, vertically and circularly polarized light are required (75). To generate these polarizations, the FR1 was rotated through 22.5° thereby rotating the plane of polarization by 45° . Circularly polarized light was produced, via a 90° relative phase shift, from the FR2 by a series of two 54.6° internal reflections. Linear polarization was returned by a subsequent rotation of FR1 back to 0° or to 45° about the optical axis, in which, the associated polarization angle underwent no retardation through the third Fresnel rhomb.

Several advantages are evident from this arrangement. First, there are sixteen selectable polarization directions consisting of eight linear and eight circular positions. Although for the measurements presented in this dissertation, linear and circular light was selected by an

alternate 0° to 22.5° rotation, respectively. Secondly, the incident plane of polarization can be rotated without suffering losses in light intensity. Finally, the variability in polarization direction can provide for photoselection applications.

In order to adjust the incident light intensity, a convex focussing lens (FR1), $f=50\text{cm}$, was placed near the sample cuvette (S). A second focussing lens was placed at right angles to the beam propagation direction for the purpose of fluorescence collection and focussing into the photomultiplier tube (PMT) assembly.

The PMT assembly consisted of a combination of a -25°C thermoelectric cooled R928 Hamamatsu photomultiplier tube and H-10 Instruments S. A. single monochromator. For the majority of the samples investigated, no slits were used in this monochromator. Only for those samples with fluorescence wavelength maxima greater than 350nm were 2mm slits used. The approximate bandpass of a slitless H-10 monochromator was determined qualitatively to be about 30nm . Prior to the monochromator, two Schott $50.8 \times 50.8 \times 1\text{ mm}$ UG-11 UV pass filters were used to further discriminate the laser light from the fluorescence signal.

Finally, for the two-photon profile measurements, a reference combination of a integrating sphere coated with magnesium oxide or three Oriel Lambertian diffusing disks (D) were used before a 3g/l Rhodamine B quantum counter (QC) and photodiode assembly (PD). The schematics and design for this photodiode assembly was provided by Dr. Rufus Cone of the M. S. U. Physics Department.

Power Squared Sensor

Reference detection of the type described previously was only capable of providing a unit measure proportional to the average intensity. For the two-photon profiles, this measure was squared $\langle I \rangle^2$ and divided into the signal to obtain the experimental two-photon absorption cross section. Since the two-photon cross section is proportional to the average of the squared intensity $\langle I^2 \rangle$, distortions arise as curling of the segments toward the dye ends since the $\langle I \rangle^2$ cannot compensate for changes in $\langle I^2 \rangle$. To this end, a power squared sensor was first developed by R. Jones and P. Callis based on the theory of second harmonic generation (SHG) through powder media (77,78). As shown in Figure 2, several modifications have been made to eliminate the need for corrections due to filter absorbance and to increase signal levels to the reference PMT.

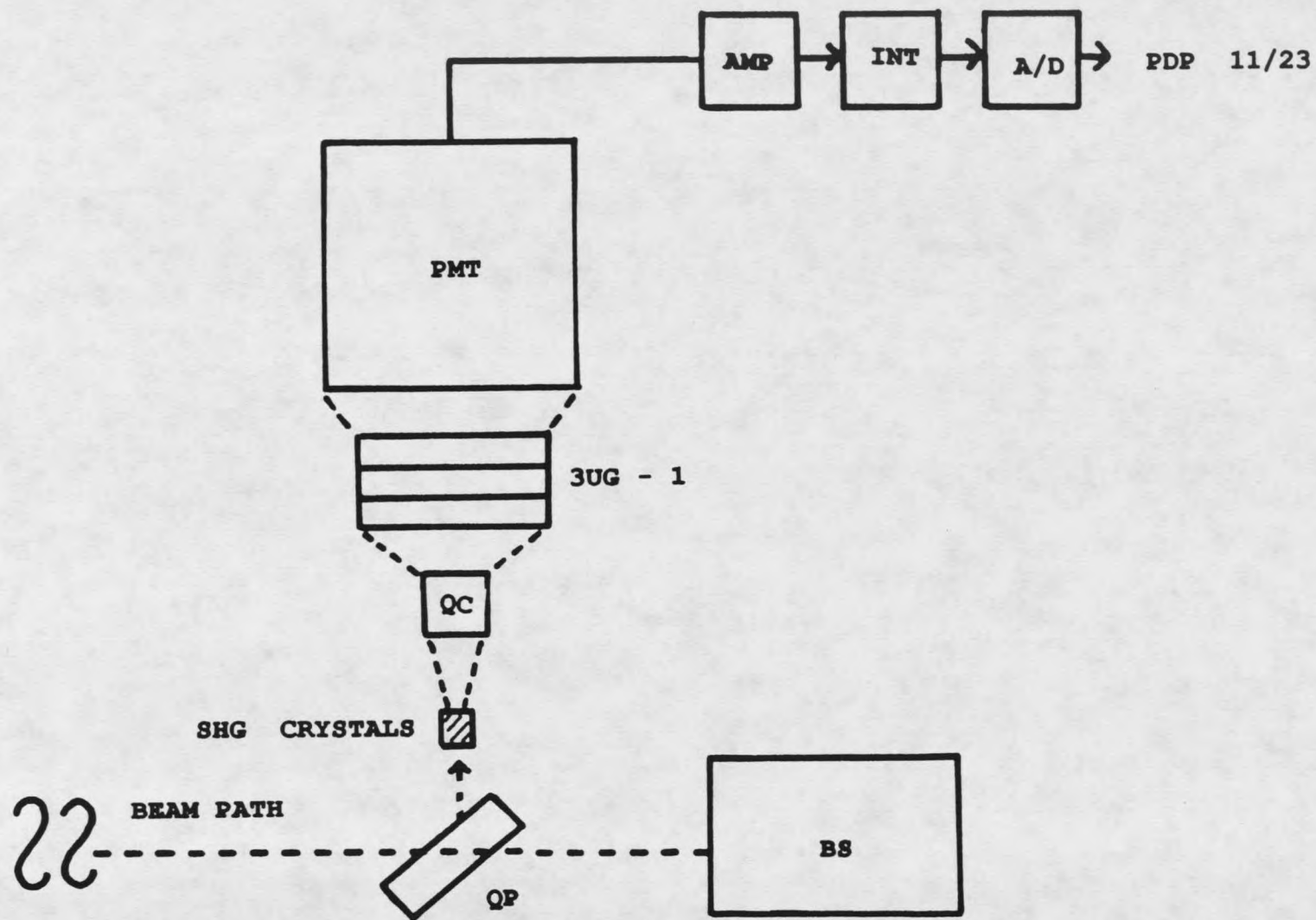


Figure 2. Power squared reference detector

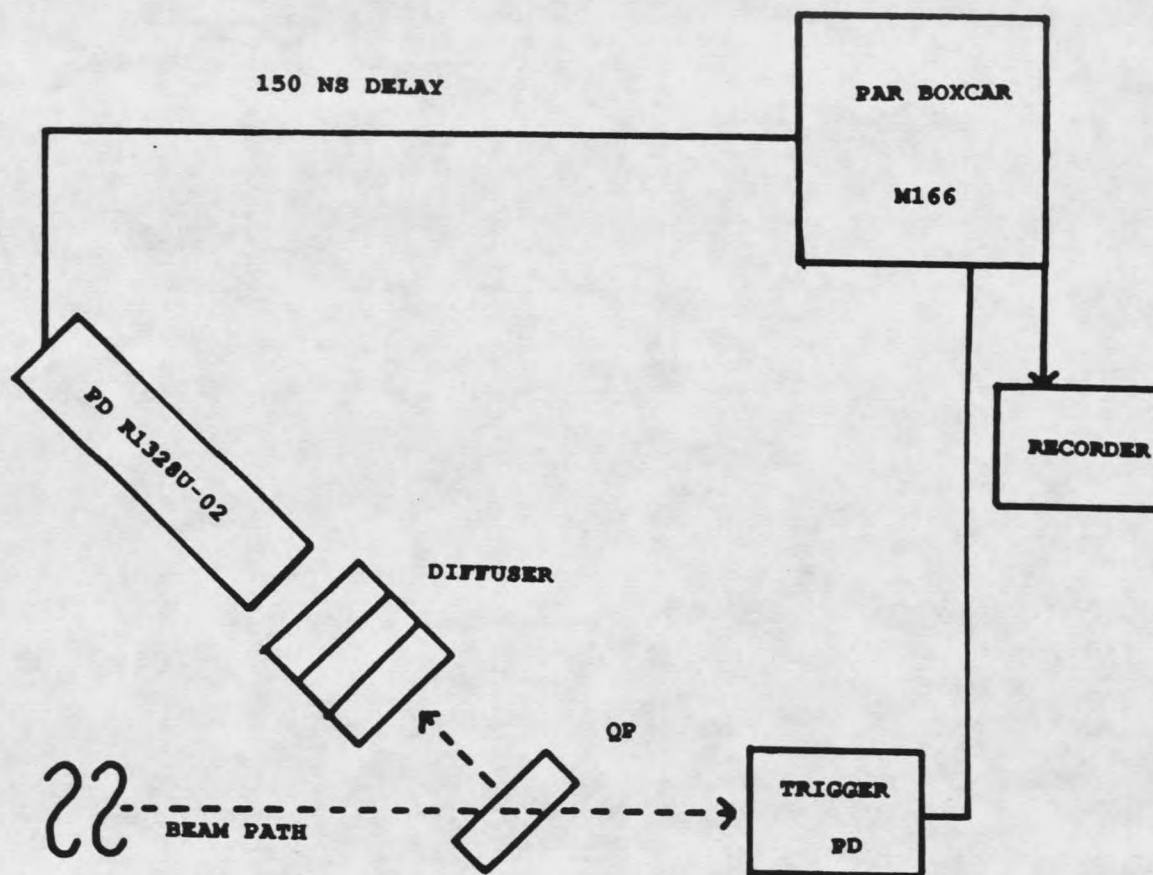


Figure 3. Temporal pulse analysis apparatus

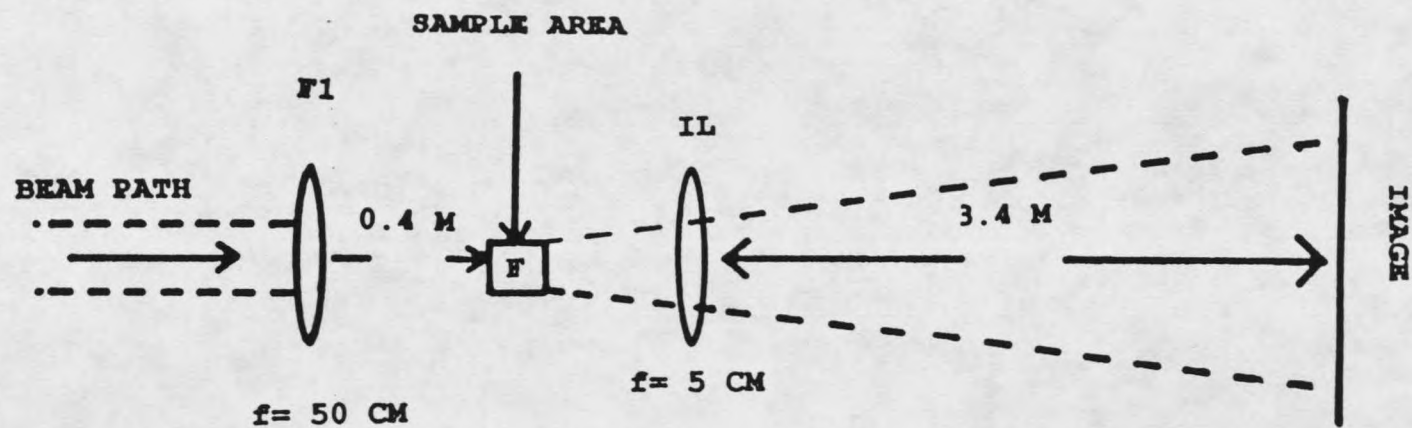


Figure 4. Spatial pulse analysis configuration

Technical assistance and advice on the application of skatol were provided by Mr. Aden Rehms of this laboratory.

The SHG powder used varied with the range of wavelength. Urea was used for the blue dyes (480-600nm) and KDP for the red dyes (590-650nm). Urea was reported to have wavelength independent conversion efficiencies from 490-600nm reducing the need for further correction (79). Obtained from Merck, the urea powder was ground by mortar and pestle and sifted to separate nearly uniform 75 μ m sized crystals. To minimize scatter of incident light, these particles were uniformly layered into a 2mm quartz cuvette (NSG, Co.) filled with a refractive index matching solvent of decahydronaphthalene (Burdick and Jackson, Inc.).

Incident laser light was split by a quartz plate (QP) into this powder matrix (see Figure 2). The SHG generated signal was absorbed by a 1g/l Skatol (Sigma) in butanol quantum counter (QC) which has been shown to have a wavelength independent quantum yield from 210-310nm (80). The Skatol emission was discriminated through three UG-1 (Schott) filters onto a R928 Hamamatsu PMT.

Pulse Width Measurements and Spatial Imaging

Figure 3 outlines the apparatus used to monitor the temporal properties of the laser pulse with wavelength.

Again, the incident beam was split by a 1/4" quartz plate. All components upfield from the beam splitter are as diagrammed in Figure 1. The split beam was directed to a diffuser assembly containing three Lambertian diffusing disks. A Hamamatsu R-1328U-02 end-on photodiode with a 75ps rise time was used as the detection device. This photodiode was biased by +1000V and gave photocurrent directly proportional to an increase in light intensity, thereby demonstrating that the phototube was unsaturated under these conditions. The beam energy was kept constant throughout the measurements with crossed polarizers.

Signal collection and analysis were performed on two instruments. The first, as listed on Figure 3, was a Princeton Applied Research (PAR) boxcar with a M163 sample integrator attachment equipped with a type S-5 sampling head. An aperture delay range of 100ns was selected with an aperture gate of 75ps (limited by the sampling head). Since the PAR boxcar requires 75ns of internal electronic delay, the signal must be held relative to the trigger pulse. To achieve sufficient delay, 100ft. of coax cable (equivalent to approximately 150nsec delay) was inserted between the phototube and the boxcar.

Temporal profiles were generated with a Houston X-Y recorder generously loaned by Dr. Richard Geer.

The second method was made possible by application of a Tektronix 7912 AD pulse digitizer (Osc.) provided by Dr. Rufus Cone of the M. S. U. Physics Department and to whom this author was most grateful. This device had the same placement as the boxcar only the delay line was removed due to the faster internal electronic delay. After the trigger pulse was received, the laser pulse profile was simultaneously monitored with resolution approaching 1ns. Pulse profiles were photographed on ASA 400 color film (Kodak). The shutter speed of a Pentax P3 35mm camera was set at one frame per 1/30 seconds. Faster shutter speed settings would have resulted in an incomplete picture due to the sweep time of the oscilloscope screen.

Once a profile was obtained, the width at FWHM was recorded along with an approximate measure of the second order coherence by a numerical integration procedure (see Table 3).

The procedure for imaging the spatial profile of the laser beam was designed on advice from Dr. John Carlsten of the M. S. U. Physics Department. As shown in Figure 4, the spatial composition of the laser beam was imaged on a wall

3.4 meters away from the imaging lens. The beam sample area was approximated to be equal to that imposed on the prepared samples discussed in this dissertation. The focussing lens, FL1, was reintroduced to the apparatus to achieve this condition. Spatial profiles were recorded on ASA 400 color film by use of a Pentax P3 35mm camera positioned in front of the image several degrees off normal. A 1/15 seconds camera shutter speed was set to take the picture of one pulse of the dye laser. In addition, the beam energy was kept constant by attenuation via crossed polarizers as monitored by a calorimeter positioned on the image side of the imaging lens.

Sample Preparation

Preparation of the selected nucleic acid bases for study under two-photon excitation were given the same care as with the thymine samples prepared in the one-photon measurements. There were some modifications, however, which improved sample purity and reduced light scatter by particulate matter in the solvent. Due to contamination of the "in-house" glass distilled water source, distilled water was purchased through Big Springs Distilled Water of Lewistown, Montana in one gallon containers and stored in a glass flask.

Table 3. Temporal Pulse Profile Characteristics.

Dye	λ	$\frac{\langle I^2 \rangle}{\langle I \rangle^2}$		FWHM (ns)	
		PAR	Osc.	PAR	Osc.
MSB/DPS	400	2.6	2.5	4.1	2.2b
	415	2.2	1.5	5.5	6.4
	434	2.5	1.6	4.1	4.5b
S420	415	2.2	1.4	5.3	5.5b
	425	2.2	1.4	4.9	6.4
	430	2.1	1.4	6.0	6.9
	447	2.4	1.5	5.3	5.5b
C440	427	2.3	1.5	5.4	5.6b
	440	2.3	1.3	5.7	7.1
	455	2.2	1.5	6.1	5.5b
C460	448	2.2	1.4	6.2	4.9b
	465	2.3	1.2	6.0	8.0
	475	2.2	1.4	6.0	6.0b
	487	2.2	1.5	6.1	3.6b
C480	462	2.1	1.4	6.5	2.6b
	485	2.1	X	6.7	X
	475	X	1.2	X	6.7
	504	1.9	1.3	6.7	5.8b
C500	495	2.0	1.3	6.0	6.4
	530	2.0	1.3	6.0	6.6
	550	2.3	1.3	5.4	4.7
C540A	523	1.7	1.6	3.3	3.6b
	545	2.1	1.3	5.9	7.6
	580	2.1	X	5.7	X
	590	X	1.4	X	4.9
R590+610	566	2.3	1.7	4.0	2.7b
	585	2.0	1.3	6.0	6.4
	598	1.9	1.3	7.0	5.3b

b refers to detection of significant mode dynamics

This source was found to be nearly 60:1 Raman to fluorescence pure with no obvious fluorescence impurities. This distilled water was further purified before use by a C₁₈ Sep Pak produced by the Waters Corporation.

Table 4 shows the selected nucleic acid bases used without further purification and the source from which they were obtained. Typical concentrations were 0.02M, yet ranged from 0.1M to 0.01M without change in one-photon fluorescence properties with excitation wavelength.

Table 4. Chemicals.

Compound	Typical Concentration (M)	Source	Lot Number
AMP	0.02	SIGMA	57C-7150
GMP	0.02	CALBIOCHEM	300712
CMP	0.02	SIGMA	66F-0488
TMP	0.02	SIGMA	85F-7145
THYMINE	0.02	SIGMA	11F-0321
CYTOSINE (A)	0.02	TOKYO-KASEI	C528
CYTOSINE (B)	0.02	CALBIOCHEM	405641
CYTOSINE (C)	0.05	ALDRICH	UNMARKED
PYRIMIDINE	0.1-0.5	SIGMA	24F-3517
PURINE	0.02	SIGMA	55F-4045
BENZENE	0.1	BAKER	435602

The two-photon excitation properties for the nucleotides were also independent of concentration in the 0.01- 0.1M range.

These compounds were dissolved in either water, glycerol (Aldrich), or 0.05M phosphate buffer (pH 2,7). Thymine and cytosine required gentle heating in order to dissolve. After each solution was prepared, the samples were filtered directly into a labeled quartz cuvette by a twice rinsed 0.22 μ m Millipore Millex-GS filter to remove particulate matter and further sterilize the preparation. A cuvette containing just the solvent in the absence of solute was treated in a similar manner. Pyrimidine was dissolved in both hexane (Aldrich) and cyclohexane (Aldrich) without further purification. Benzene was also dissolved without further purification into Photrex (Baker) methanol. When not being analyzed, all of these samples were stored in an air-tight container at 5C°. Finally, since these molecules have characteristically low quantum yields for fluorescence, both the cuvettes and glassware used in the preparation were dedicated only to this project and never exposed to other molecular systems.

Sample purity, fluorescence emission maxima, and sample pH were determined for each sample before and after a complete two-photon experiment with the Spex apparatus and procedure described for the one-photon measurements. In addition, thermal desorption mass spectra, courtesy of Dr.

Joe Sears of the M.S.U. Mass Spectrometry Facility, on a sample of thymine and cytosine after a two-photon experiment confirmed purity to 99% without revealing any breakdown or photochemically derived reaction products. Figures 5-13 are complete one-photon fluorescence and polarization measurements on samples listed in Table 4 in order to establish sample integrity. In all cases but two, the fluorescence and polarization anisotropies were characteristic of a single chromophore in solution. The multicomponent fluorescence of the AMP (Figure 5) and cytosine (Figure 10) solution, however, was troublesome and is addressed.

The drop in anisotropy (R) to the red of the fluorescence emission maximum of cytosine (Figure 10) was in response to a shoulder centered at about 350nm. A similar drop in R was observed on the red edge of the absorption band. Since cytosine was believed to contain only one transition under the first absorption band and previous fluorescence studies on cytosine fail to resolve the 350nm fluorescence (19), the origin was believed to be the result of an impurity. The magnitude of this impurity fluorescence was wavelength and source dependent. Samples (sold in the disodium salt form) showing considerable amounts of impurity

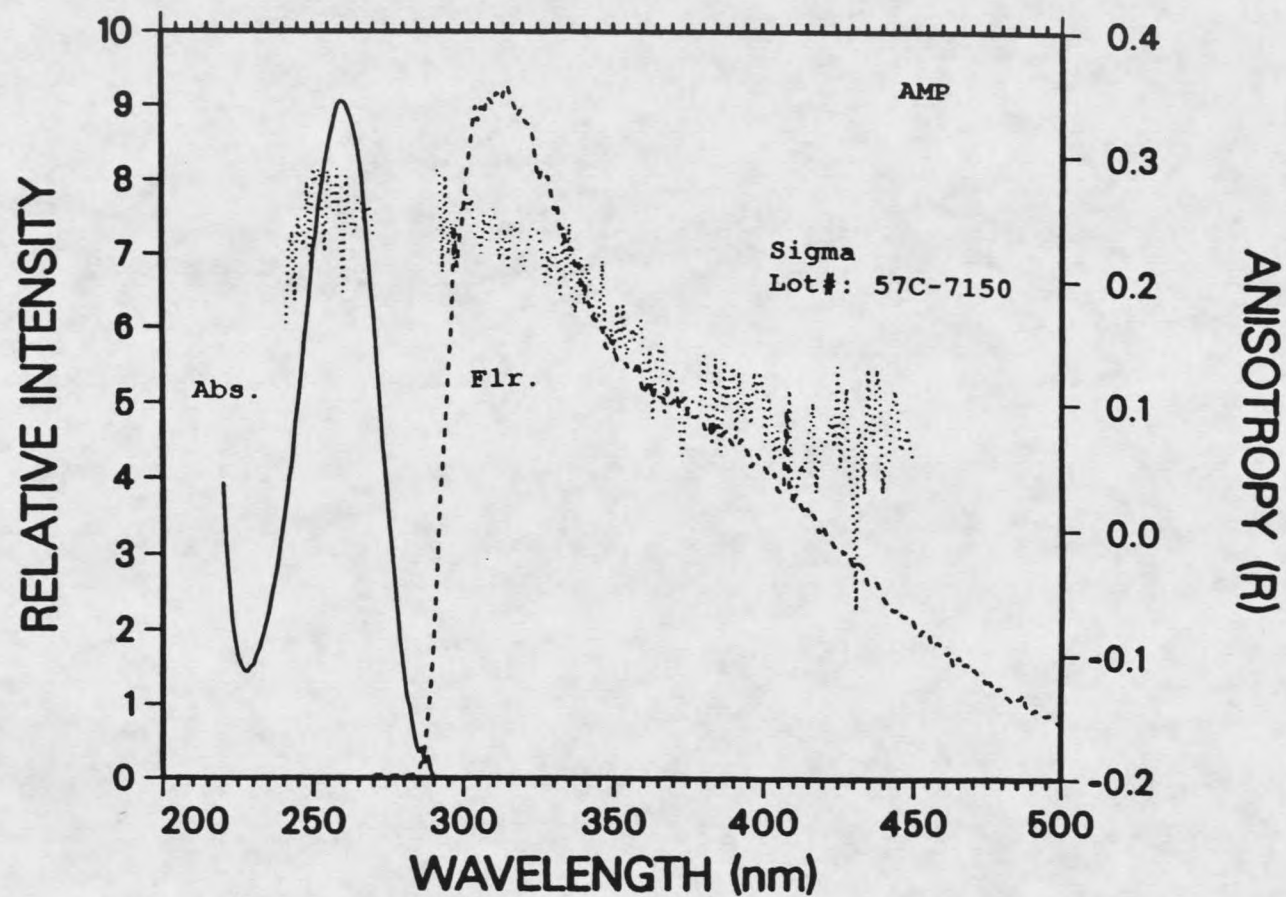


Figure 5. One-photon absorption and fluorescence spectra of adenosine-5'-monophosphate (AMP) in 0.05M phosphate buffer, pH7.0. Absorption (-); Fluorescence emission (---); Anisotropy (....).

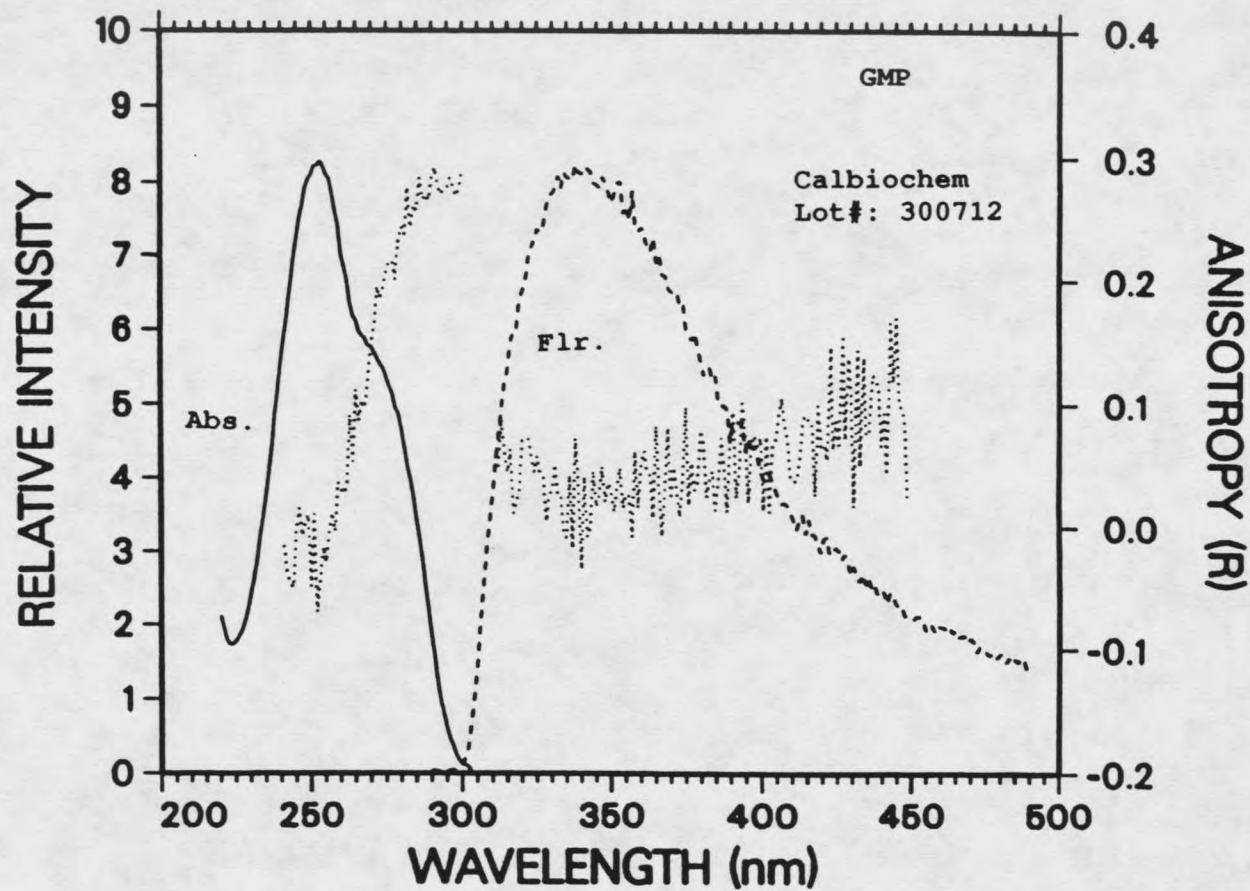


Figure 6. One-photon absorption and fluorescence spectra of guanosine-5'-monophosphate (GMP) in 0.05M phosphate buffer, pH7.0. Absorption (-); Fluorescence emission (---); Anisotropy (•••).

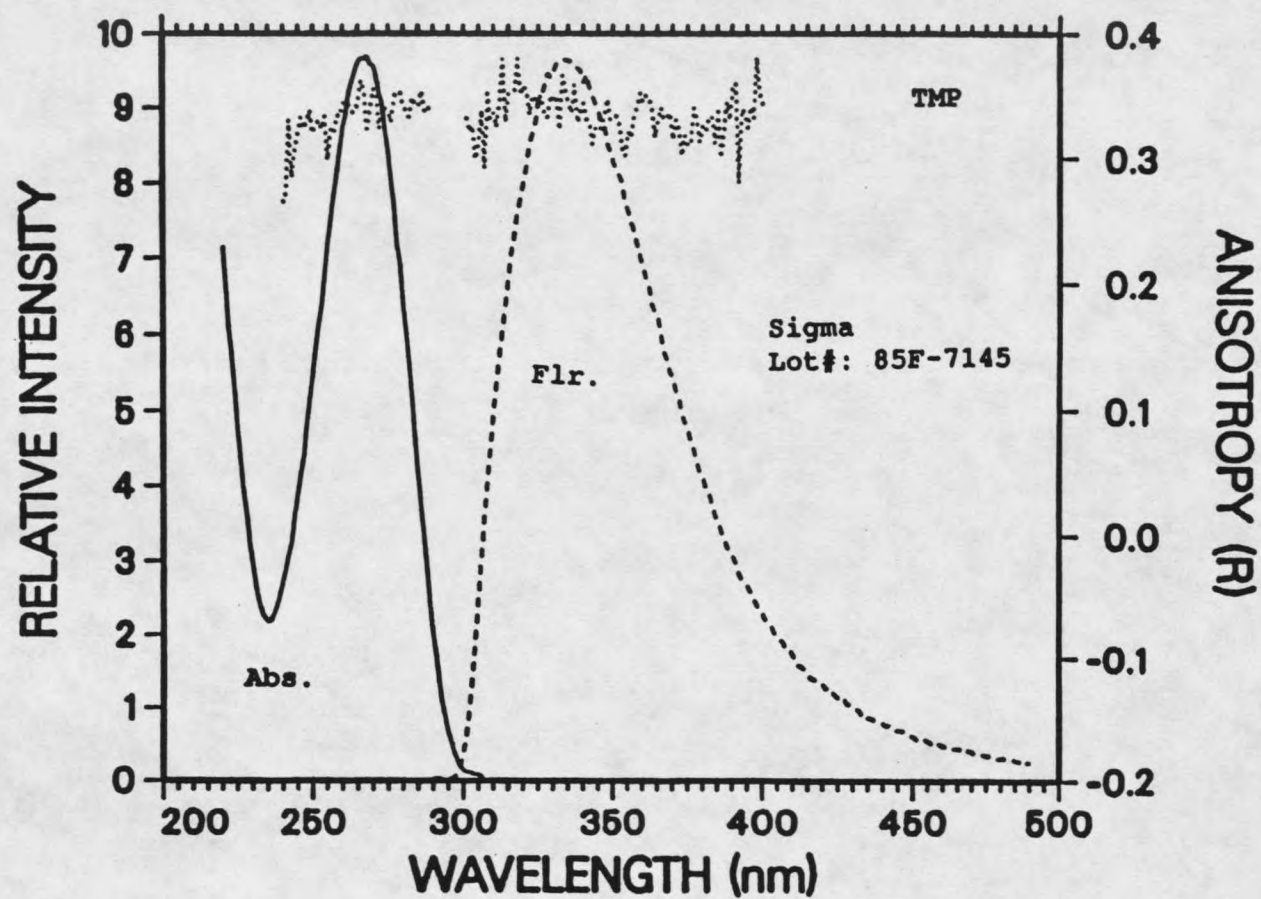


Figure 7. One-photon absorption and fluorescence spectra of thymidine-5'-monophosphate (TMP) in 0.05M phosphate buffer, pH7.0. Absorption (-); Fluorescence emission (---); Anisotropy (....).

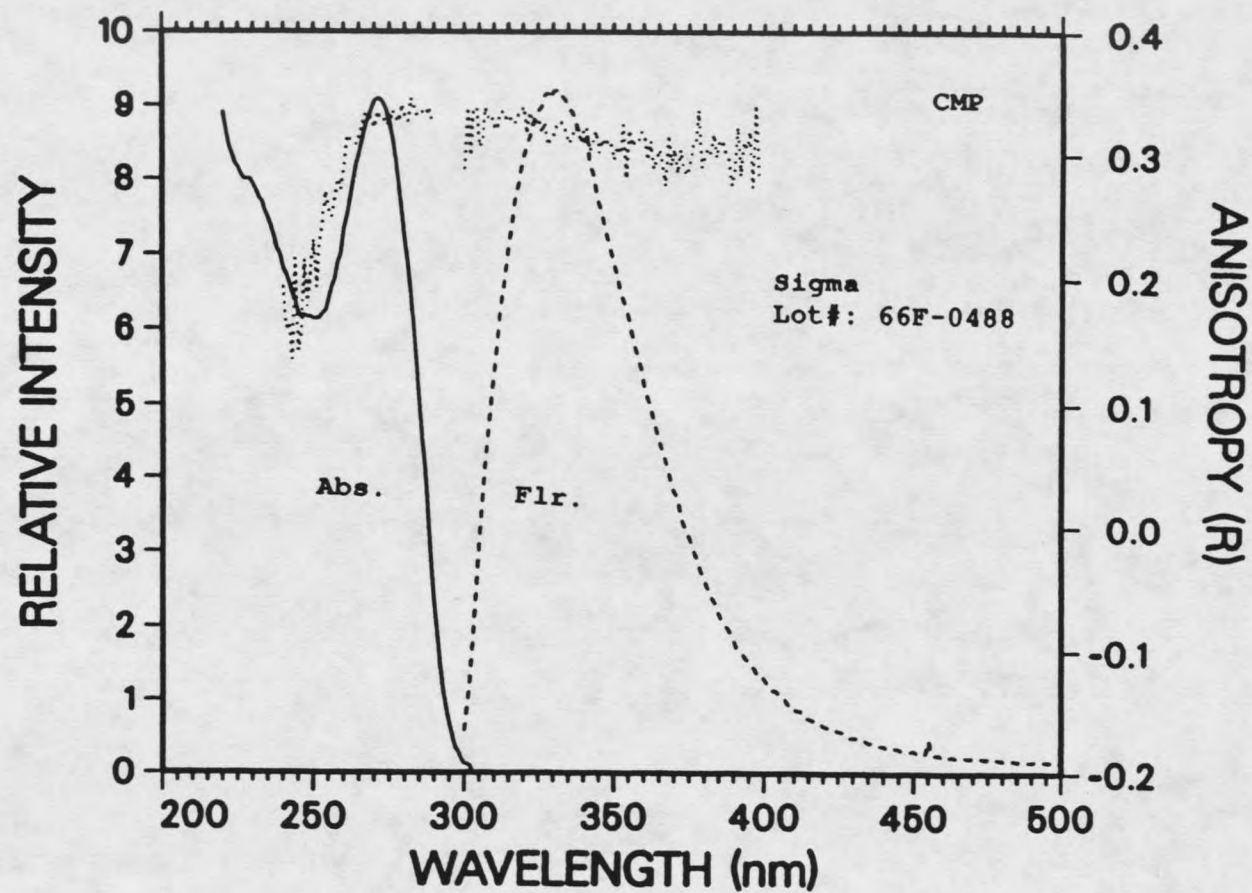


Figure 8. One-photon absorption and fluorescence spectra of cytidine-5'-monophosphate (CMP) in 0.05M phosphate buffer, pH7.0. Absorption (-); Fluorescence emission (---); Anisotropy (•••).

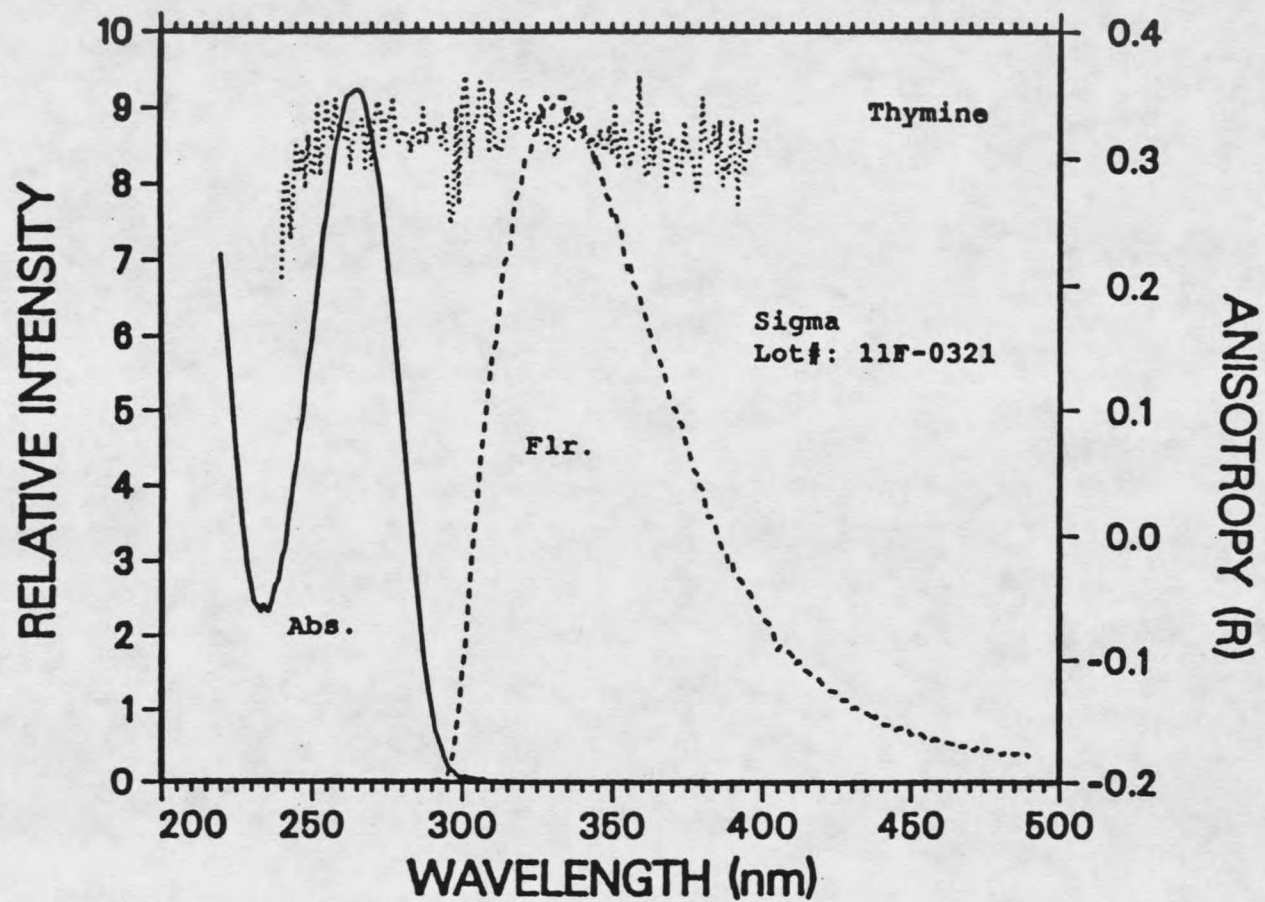


Figure 9. One-photon absorption and fluorescence spectra of thymine in 0.05M phosphate buffer, pH7.0. Absorption (-); Fluorescence emission (---); Anisotropy (···).

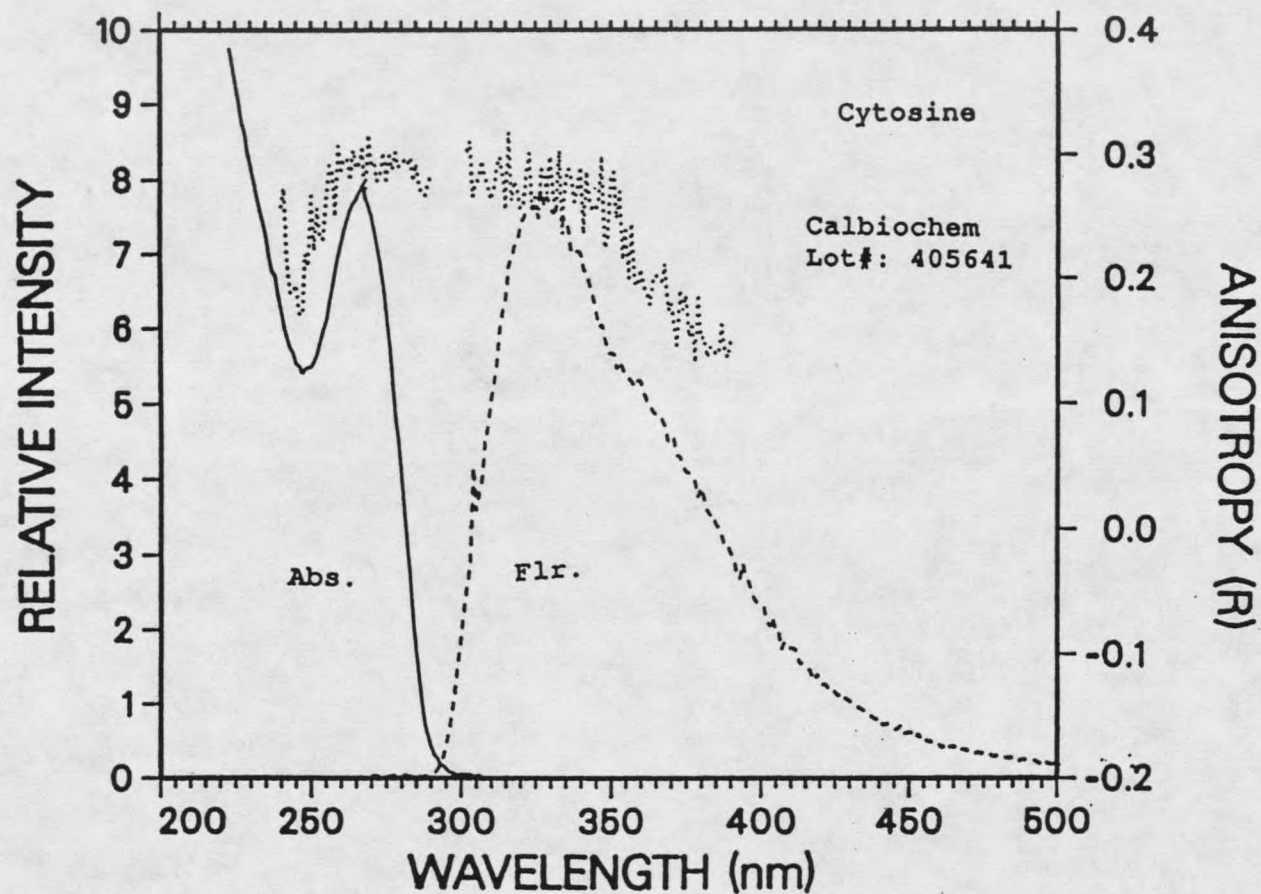


Figure 10. One-photon absorption and fluorescence spectra of cytosine in 0.05M phosphate buffer, pH7.0. Absorption (-); Fluorescence emission (---); Anisotropy (***).

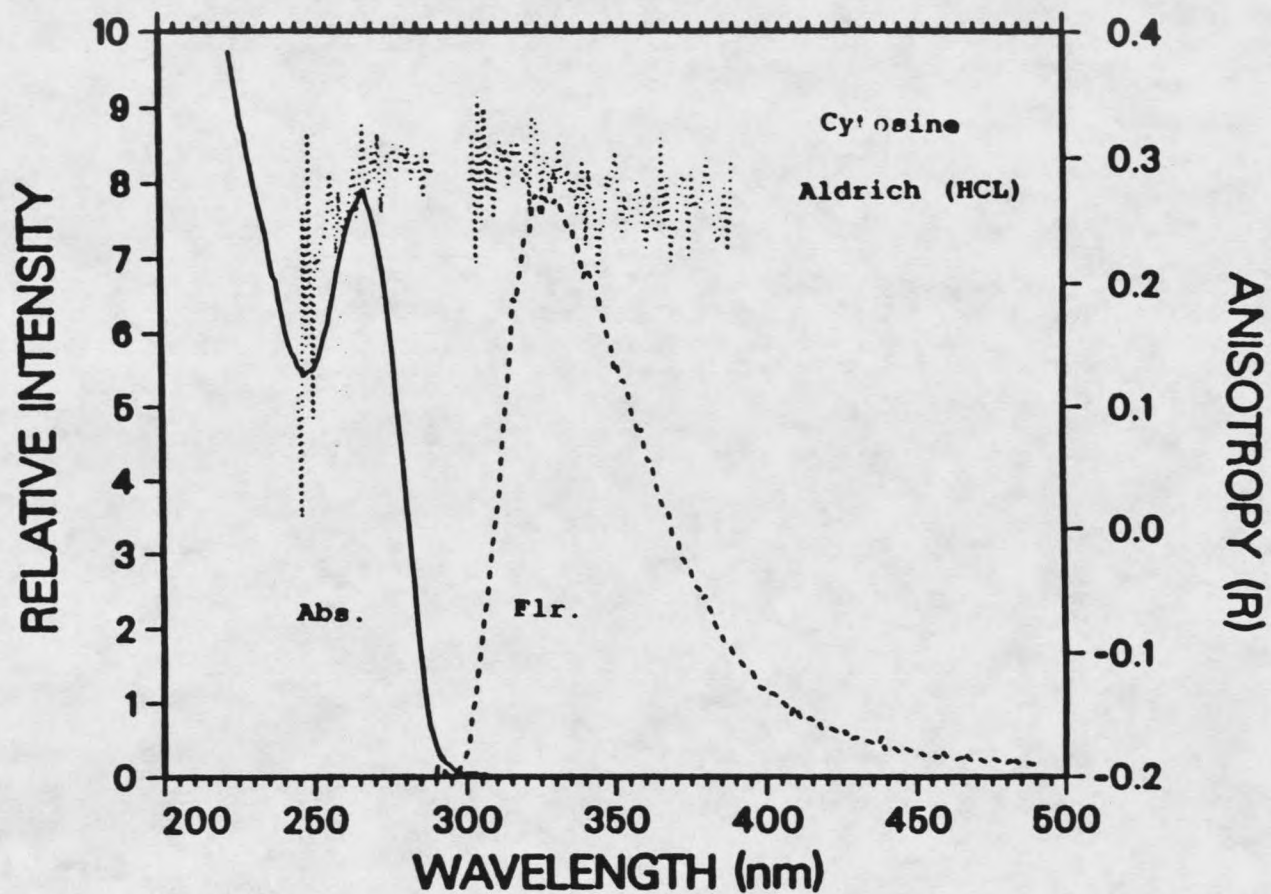


Figure 11. One-photon absorption and fluorescence spectra of cytosine in 0.05M phosphate buffer, pH7.0. Absorption (-); Fluorescence emission (---); Anisotropy (***).

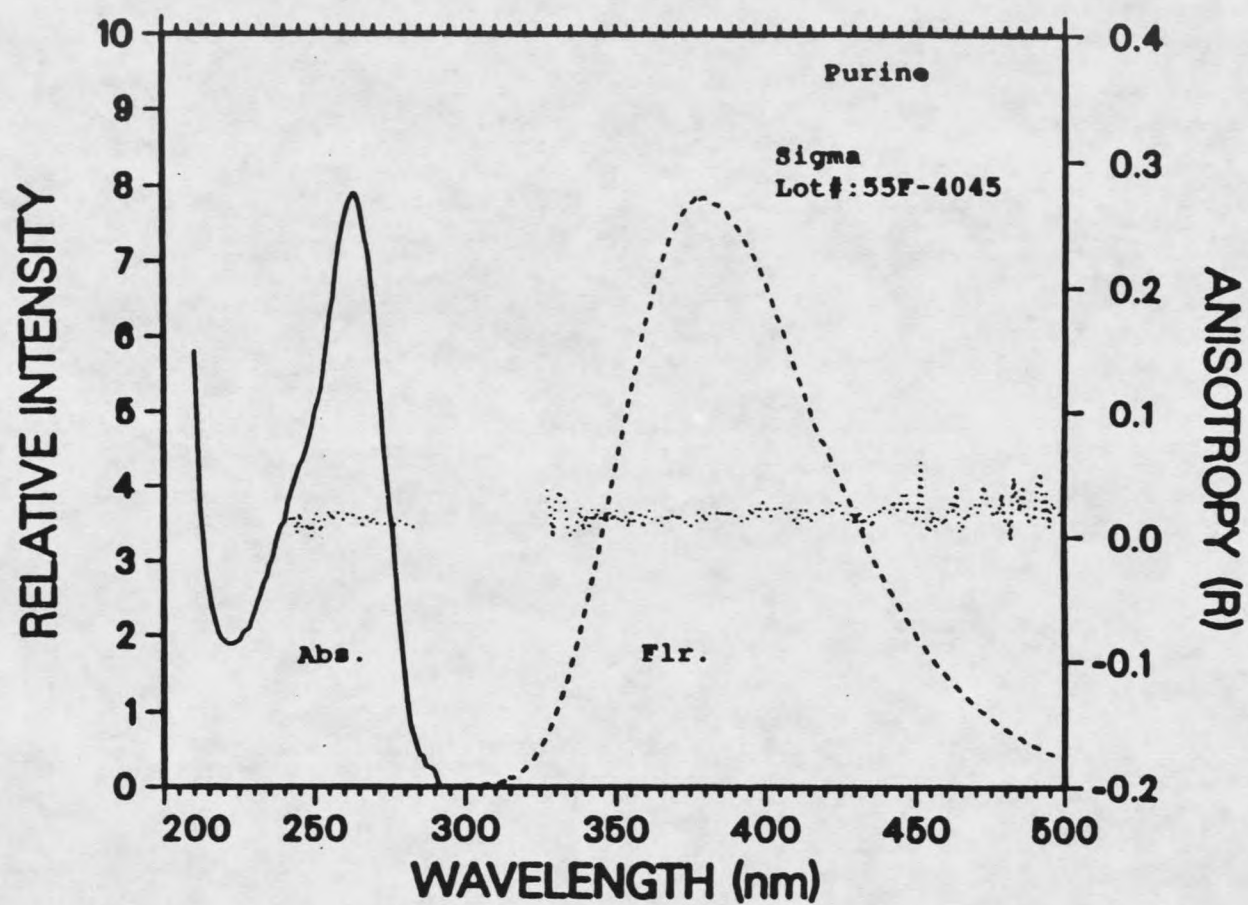


Figure 12. One-photon absorption and fluorescence spectra of purine in 0.05M phosphate buffer, pH7.0. Absorption (—); Fluorescence emission (---); Anisotropy (***).

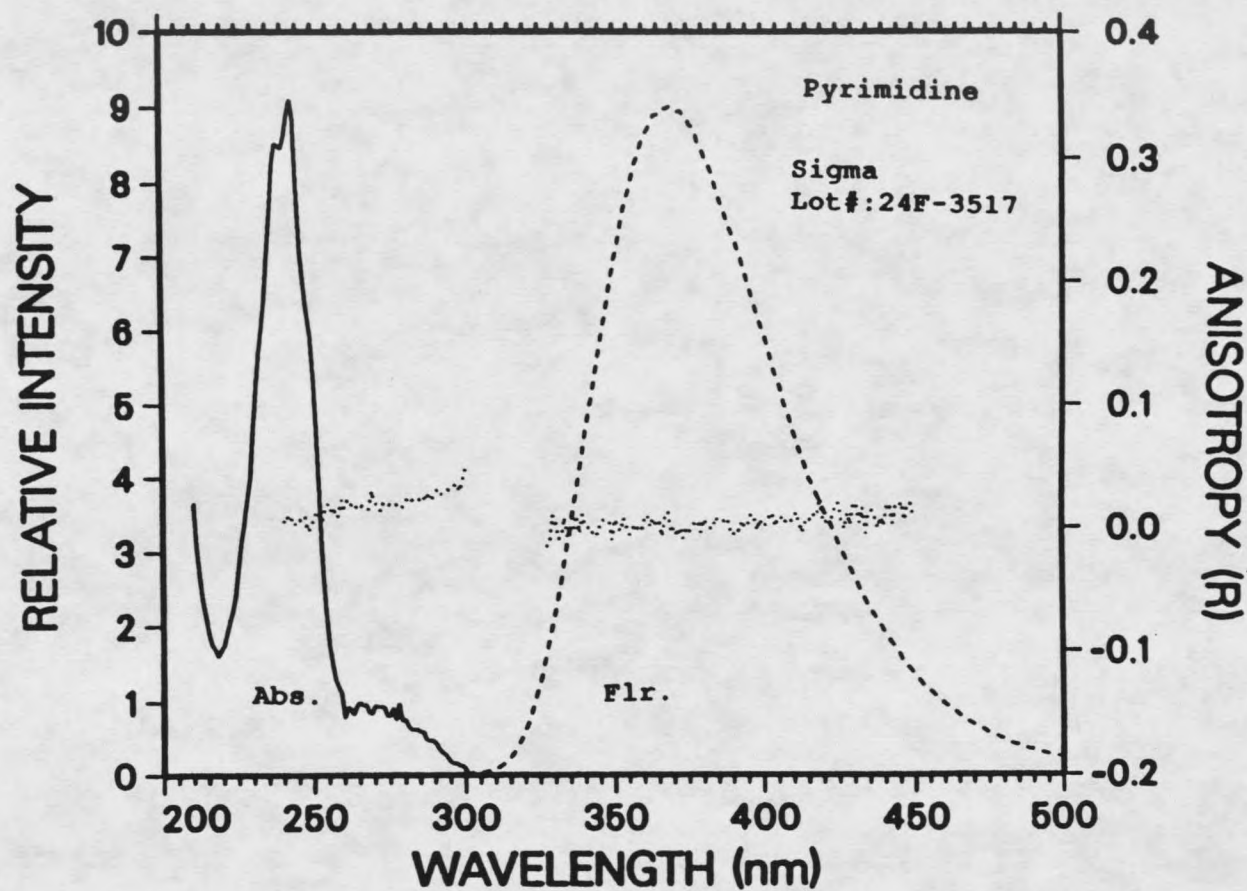


Figure 13. One-photon absorption and fluorescence spectra of pyrimidine in 0.05M phosphate buffer, pH7.0. Absorption (-); Fluorescence emission (---); Anisotropy (***).

are Calbiochem (B), Sigma (data not shown) and particularly the Tokyo-Kasei source (data not shown) where the magnitude of the 350nm impurity was nearly equal to the cytosine fluorescence. Only cytosine sold in the HCL form (Aldrich) and readjusted to pH 7.0 are void of this impurity as shown in Figure 11. The Tokyo-Kasei source was observed to be substantially hydrated upon opening and perhaps facilitated the formation of breakdown products. The Calbiochem sample (Figure 10) was analyzed by mass spectrometry and found to be 99% pure. Therefore, this impurity must be highly fluorescent compared to cytosine and in solution in less than 1%. For the two-photon studies both the Calbiochem (B) and Aldrich (C) sources were chosen for study.

Solutions of AMP from several sources were also analyzed by polarized one-photon fluorescence. Comparison of these sources revealed that the ratio of the relative intensities varied between the AMP peak at 315nm and the shoulder at 380nm. This 380nm shoulder had a wavelength maximum corresponding to the purine riboside and may have been the contaminant present. Since both cytosine and adenine have an amine substitution, a common deamination process may be responsible for the impurities found with these samples.

AMP was still used as a two-photon sample and the fluorescence wavelength selected corresponds to the 315nm maximum expected for the pure AMP molecule.

Measurements

As outlined under two-photon theory, the signal resulting from a two-photon process is proportional to the square of the incident intensity. This quadratic behavior was achieved by adjustment of the focal spot size, and therefore the power density, by the FL1 focussing lens (Figure 1). For all of the nucleic acid bases, a quadratic signal was achieved with spot diameters ranging from 1.0mm to 10mm. The signal of these samples remain quadratic under such a tightly focussed beam for two reasons; (1) the fluorescence lifetime is much shorter than the laser pulse width; (2) the fraction of molecules excited in the irradiation volume per pulse is less than one percent. For benzene, however, the fluorescence lifetime approaches the temporal duration of the pulse. Therefore, such a tight focus would have increased the probability for multiphoton ionization. Quadratic response is lost in the event of such a process. Consequently, the spot size was expanded for molecules such as benzene to 2 to 3mm in diameter.

A typical profile was generated using 400 shots per point-per polarization with the YAG laser tuned to 20 shots/second. The two-photon fluorescence signal and reference response were analyzed through an identical array of integrator, amplifier and A/D conversion electronics before being stored into the PDP 11/23 (Digital) computer (see Figure 1). The software for data acquisition and manipulation was modified by Dr. Patrik Callis and Mr. David Theiste of this laboratory for interface to the Lumonics hardware. The data acquisition electronics is described in detail elsewhere (81).

Through the course of study, this author has had to repair or replace nearly all electrical components critical to data acquisition. During these repairs a detailed manual was prepared detailing electronic component, specifications, modifications, operation theory and instruction which was entitled, "The Home Grown Electronics Guide to Better Science". Included in this manual is a complete laser, computer repair and diagnostics protocol to be used in conjunction with existing manuals. A complete and updated version can be found in the laboratory of Dr. Patrik Callis.

THEORY AND CONCEPTS

One-Photon Fluorescence

Once a molecular system is elevated to an upper energy state by an induced perturbation, several mechanisms of relaxation take place which shunt the energy and returns the system to a more stable electronic configuration. These mechanisms are grouped according to two classes, radiative and non-radiative decay.

For non-radiative decay, the excess energy is transferred to the media as heat, or phonon emission, either between the ground and first excited state or between vibronically coupled excited states. As shown in Figure 14, the non-radiative decay between states of similar multiplicity is called internal conversion (IC). A basically similar mechanism arising between states of different multiplicity is known as intersystem crossing (ISC). Since the focus in this dissertation is on solvated molecules, the rules for these events are simplified.

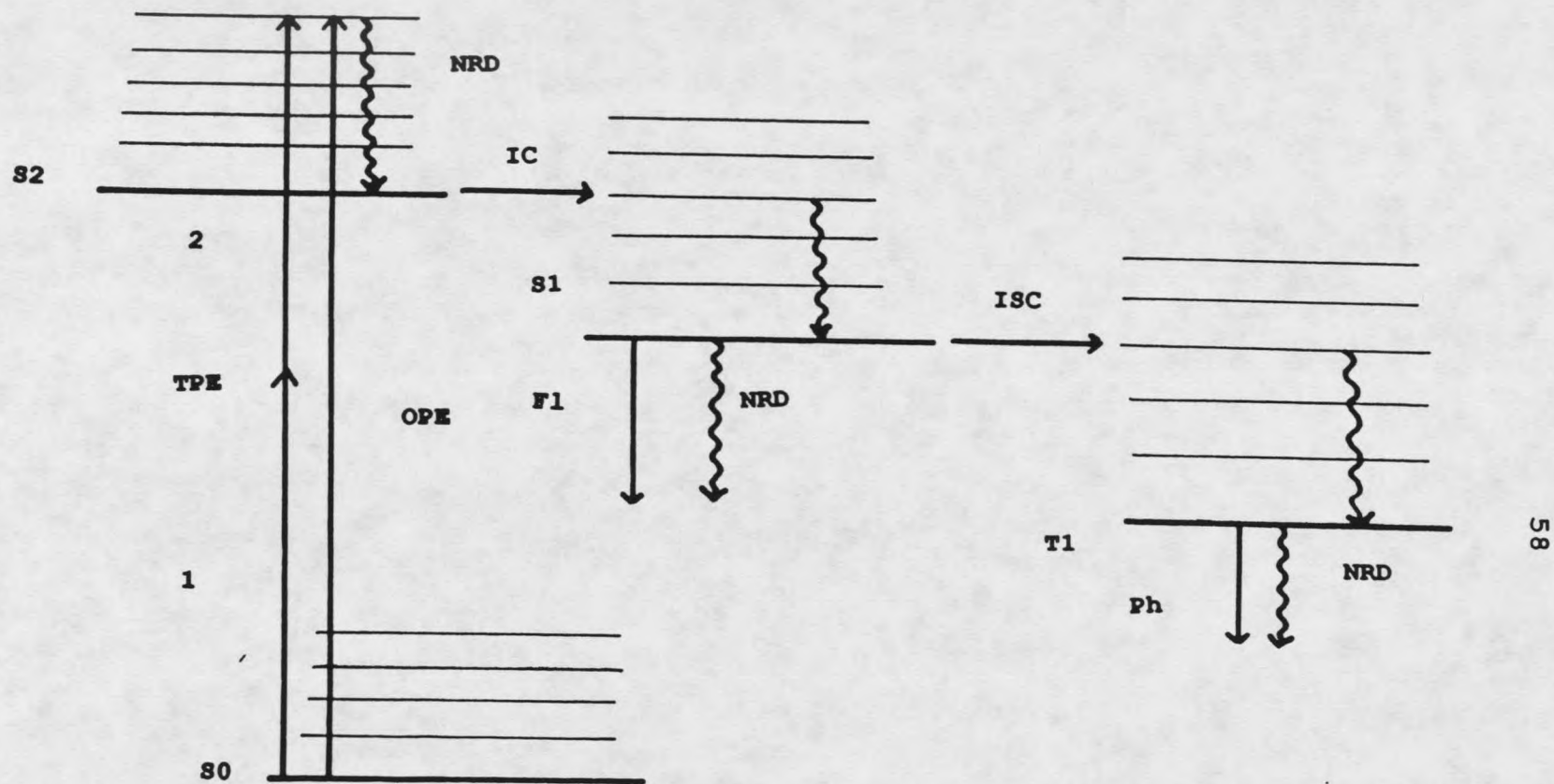


Figure 14. Radiative and non-radiative pathways

For a solvated molecule undergoing an excitation between singlet states, $S_0 \rightarrow S_2$ for example, the excess vibrational energy is transferred to the solvent through collisions such that non-radiative relaxation (Figure 14, A) occurs to some lower vibrational energy. If this vibrational state is coupled to another singlet manifold, S_1 , then internal conversion and non-radiative relaxation further (B) stabilizes the energy to some lower vibrational state in S_1 . During the course of this relaxation in S_1 , the energy can be directed into two additional pathways.

First, in systems like the nucleic acid bases, a triplet manifold T_1 may be vibrationally coupled and offers an avenue for further stabilization. Since such an intersystem crossing mechanism requires spin inversion, however, the matrix elements coupling the two states through a spin-orbit operation must be non-zero (83). That is, the product of the two coupled states must have the same symmetry as one of the point groups rotational components. For the nucleic acids, which contain only C_2 symmetry, all of these elements are potentially non-zero and provide an alternate route. This process may also be facilitated through an increase in the density of states.

If the molecule relaxes through strictly singlet pathways, then the energy of the system resides in the lowest vibrational level of the lowest excited singlet state, S_1 . From this level, the molecule can non-radiatively decay to the ground state (NRD) or can radiatively emit the energy as fluorescence, F_1 . If the fluorescence quantum yield is independent of excitation wavelength, then the non-radiative decay to the lowest excited state must have constant efficiency and is said to obey Vavilov's Law (82). Closely related to Vavilov's Law is a principle concerning the nature of the absorbing and emitting states.

For absorption and the emission arising into and from the same excited state, the fluorescence emission profile will be an exact mirror image of the absorption. This mirror image property, see Figure 9 of thymine for an example, is a consequence of the faster non-radiative decay between states $S_N \rightarrow S_1$ and vibrational relaxation within S_1 relative to the radiative rates. Such a relationship between decay rates is known as Kasha's rule.

A sensitive way of resolving band composition is to measure the angle between the absorption and emission transition moments for molecules with radiative lifetimes

several orders of magnitude faster than the rotational motion. This can also be achieved with molecules immersed in a cooled viscous solvent. If each excited state has a different transition moment direction relative to a fixed molecular axis, polarization measurements can resolve each state even if several states reside under a single absorption envelope.

One-Photon Fluorescence Polarization

Suppose a molecule with a single transition, $S_0 \rightarrow S_1$, has that transition moment oriented at some angle, θ , from the OZ axis (see Figure 15) at the time of excitation by an electric vector parallel to the OZ axis (84). The probability that an absorption will take place is proportional to the square of the cosine of the angle between the polarized electric vector and the transition moment. A $\cos^2\theta$ angular dependence is derived from the dot product between the transition moment and polarized electric field vector. Qualitatively, such a relationship is not difficult to comprehend.

If the light vector is oriented parallel with the transition moment, the probability for absorption is high. Conversely, if they are opposed by right angles, $\theta = 90^\circ$,

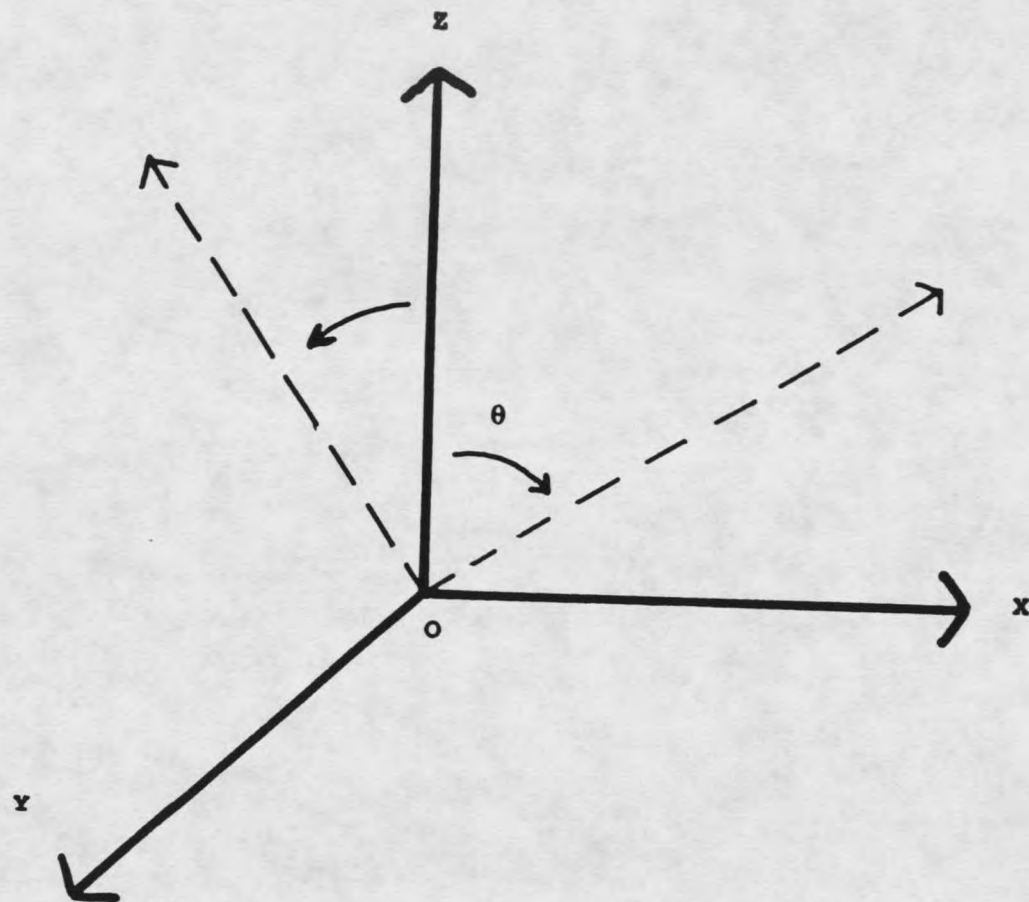


Figure 15. Cartesian coordinate system

then there would be little chance of an absorption taking place. For an observer along the Y axis and looking through a polarizer passing only vertically polarized light, the subsequent emission intensity along the OZ axis would have the same angular dependency. That is, the emission intensity observed would be greatest at Θ equal to 0° and non-existent at $\Theta = 90^\circ$.

For a solution of randomly oriented molecules, the one-photon polarization relationship or anisotropy (R) between the absorption and emission transition moments is proportional to a second order Legendre polynomial, equation 2,

$$R = (3/5) (\langle \cos^2 \Theta \rangle - (1/3)) \quad (2)$$

The limits of the anisotropy are easy to derive from equation 2. For an angle between parallel moments, the value of R would be 2/5 or 0.4. Conversely, orthogonally oriented moments would display an R value equal to -0.2. For reasons not yet identified, neither of these theoretical limits have been experimentally observed; however, thymine and TMP, Figures 7 and 9, approach the 0.4 upper limit. A value of $R = 0$ can be obtained by either of two processes. One requires that the angle between moments be exactly equal

to the magic angle of 54.6° . The second is if the molecule has sufficient time to rotate during the excited state lifetime, see Figure 13 for an example.

With the laboratory cartesian axis system as a guide, a formula (see equation 3) can be derived to calculate the anisotropy from the measure of intensities obtained by a pair of polarizers, one in the excitation path and the second in the emission path (85).

$$R = \frac{I_v - I_H}{I_v + 2I_H} \quad (3)$$

In the apparatus described in Chapter 2, only a single polarizer was used in the emission monochromator.

Therefore, equation 3 needed to be modified to account for excitation by natural light. To calculate R, for the discrimination through only one polarizer, equation 4 is used.

$$R = \frac{I_v - I_H}{I_v + 0.5 I_H} \quad (4)$$

The sensitivity of the polarization technique can be demonstrated by comparison of thymine, Figure 9, with one

transition in the first absorption and GMP, Figure 6, which contains more than one such transition.

Two-Photon Spectroscopy

In 1931, M. Goppert-Mayer proposed that two photons can be simultaneously absorbed in the same quantum event provided that the photon flux be sufficiently intense (68). The result of such absorption would be the population of an excited state equal in energy to the sum of two photons, (Figure 14, TPA). The formulations describing this process can be obtained by extension of the wavefunction to second order via perturbation theory (27).

Through the use of time-ordered Feynman diagrams and full expression of the perturbation Hamiltonian, equations describing all one- and two-photon events, for example absorption and scattering, can be derived. For the two photon absorption, an expression for the strength of a transition is called the two photon cross section and is shown below,

$$\delta = K |E|^4 g(\nu) |S_{gf}|^2 \quad (5)$$

where K is a set of constants, E is the amplitude of the electric field, g(ν) is a geometric lineshape function and

S_{gf} is the two photon tensor for two-photon excitation between the ground (g) and final (f) states. Evident from equation 5 is the intensity squared dependency of the two photon event as opposed to the direct intensity relationship of one photon absorption. This intensity squared dependency can be exploited to identify the process as a two photon absorption.

The portion of the tensor describing the intrinsic molecular properties which govern the absorption of two identical photons can be written in the general form (27),

$$(S_{gf})_{\alpha\beta} = \sum_k \frac{[<g|\alpha|k><k|\beta|f>]}{\omega_{kg} - \omega} \quad (6)$$

in which α and β are the cartesian coordinates, summation is over the intermediate states (k), ω_{kg} is the circular frequency separation between states k and g, ω is the circular frequency of the laser photon, and g and f represent the ground and final states, respectively. The intermediate state(s) is defined as the state(s) participating in the transition which provide a significant contribution to the sum (27). Such states can be virtual, and therefore, be intermediate in time and not space, or they may represent real eigenstates including the ground and

final state.

The experimental parameter for the two photon cross section can be derived from the intensity relationship of the emission to the incident beam as shown in equation 7 (86);

$$\delta(\omega) = \frac{I_E}{I_0^2(\omega)} \quad (7)$$

where I_E is the two-photon fluorescence emission intensity and I_0 is the incident intensity.

The selection rules for two photon absorption can also be surmised from the form of the two photon tensor. For a molecule with a center of symmetry, two states are coupled by simultaneous two-photon absorption only if they are of similar inversion parity; i. e., $g \rightarrow g$ or $u \rightarrow u$. Qualitatively, the product of a pair of one photon events, $g \rightarrow u$, would result in an overall transition equivalent of $g \rightarrow g$. For the molecules presented in this work, only benzene follows these requirements since the nucleic acid bases do not contain higher order symmetry. Consequently, all states for the DNA bases are potentially coupled to the ground state under a two-photon perturbation.

Another important property, in addition to the selection rules, is noted upon inspection of the tensor. The way the two photons are absorbed in the transition event are rigorously polarization dependent. This property allows room temperature polarization information to be derived in solution which otherwise would be unobtainable under alternate methods. Two-photon polarization information can be used in a way similar to the one photon anisotropy and is described in the next section.

Two-Photon Polarization

The 3 X 3 transition tensorial properties of the two-photon event can be derived by performing a coordinate transformation which expresses the α or β of equation 6 in the molecular frame (27). This property, for a set of uniform and aligned molecules, would yield nine independent measurable parameters. For one-photon absorption under a similar constraint, only three measurable parameters exist. A wide variety of light polarizations, used creatively, could ideally resolve the complete electronic structure of any system. However, if the molecules are allowed to tumble and reorient randomly, then some information is lost to orientational averaging.

For a randomly oriented sample under one photon excitation only the magnitude of the transition moments survives such averaging. Memory of the orientation of exciting light relative to the molecule is not retained. After performing a coordinate transformation on δ for the absorption of two photons and averaging over all possible permutations of the direction cosines, three sets of permutations survive. In addition to the three laboratory polarization parameters (F, H and G), the three molecular parameters are designated,

$$\delta_F = K [(\sum S_{\alpha\alpha}) \cdot (\sum S_{\beta\beta})] \quad (8)$$

$$\delta_G = K [(\sum S_{\alpha\beta} \cdot S_{\alpha\beta}^*)] \quad (9)$$

$$\delta_H = K [(\sum S_{\alpha\beta} \cdot S_{\beta\alpha}^*)] \quad (10)$$

The average two-photon cross section is then expressed,

$$\langle \delta \rangle = \delta_F F + \delta_G G + \delta_H H . \quad (11)$$

If the two photons are identical energy in energy and polarization then the expression collapses further since $\delta_G = \delta_H$.

The experimental two-photon polarization, which is used to characterize these two independent molecular parameters, is given by equation 12,

$$\Omega(\omega) = \frac{\delta_{\text{cir}}}{\delta_{\text{lin}}} = \frac{-\delta_F + 3\delta_G}{\delta_F + 2\delta_G} \quad (12)$$

where δ_{cir} , δ_{lin} are the circular and linear cross sections experimentally obtained by the designated incident polarization, respectively. Hence, the elucidation of an excited state(s) can be achieved through the judicious use of various light polarizations.

The ideal limits of Ω are derived below, and like the one photon anisotropy, the lower and upper limits of Ω have never been experimentally observed (27). For identical photons, the measurement of the lower limit seems possible; and yet, the experimentally observed lower limit of $\Omega=0.25$ (in the case of benzene) may only be observed because of the inequality of the out-of-plane zz tensor component (89). A similar reasoning for the failure to observe the upper limit may apply to the off-diagonal αz components of the tensor. An example of an upper experimental limit may be the B_1 transition of pyrimidine with $\Omega=1.3$ (36). The tensor which represents the theoretical limits of Ω is of the form,

$$\begin{pmatrix} a & b & c \\ d & e & f \\ g & h & i \end{pmatrix}$$

where each letter can have any value. The upper limit of 1.5 is realized from the trace of a symmetric tensor equal to zero ($a+e+i=0.0$). The lower limit of zero is obtained when a, o and i are all equal in value and all off-diagonal elements are zero. Predicted tensor patterns corresponding to different point groups can be derived and are accumulated elsewhere (87). For the all point groups including the C_s group, the transitions between symmetric states (such as $\sigma\sigma^*$ or $\pi\pi^*$) can have any value for Ω between the zero and 1.5. The only transition, therefore, that may be distinguished by two-photon polarization values for the C_s group is the non-symmetric $\sigma\pi^*$ transition which must have a value of Ω equal to 1.5.

The characteristics attributed to the polarization value previously described for a two-photon transition depends on the form of the tensor. Isotropic fluorescence emission is assumed when carrying out the averaging procedure. For the DNA bases, the fluorescence emission is not isotropic, therefore, the values of Ω will depend on both the tensor form and the relative direction of the fluorescence transition moment vector. A detailed derivation on the relationship between these two position elements can be found in the literature (88).

Permanent Dipole Effects

When a large change in permanent dipole for a molecule accompanies a transition with large oscillator strength, the ground and final states contribute a large term to the tensor expression (89,98). The DNA bases are believed to have these properties for many of their transitions. To illustrate this point, the two-photon tensor can be re-written in the form,

$$S \equiv h\omega_1 h\omega_2 \sum \left(\frac{A_{fk} B_{kg}}{\omega_{kg} - \omega_1} + \frac{B_{fk} A_{kg}}{\omega_{kg} - \omega} \right) \quad (13)$$

Consider the case where k is equal to f and g , the final and ground states, respectively. Since $\omega_{gg} = \omega_{ff} = \text{zero}$ and the resonance conditions applies, $\omega_{fg} = \omega_1 \pm \omega_2$, then part of the tensor describing the dipole contribution can be given by,

$$S_{\text{dipole}} \equiv \frac{A_{fg} (B_{ff} - B_{gg})}{\omega} \quad (14)$$

where identical photons are assumed. As is suggested from equation 14, the value of the S_{dipole} depends on the dot product between the transition dipole vector and change in permanent dipole moments; and therefore, the tensor value

will depend also upon the cosine of the angle between these moments. Consequently, this term may increase or decrease the two-photon absorptivity.

RESULTS AND DISCUSSION

One-Photon Fluorescence Polarization of Thymine and Uracil at Room Temperature

The notion of a $n\pi^*$ state creating the drop in one-photon fluorescence anisotropy observed by Morgan and Daniels (MD) for thymine has some merit in theory (63). There appears to be no dispute between MD and Callis (19) that the fluorescence, as shown by the high and constant fluorescence anisotropy (R), originates from the lowest excited $\pi\pi^*$ singlet state when thymine is dissolved in a polar solvent. If there is an isolated $n\pi^*$ transition residing at energies greater than the $\pi\pi^*$ state, then excitation into this manifold will result in a decrease of R close to the theoretical minimum of -0.2 since the two transition moments have near perpendicular orientations. To have these two transition moments mixed such that the $n\pi^*$ state was centered slightly off to the red of the $\pi\pi^*$ state, the values of R observed would depend on the weighted average of each with wavelength. The influence of the weakly absorbing $n\pi^*$ transition would only become noticeable

at the red edge as the $\pi\pi^*$ state waned in intensity.

Although such an explanation is plausible, there are remaining possibilities which were not considered by MD. An alternate suggestion would be that there are two transitions under the first absorption envelope, but that the second weaker state is also a $\pi\pi^*$ state of different polarization. However, for either type of state to influence the fluorescence excitation profile at the red edge, the band shape of the second transition would have to be steeper on the onset than the strong $\pi\pi^*$ band. Solvent impurities could also begin to add into the fluorescence excitation spectrum at the far red edge of the profile. Along the same line, the possible difference in pH between the MD and Callis experiments, suggests that the drop in R with wavelength marks the influence of the thymine anion in solution. This latter possibility was pursued because the thymine anion fluoresces strongly. The thymine anion actually exists in tautomeric equilibrium, the details of which are not pertinent but interesting to consider (17,28,33,67). In other words, only the 3-H thymine anion interferes and the analysis below assumes that the concentration ratio of the two tautomer forms remains constant.

Figure 16 shows the effect of pH on thymine excitation anisotropy (TEXA). The (o) represents TEXA for thymine in a pH 5.0 buffer. As shown, the TEXA was independent of wavelength across the entire band as previously reported by Callis (64). With the pH increased to the MD condition of 7.6, the TEXA (Δ) was dependent with wavelength dropping at the red edge close to the 3-HT⁻ fluorescence anisotropy limit. This is a nearly identical result to that published by MD (68). Further increase of pH above the basic pka of 9.9 gives a uniform R result invariant with wavelength characteristic of the anion, 3-HT⁻.

From examination of Figure 16, the fluorescence emission anisotropy remained high and constant across the region which is primarily dominated by the neutral absorption profile. Only when the anion absorption strength becomes relatively significant did the change in R occur with a pH 7.0 aqueous solution. To estimate the effect of the anion versus the neutral species, the relative quantum yield had to be determined by exciting at a fixed wavelength greater than 285nm with solutions of equal absorbance. The pH 12 "pure" anion was found to fluoresce 12 times greater than the neutral solution when excited at 295nm (data not shown). Because the average equilibrium

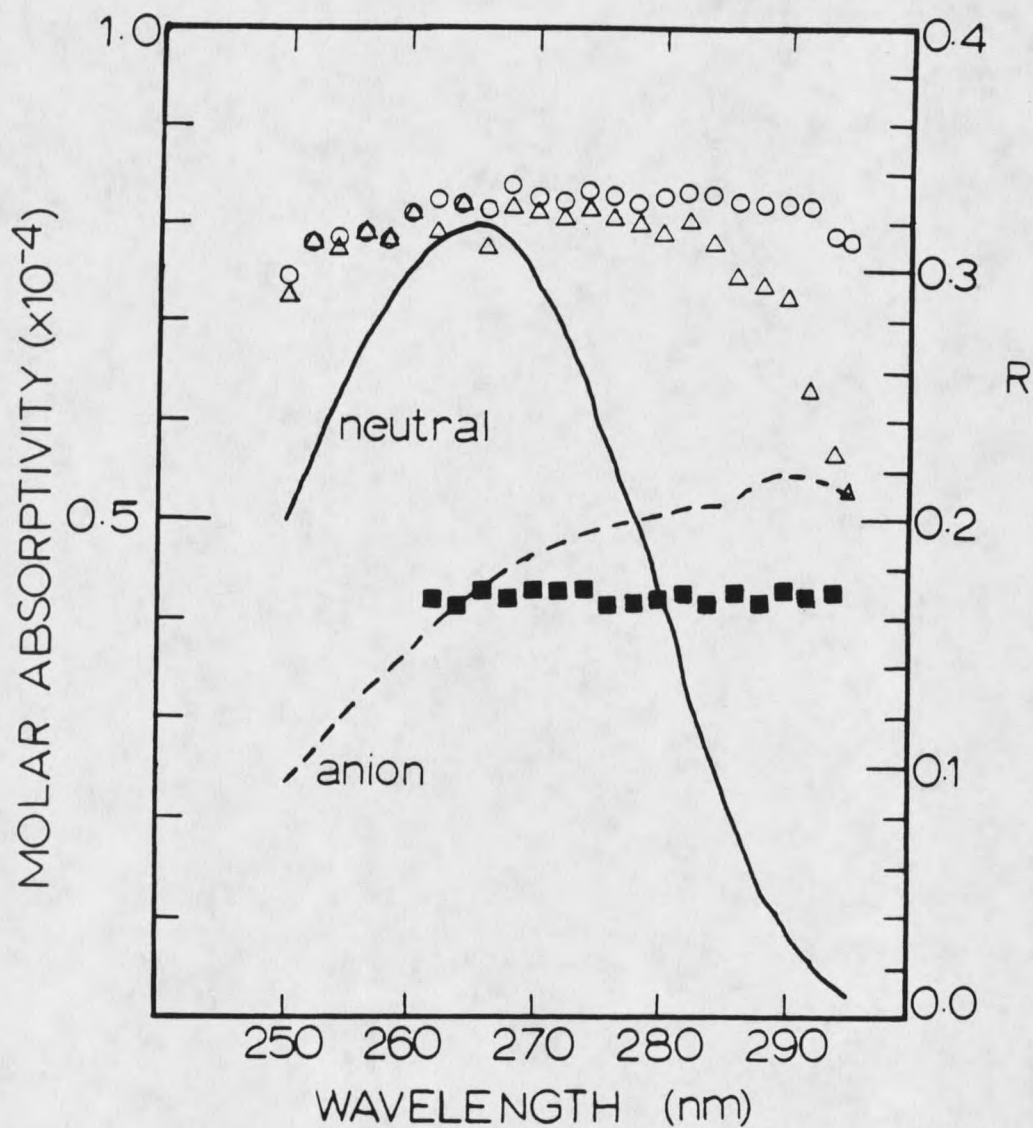


Figure 16. One-photon fluorescence excitation anisotropy for thymine with pH. Neutral UV absorption (—); Anion UV absorption (---); Anisotropy, pH5.0 (o), pH7.6 (Δ) and pH12.0 ($\blacksquare$)

concentration of the anion tautomers was the only observable in these experiments, the properties attributed to the anion solution were approximated as originating from a single species.

The molar absorptivity of the anion was obtained from Figure 16 and found to be 24 fold that of the neutral at 295nm. So, for solutions of equal concentration, the anion fluorescence would dominate in a ratio of 288:1 in the absence of excited state proton transfer. At pH 7.6, the concentration of anion species would be present at approximately 0.5%, and so, the fluorescence ratio of anion to neutral would be about 1.5 to 1. Therefore, the expected R for a solution thymine at pH 7.6 excited at 295nm would be roughly 0.24 in reasonable agreement with data shown in Figure 16.

To further support the idea that the depolarization was due to anion contamination, theoretical values of expected R with wavelength were compared to experimental data and shown in Figure 17. To calculate R_{calc} , the ratio of molar absorptivities was obtained through UV absorption profiles with solutions of known concentration. From these values, the ratio of absorbance of anion (A_a) versus absorbance of neutral (A_n) for a given concentration can be calculated

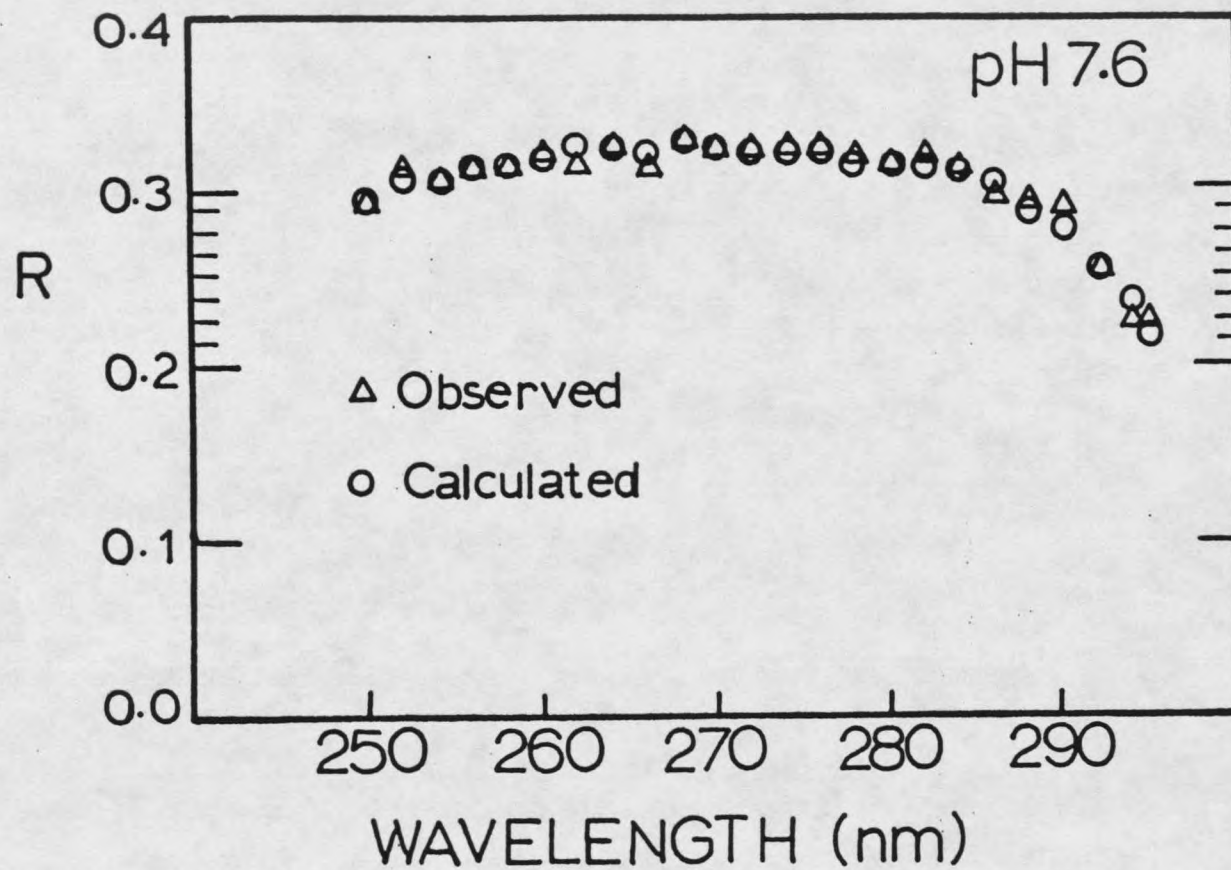


Figure 17. Calculated and experimental one-photon fluorescence excitation anisotropy of thymine, pH 7.6

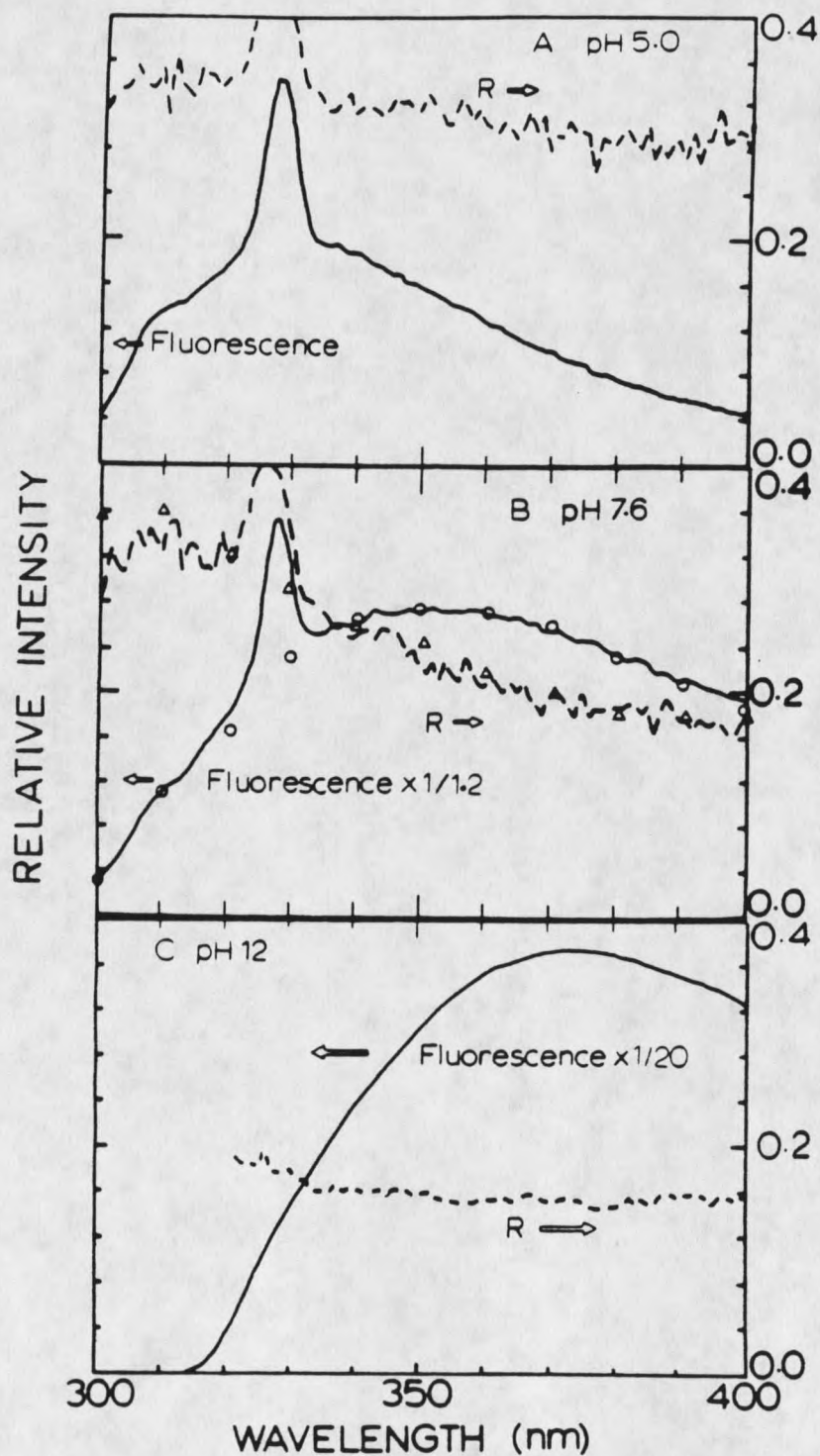


Figure 18. Fluorescence emission anisotropy for thymine with pH. Predicted anisotropy (Δ) and intensity (o) listed in panel B

given the pH of solution. Since the relative quantum yield (Q) ratio was previously determined as $Q = 12$, the ratio of the fluorescence due to anion (F_a) and neutral (F_n) would be proportional to the direct product (r) of Q with $A_a(\lambda)/A_n(\lambda)$. Then, with the relation,

$$R_{\text{calc}} = \frac{F_a R_a(\lambda) + F_n R_n(\lambda)}{F_a + F_n} \quad (15)$$

simplified by division of F_n to

$$R_{\text{calc}} = \frac{r R_a(\lambda) + R_n(\lambda)}{r + 1} \quad (16)$$

the R_{calc} can be predicted with wavelength. Evident from Figure 17 was the near one to one correspondence of the calculated and experimental TEXA.

The same evidence of anionic interference can be seen in the fluorescence emission experiments. As shown in Figure 18 panel A, thymine in pH 5.0 shows emission and anisotropy characteristics of the neutral species (64). As pH is increased to 7.6 (panel B) evidence in the emission profile of a red shifted, $\lambda_{\text{max}} = 360\text{nm}$, component begins to add into the neutral thymine emission, and the R values drop in kind. Finally, at pH 12 (panel C) the fluorescence emission and anisotropy are characteristic of the highly

fluorescent 3-HT⁻ species. To demonstrate the influence of the anion in emission, the fluorescence emission intensity and anisotropy of panels A and C were used to synthesize a theoretical profile and R_{calc} to compare to panel B.

To accomplish this, the equation of the fluorescence intensity was used,

$$I_f = K I_0 A \Phi_f \quad (16)$$

where K is a constant, I_0 is equal to the incident intensity, A is the absorbance and Φ_f is the quantum yield for fluorescence. The ratio of $\Phi_f(A)$ of the anion and the neutral $\Phi_f(N)$ can be directly related by dividing equation 16,

$$\frac{I_f(A)}{I_f(N)} = \frac{K I_0 A(A) \Phi_f(A)}{K I_0 A(N) \Phi_f(N)} = \frac{\Phi_f(A)}{\Phi_f(N)} \quad (17)$$

for an aqueous solution of equal absorbance. To derive the expression for the fluorescence with wavelength excited at 295nm,

$$F(\lambda) = G (A_a \Phi_a + A_n \Phi_n) \quad (18)$$

where G is a geometrical factor.

Since $A_{a,n}$ can be expressed in a solution mixture as

$$A_a = \frac{E_a C_a}{E_a C_a + E_n C_n} \quad (19)$$

and

$$A_n = \frac{E_n C_n}{E_a C_a + E_n C_n} \quad (20)$$

then,

$$F(\lambda) = \frac{E_a C_a \Phi_a + E_n C_n \Phi_n}{E_a C_a + E_n C_n} \quad (21)$$

Simplifying by division through C_n ,

$$F(\lambda) = \frac{E_a (C_a/C_n) \Phi_a + E_n \Phi_n}{E_a (C_a/C_n) + E_n} \quad (22)$$

gives the expression that can be used to calculate expected fluorescence profile and R. As shown in Figure 18 panel B there was a direct relationship between the profile and anisotropy demonstrating the importance of the minute but highly fluorescent presence of the anionic thymine species.

For the MD results addressed earlier (68), there had to be reason to suspect their interpretation before analyzing discrepancies. The first of these has already been mentioned in the lack of including a second, yet weakly absorbing, $\pi\pi^*$ state as a possible candidate. Secondly, their resolved $n\pi^*$ band had an exceedingly narrow full width

at half maximum (FWHM) compared to the intense $\pi\pi^*$ band. Thirdly, the oscillator strength of carbonyl $n\pi^*$ transitions are characteristically two orders of magnitude or more weaker than their $\pi\pi^*$ counterparts; consequently, the fluorescence lifetimes would be on the order of 100 picoseconds or longer if $\Phi_f = 10^{-4}$. Since the Φ_f is independent of wavelength across the first absorption band, the τ_r would have to vary in accord with τ_f given the relation $\Phi_f = (1/\tau_r)\tau_f$. Considering the relationship between τ_f and τ_r , this is not probable since Kasha's rule is obeyed here.

Through absorption studies of thymine derivatives, the possibility of tautomer interference has been suggested (17). With theoretical determinations on relative tautomer concentration not necessarily precluding experimental reality, this question requires addressing. Wilson, et al. (90) has published a "yardstick" for measuring the propensity with which a tautomer may influence optical spectra. The first of the three main criteria states that the tautomer must be at an equilibrium concentration low enough to escape detection by absorption, yet dominate the room temperature fluorescence, i.e., Φ_f greater than 100 fold that of the main tautomer. Likewise, the tautomer

absorption edge must exactly correspond to the main species to escape detection, but have an absorption maximum shifted to account for the change in overall Φ_f . Finally, the tautomer pK_a 's should reflect changes in fluorescence with pH. Using these criteria coupled with the independent nature of Φ_f and fluorescence anisotropy with wavelength, the drop in R observed by MD is not explained with a tautomer model.

Two contemporary and novel approaches have also forwarded the notion of an observable $n\pi^*$ (62,64). However, the study involving supersonic jet expansion has been reported to be the result of contamination in the sample chamber (31). The second method, involving the application of multimode vibrational theory to the five active Raman modes of UMP, has exposed some interesting questions, yet draws questionable conclusions (28).

The resonance Raman studies conducted by Turpin and Peticolas were motivated by an uncomfortably large fluorescence Stokes shift (62). They propose that this Stokes shift, about 6600cm^{-1} , is the result of emission from a weakly absorbing state, $n\pi^*$ state by implication, lying lower in energy than the $\pi\pi^*$ states. In addition to a $n\pi^*$ state model, they suggest an alternate explanation for the

Stokes shift as the result of solvent relaxation. These suggestions are in serious conflict with this work, and studies independently done now by several other investigators, which demonstrated that the emission is of $\pi\pi^*$ origin and not $n\pi^*$. The experimental evidence when comparing several other systems, for example the carotenoids, suggests that the Stokes shift observed is not exceedingly large (91).

Table 5. Fluorescence Anisotropies (R) of Uracils in Aqueous Solution Near 20°C.^a

Compound	$R(\lambda_{\max})$	$(R\lambda_{\text{edge}})$	Reference
Uracil	0.34	0.32	this work, (28)
UMP	0.35	0.34	this work, (28)
Thymine	0.32	0.31	this work, (28)
Thymine	0.30	----	92
Thymine	0.34	0.33	19
Thymine	0.30	0.17	63
Thymine	0.34	----	60
Thymidine	0.32	----	92
Thymidine	0.34	----	60
TMP	0.33	----	92
TMP	0.34	----	60

a $\lambda_{\max}$ refers to excitation near the absorption maximum, near 260nm; λ_{edge} refers to excitation at the absorption edge where absorption is 10% that at $\lambda_{\max}$. This varies from 285-290nm depending on the compound.

One-photon polarized fluorescence measurements were conducted on uracil and UMP for the first time, see Table 5, and compared to some previously reported values for thymine.

The high anisotropy was constant across the first absorption band, consistent with the thymine results, provided that the pH was six or below. The slightly higher R value for uracil is presumably a consequence of shorter τ_f relative to thymine (28). In addition to the R values, the fluorescence maximum for uracil was found to be 319nm in agreement with the relative absorption profile of uracil and thymine, and yet, in disagreement with 305nm reported previously (21).

Two-photon Fluorescence Excitation and Polarization of Thymine, Cytosine and Nucleotides of DNA at Room Temperature

Figure 19 illustrates the polarization (Ω) properties of each nucleotide in neutral aqueous solution. Although not with the variation in Ω seen in the case of pyrimidine (PYR) in cyclohexane, the polarization of each nucleotide was found to be unique and distinctive. The INDO/S calculated values of Ω are also in **relative** agreement with respect to the first absorption bands of each nucleotide. For example, Thymine (and TMP by implication) was predicted to be lower in relation to any of the other bases. Provided for the comparison between theory and experiment, Table 6 outlines the results of this work with the INDO/S values tabulated by Dr. Patrik Callis of this laboratory.

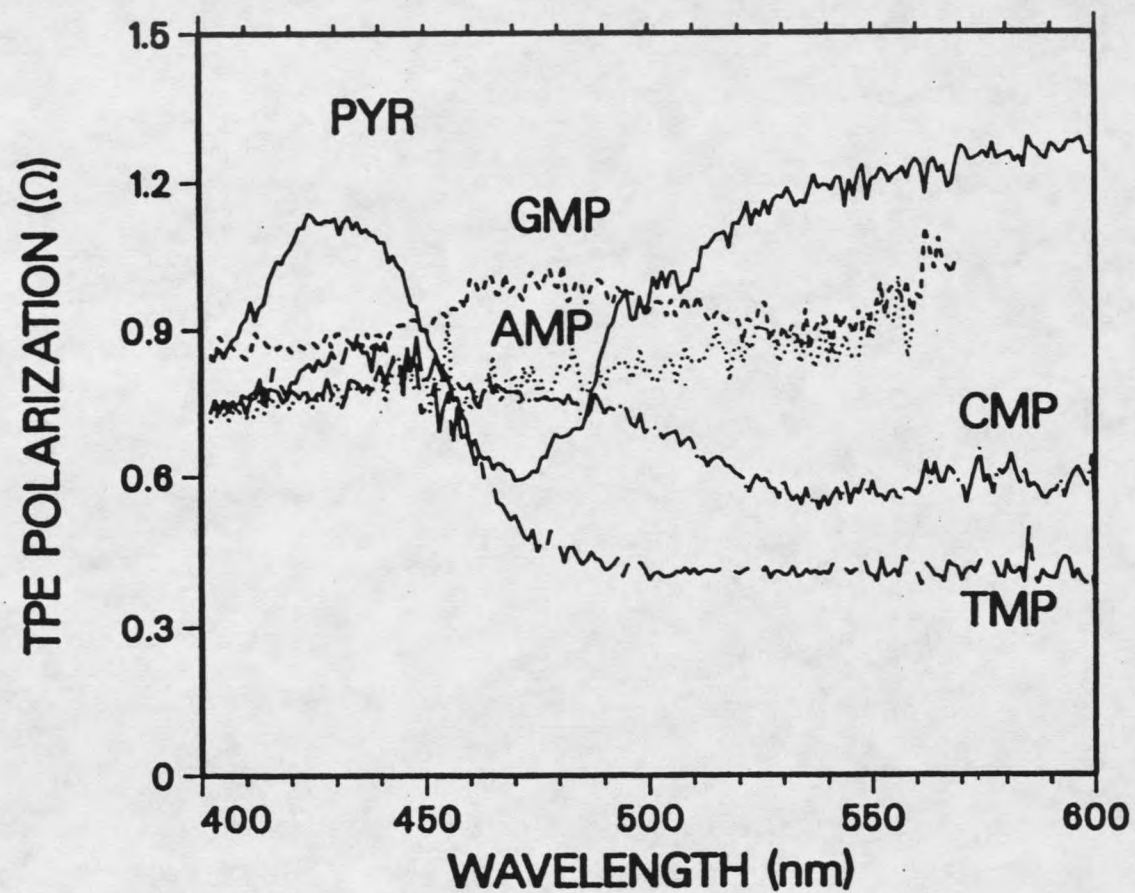


Figure 19. Two-photon excitation polarizations for selected nucleotides and pyrimidine (in cyclohexane)

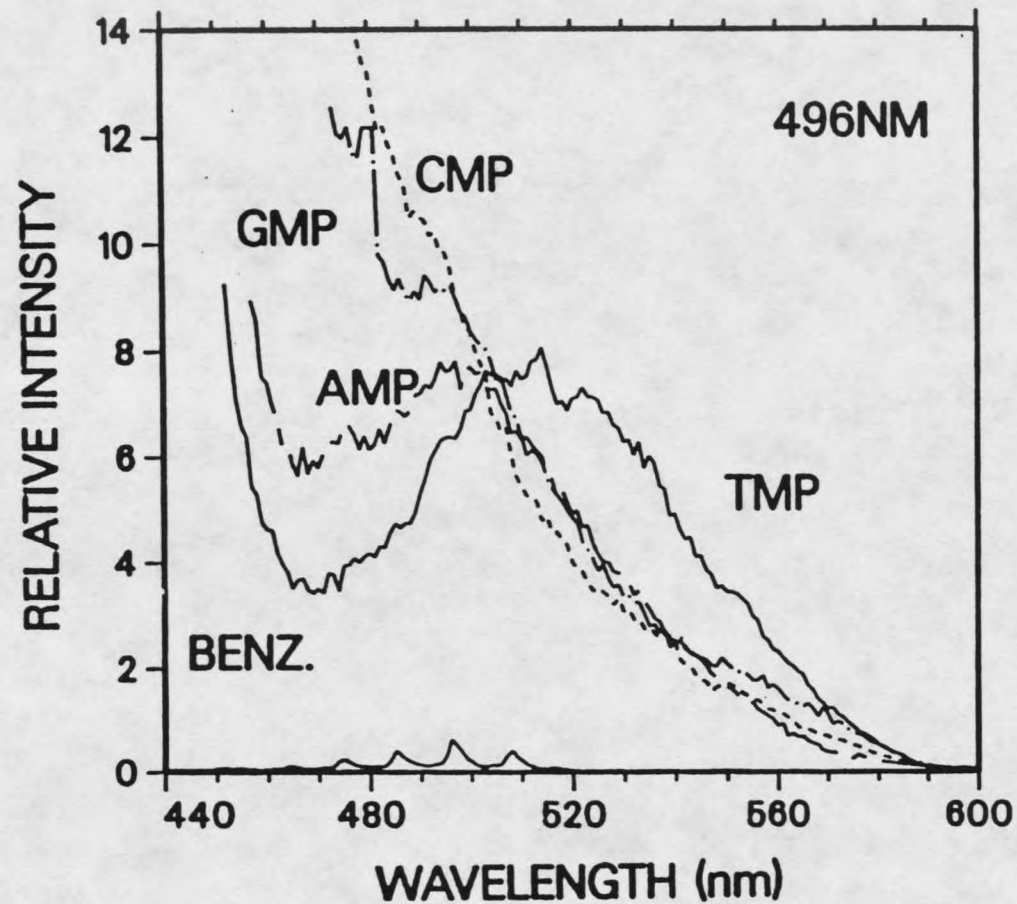


Figure 20. Relative two-photon absorption cross sections for selected nucleotides. Two-photon cross sections relative to TMP at 496nm

Table 6. One- and Two-Photon $\pi\pi^*$ Absorption Strengths for Selected DNA Bases, Calculated from INDO/S.

Molecule	λ (nm)	OPA	TPA		
		f	δ_{lin}	Ω_{th}	Ω_{exp}
Benzene					
Lb (V ₁₄)	255	0.00	0.66	0.25	0.24
Uracil					
I	233	0.31	44.0	0.56	0.40
II	193	0.10	38.0	1.17	
III	186	0.21	65.0	0.43	
IV	170	0.59	23.0	0.38	
Cytosine					
I	264	0.08	18.0	1.05	0.60
II	223	0.31	44.0	1.50	
III	190	0.87	22.0	1.46	
IV	182	0.20	14.0	1.46	
Adenine (9H)					
I	255	0.05	7.0	1.45	0.90
II	232	0.19	15.0	1.00	
III	202	0.09	77.0	1.10	
IV	185	0.46	22.0	0.98	
Guanine (9H)					
I	274	0.22	12.0	1.47	0.90
II	237	0.22	14.0	0.62	
III	223	0.01	202.0	0.71	
IV	185	0.10	55.0	1.06	
V	173	0.51	65.0	0.49	

The INDO/S values were generated from application of an all- π electron INDO/S semiempirical molecular orbital method employing 150 singly and doubly excited configurations and Ohno-Klopman electron repulsion integrals. TPA cross

section values are in units of $10^{-50} \text{ cm}^4 \text{ sec}^{-1} \text{ molecule}^{-1}$ assuming a 3000 cm^{-1} FWHM Lorentzian line shape. In addition to polarization comparisons, the relative two-photon cross sections (δ) of the nucleotides to TMP were measured and graphed in Figure 20. Again, there are distinct differences between the profile structures of several bases, such as the difference of TMP and CMP. The experimental relative absorption strengths appear to be nearly equivalent without evidence of the variation proposed by the calculated values. However, in agreement with theory, qualitative estimations for the two-photon absorption strength of each nucleotide were at least an order of magnitude greater than benzene in solution.

TMP and Thymine

Figures 21 and 22 are the two-photon fluorescence excitation and polarization characteristics of TMP and thymine, respectively, in neutral aqueous solution. Note, that in all subsequent figures, the one-photon spectra are graphed at one-half their absorption wavelength (plotted along the laser wavelength). As might be expected from the common one-photon and structural properties, the profiles and values in Ω followed a similar trend with wavelength. The polarization value for each case was found to be low

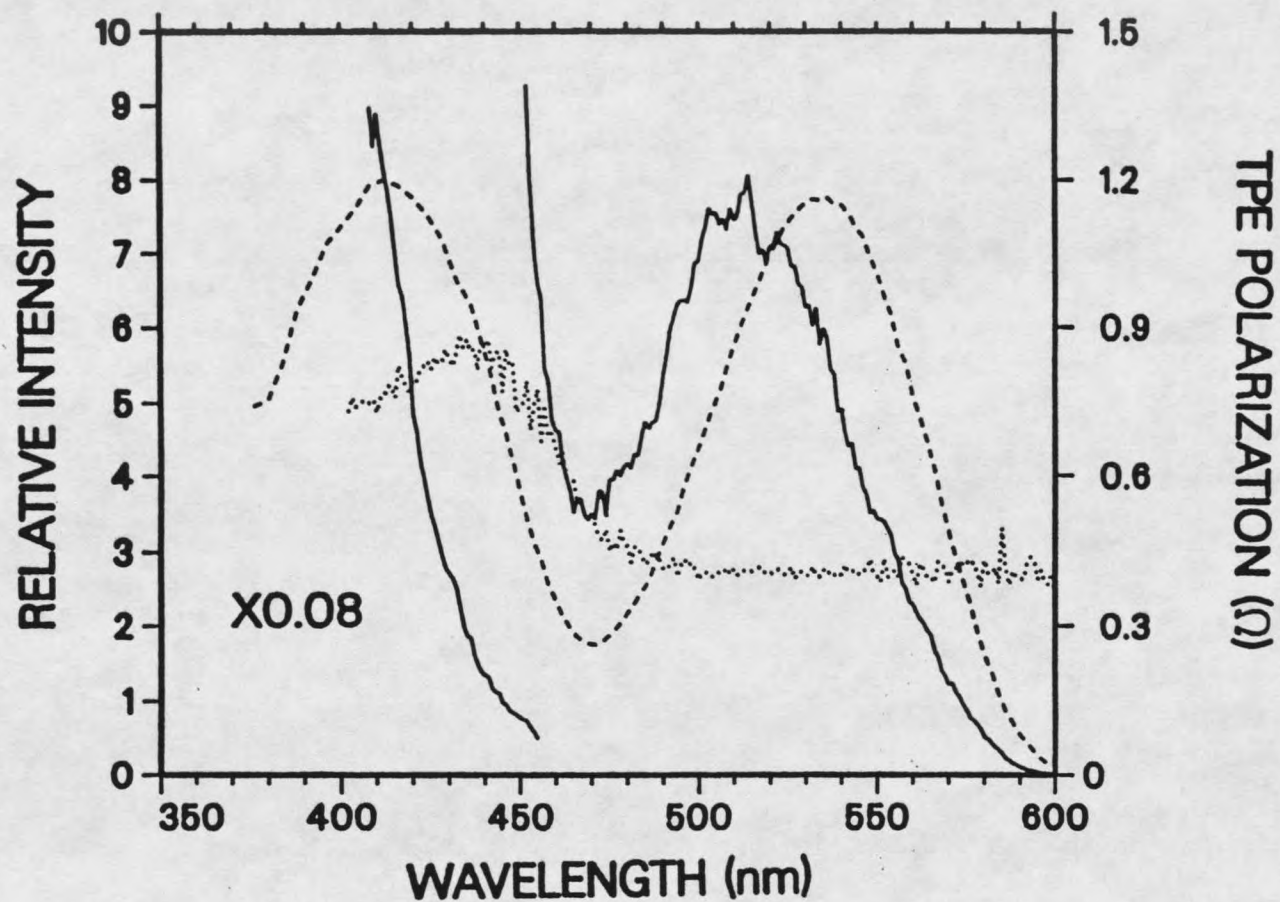


Figure 21. Two-photon fluorescence excitation spectrum and polarization of thymidine-5'-monophosphate in 0.05M phosphate buffer, pH7.0. Linear polarized two-photon profile (-); One-photon absorption profile (---); two-photon polarization (···)

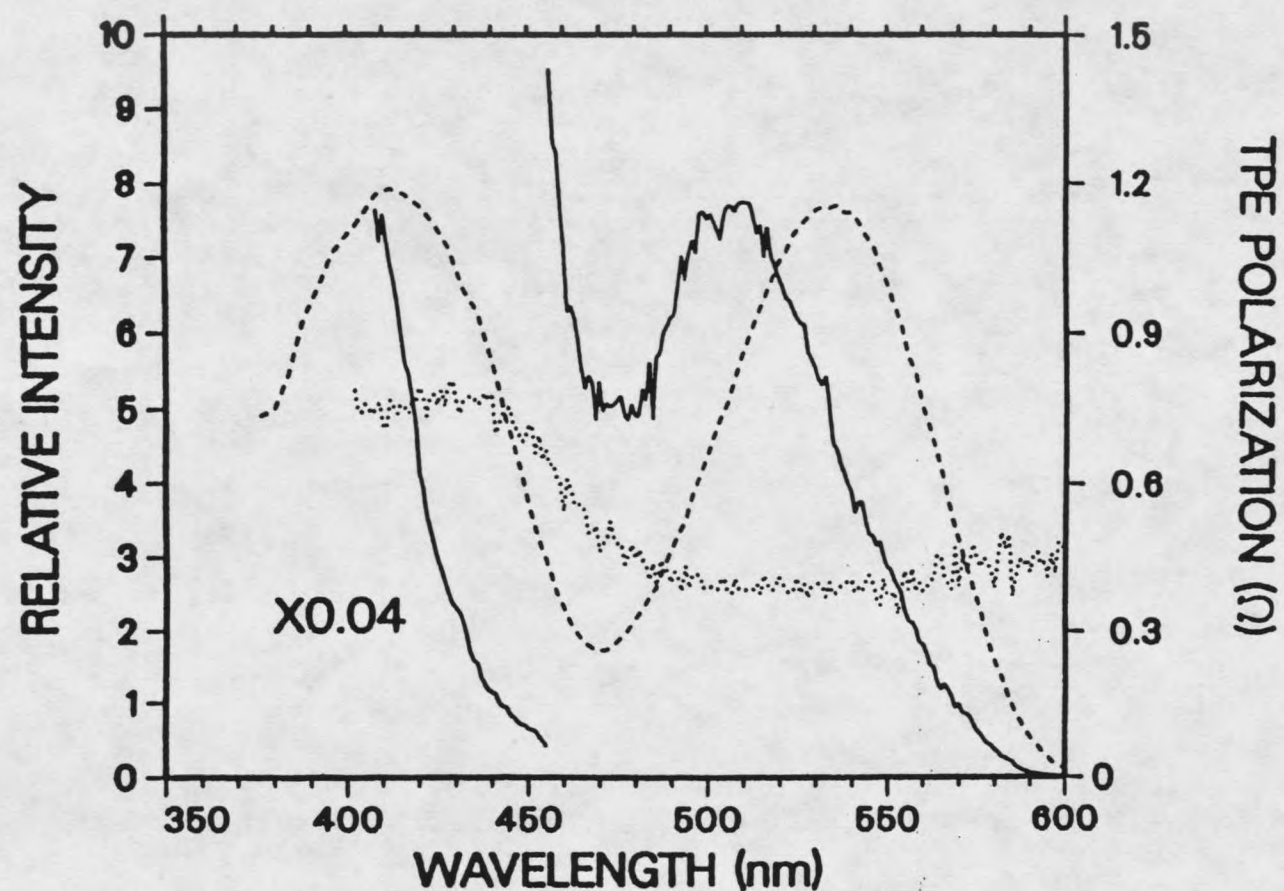


Figure 22. Two-photon fluorescence excitation spectrum and polarization of thymine in 0.05M phosphate buffer, pH7.0. Linear polarized two-photon profile (-); One-photon absorption profile (---); two-photon polarization (···)

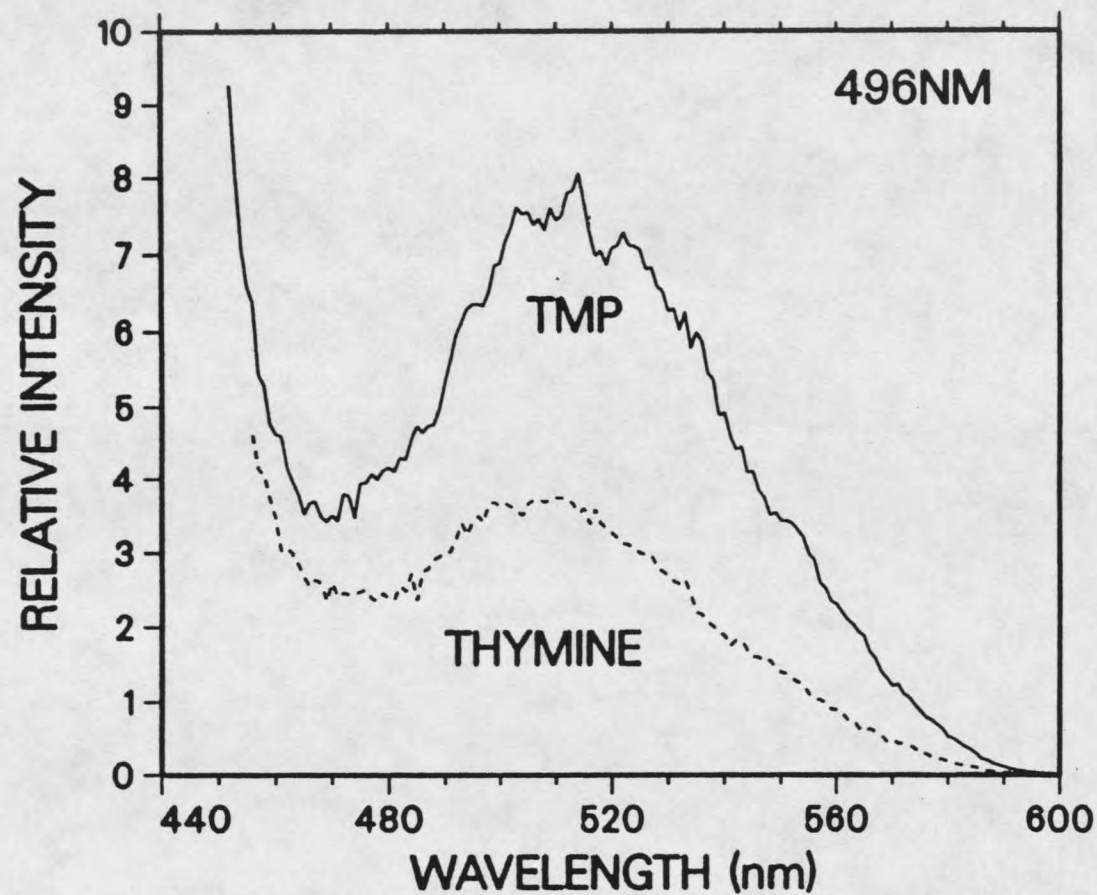


Figure 23. Relative two-photon cross sections of TMP and thymine, pH7.0

($\Omega=0.4$) and constant across the first UV band. At the red edge of the second absorption envelope (about 460nm), the two cases diverge with increasing energy. For TMP, there was a definite and reproducible inflection in Ω about 440nm. TMP polarization values rose to about 0.9, and subsequently decreased to roughly 0.7 at 400nm. No inflection was observed for thymine which remained constant at $\Omega \approx 0.75$ in the same region.

The two-photon profiles for each molecule are similar in that they both are shifted about 1500cm^{-1} to the blue relative to the one-photon spectrum. With increasing energy, both systems exhibit a minimum which roughly corresponds to the UV minimum, and then, "explode" in transition strength in the region corresponding to the second absorption band. Since the relative two-photon cross section was found to be twice as strong for TMP over thymine at the first absorption band (see Figure 23), the relative strengths of this second dominant band are nearly equal with both molecules.

The difference in relative strengths of the first two-photon band between TMP and thymine was not predicted by INDO/S methods (data not shown). From a theoretical perspective, substitution of a methyl group does not

represent a strong perturbation, and therefore, might not be expected to significantly influence the transition density pattern. Experimentally, however, substitution at N1 by a methyl group does increase the one-photon absorptivity by roughly twenty percent, and therefore, lends evidence in support for the variation in transition strength observed under two-photon excitation. The two-photon excited fluorescence emission spectrum of TMP, see Figure 24, also coincided with the one-photon fluorescence emission spectrum independent of excitation wavelength in aqueous solution. Therefore, the molecule exhibiting the fluorescence under two-photon excitation was established as neutral TMP. Aspects concerning the excited state structure of thymine and its relatives can now be addressed in the context of the two-photon information obtained.

Focussing on just the first absorption band, it would appear that the two-photon polarization values obtained for TMP and thymine are in harmony with the considerable body of one-photon polarization work in assigning a single $\pi\pi^*$ transition to this band. With such a constant trend in Ω across the entire band, a second transition would either have to have polarization characteristics equal to the "main" band or be too weak to influence the polarization

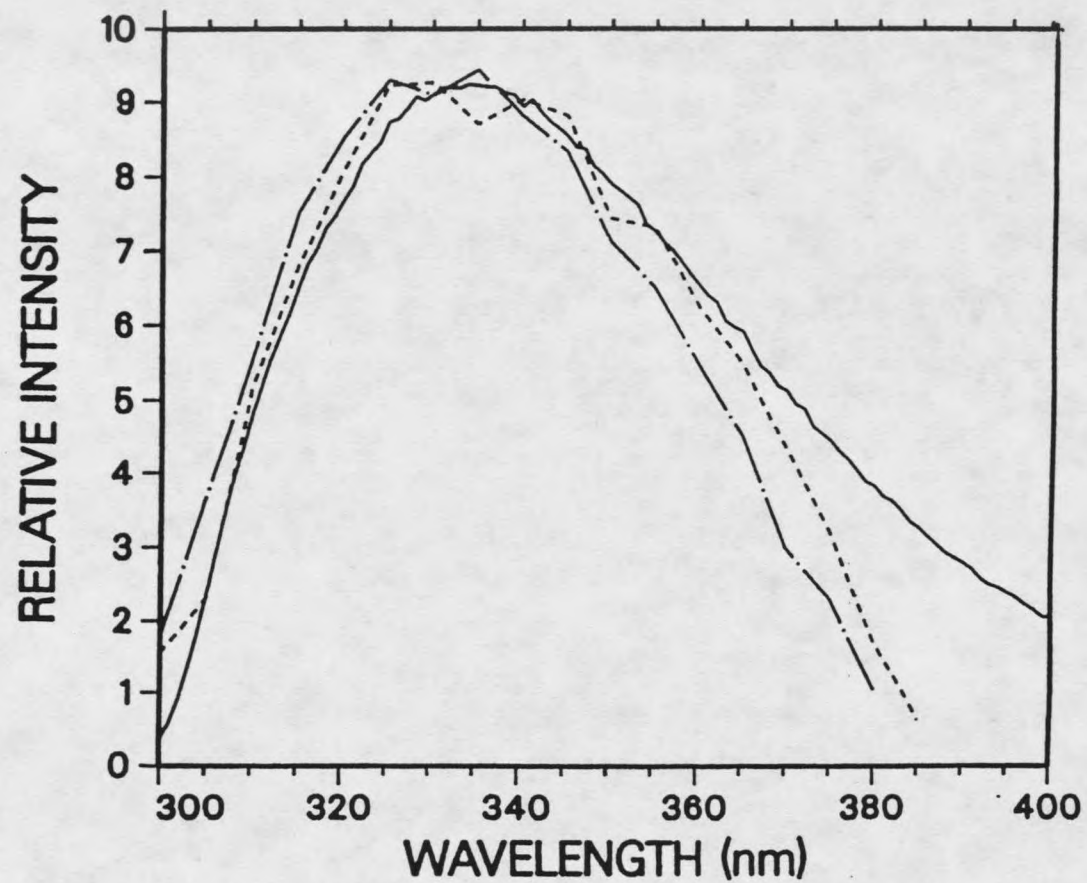


Figure 24. Two-photon fluorescence emission of TMP, pH7.0. Excitation at 425nm (---); 520nm (-•-); One-photon fluorescence emission when excited at 265nm

ratio. The two-photon cross sections with wavelength of TMP and thymine may suggest another interpretation.

Since the two-photon maximum was slightly shifted within the first absorption envelope relative to the one-photon maximum in each case, one explanation might be that the polarization values observed are the result of a single transition, but that this transition is not the same transition responsible for the one-photon event. That is, there are two transitions under the first absorption band of thymine, for example, which completely reverse in absorption strength under the two types of excitation. With all transitions being allowed in C_s for both spectroscopies, the complete domination of one transition moment with the mode of excitation seems unlikely. Moreover, the INDO/S semiempirical method fails to offer evidence of a composite picture for the first absorption band using both forms of excitation. Other explanations concerning the reason for the observed shift will be presented in the section describing the analysis of the two-photon response.

Arguments supporting the composite nature of the second absorption band are strengthened by the change in Ω observed for TMP, see Figure 21. The inflection of Ω for TMP within this second envelope defines at least two bands which fail

to resolve under one-photon polarized experiments. The wavelength of maximum Ω for TMP at 440nm (220nm) corresponds directly to the location of a weak transition suggested by other methods. In INDO/S calculations using both singly and doubly excited configurations (SEC and DEC's), two states are also predicted (see Table 6 for uracil, bands II and III). The state presently assigned as responsible for the bulk of the one-photon absorption (band III) has the transition density pattern equally directed along each allyl ($C_5-C_4=O_4$, for example) fragment. The second, one-photon weak, transition (band II) has the majority of the transition density localized along the $N_3-C_4=O_4$ fragment (65). The predicted two-photon polarization values are also listed in Table 6 for these transitions.

With respect to the predicted values for Ω with each excited state, the experimental Ω data from TMP could be correlated with these two transition moments. That is, the band II type transition state, with $\Omega \approx 1.0$, corresponded to the rise in Ω observed on the red edge of the second absorption band for TMP. A drop in Ω with decreasing wavelength illustrated the influence of the "double allyl" band III type transition with its predicted low two-photon polarization ratio.

For thymine and TMP, the calculated order of these two transition moments are reversed regardless of INDO parameters used. Although band III was found to be lower than band II for these molecules, the separation in energy is small. The semiempirical methods employed are not expected to correctly predict the order of such closely spaced transitions (65). As opposed to TMP where a definite change is observed, the two-photon polarization of thymine remains constant across this second band. Therefore, the experimentally observed value of $\Omega \approx 0.75$ could represent the weighted average of the now degenerate transition pair with excited state characteristics comparable to that calculated for bands II and III. The addition of a methyl group to the N1 position must represent a sufficient enough perturbation to break this degeneracy, and therefore, resolve the two transitions in the second absorption band of TMP.

When comparing the two-photon polarization values of each of the nucleotides with theory, the experimental values of Ω tend to be much lower in all cases, see Table 6. The general theory for the fluorescence detection of the two-photon signal, and polarization ratio by implication, depend only on the pattern of the two-photon tensor. Provided that fluorescence emission is isotropic and from an isolated

molecular transition, the values of Ω should be invariant to the choice of polarization direction of the linearly polarized light. Theoretical INDO methods for calculating the two-photon properties assume that the molecule conforms to this class. However, the nucleotide solutions are not isotropic emitters because of the short fluorescence lifetimes. For such anisotropic emitters, the polarization ratio now depends on two variables, the two-photon tensor and the direction of the fluorescence emission vector. Therefore, the discrepancy in Ω between theory and experiment could be explained in the context of a photoselection mechanism.

Figure 25 illustrates the effect on Ω with the choice of linear polarization for TMP. As shown, the polarization was found to depend on the type of polarized linear light. The ratio of circular to horizontal (C/H) polarization was found to be much higher ($\Omega=0.9$) than theoretically expected, $\Omega=0.6$. In contrast, circular versus vertical measurements (C/V) of Ω , which were exclusively used throughout this study, resulted in values comparably less than $\Omega=0.6$. Qualitatively, the average of the two results was close to the expected value. A test case simulating thymine, with a tensor pattern having only one diagonal component and

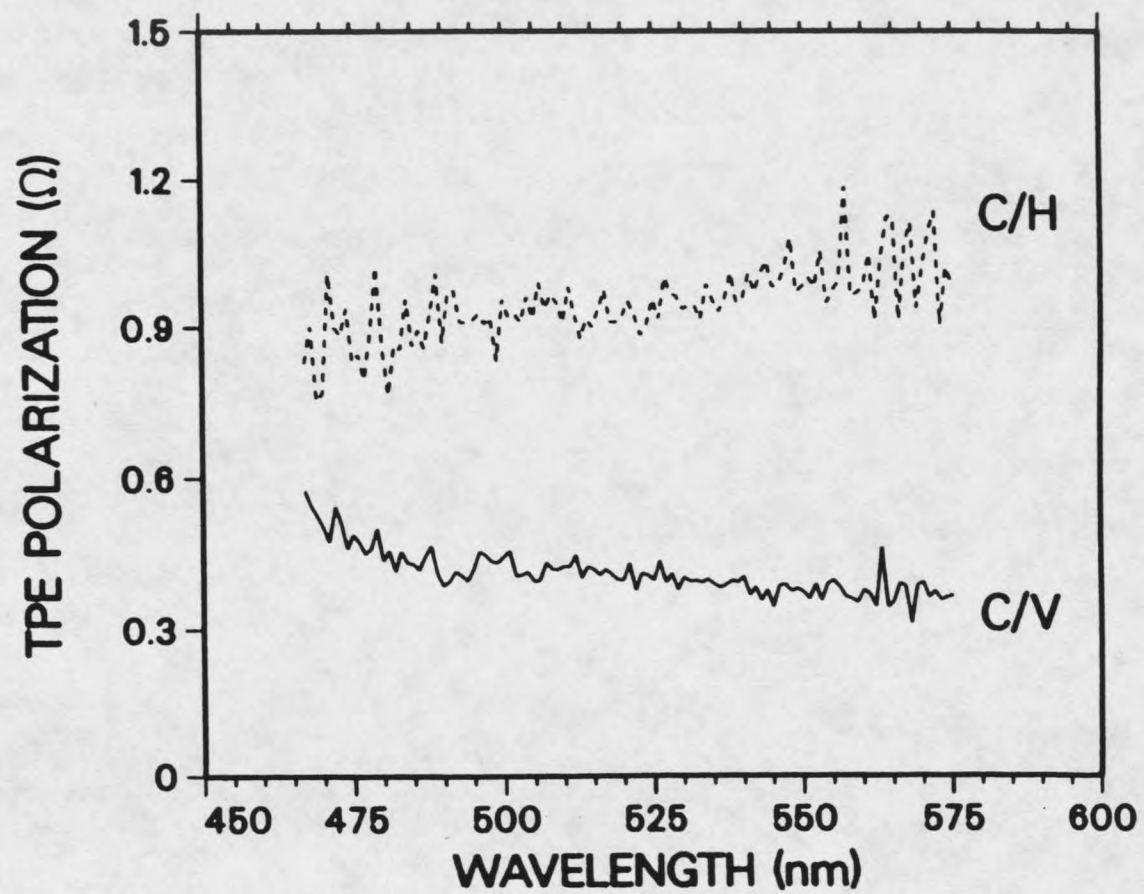


Figure 25. Two-photon photoselection experiment with TMP, pH7.0

transition vector oriented along a single cartesian direction, was applied to the photoselection formula for calculating Ω . For the isotropic fluorescence emitter, the value of Ω expected would be 0.67 given only the tensor pattern. In the case just described for the anisotropic emitter, the value of Ω for vertical polarized light would be about 0.4 in agreement with experiment. However, when calculating the two-photon polarization for horizontally polarized light a value of about 0.6 is obtained for reasons which are not yet clear. Under the condition of an anisotropic fluorescence emitter, the theoretical calculation methods would require modification to account for the relative orientation of the fluorescence transition moment. From an experimental viewpoint, the two-photon polarization differences between states may be further accentuated by taking advantage of the process of photoselection for these anisotropic emitting molecules.

A comparison of the two-photon spectral characteristics of thymine and 5-methyl cytosine with pH further authenticates the response of these nucleic acid systems under two-photon excitation. Shown in panel A of Figure 26, the two-photon profile and polarization of 5-methyl cytosine was found to be comparable to cytosine

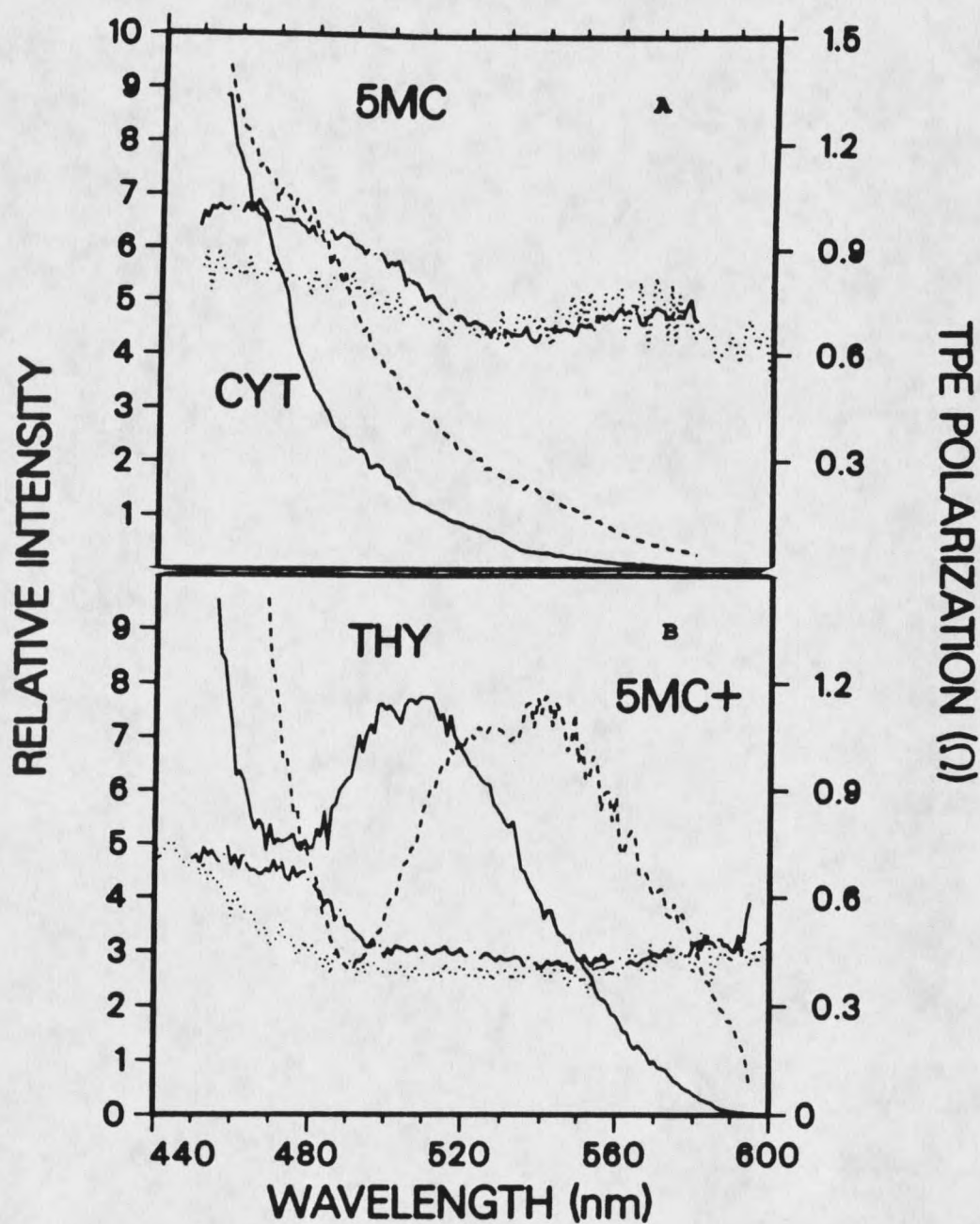


Figure 26. Two-photon fluorescence excitation spectrum and polarization of 5-methyl cytosine and thymine with pH. 5-methyl cytosine polarization in both panels (---)

(detailed in the next section) in neutral conditions. Again, the perturbation of the methyl group on cytosine would not be expected to alter the spectral features in accord with this finding. However, much like with TMP and thymine, certain radiative distinctions are observed between the two molecules (11,58). Upon dropping the pH below 4.6, the two-photon spectral features of 5-methyl cytosine become coincident with neutral thymine instead of neutral cytosine. The two most striking feature similarities are the relative shape and position of the two-photon profile with the one-photon spectra, and the convergence of Ω to values of 0.4. Explanation of this result can be reconciled by the isoelectronic equivalence of 5-methyl cytosine cation and neutral thymine. The electronic evolution of 5-methyl cytosine to thymine can be qualitatively pictured as the removal of one amine proton and subsequently depositing the other into the amine nitrogen. The net result of such an operation would have a small effect on the π electrons.

CMP and Cytosine

Much like thymine, the electronic picture of cytosine and relevant derivatives suffers from the same confusion over the number rather than the type of electronic states. Regardless of the parameterization or theoretical method

applied to cytosine, the results are unanimous in assigning three $\pi\pi^*$ transitions within the 195 to 300nm range (65,93-95). Circular dichroism experiments and recent application of reflectance spectroscopy on cytosine monohydrate crystal have expanded the number of possible $\pi\pi^*$ states to four within the same range (10,96). Although not resolved explicitly, the latter study inferred four bands using a gaussian fitting technique to their crystal absorption data (96). In addition, two peaks and two shoulders are seen by inspection of the one-photon absorption profile for cytosine in aqueous solvents. The standoff between theory and experiment rests on whether the band III (212nm) is properly assigned. Polarized one-photon experiments may have difficulty in resolving all four states since the polarization directions of band III and band IV (197nm) may be collinear (96). The two-photon spectra of CMP and cytosine were obtained down to 400nm (200nm) in an effort to resolve this question.

The two-photon polarization values for CMP, see Figure 27, did appear to coincide with the proposed resolution of four bands (96). As shown, the values of Ω average close to 0.6 in response to band I. With decreasing wavelength, band II (with $\lambda_{\text{max}} \cong 230\text{nm}$) begins to exert influence, according

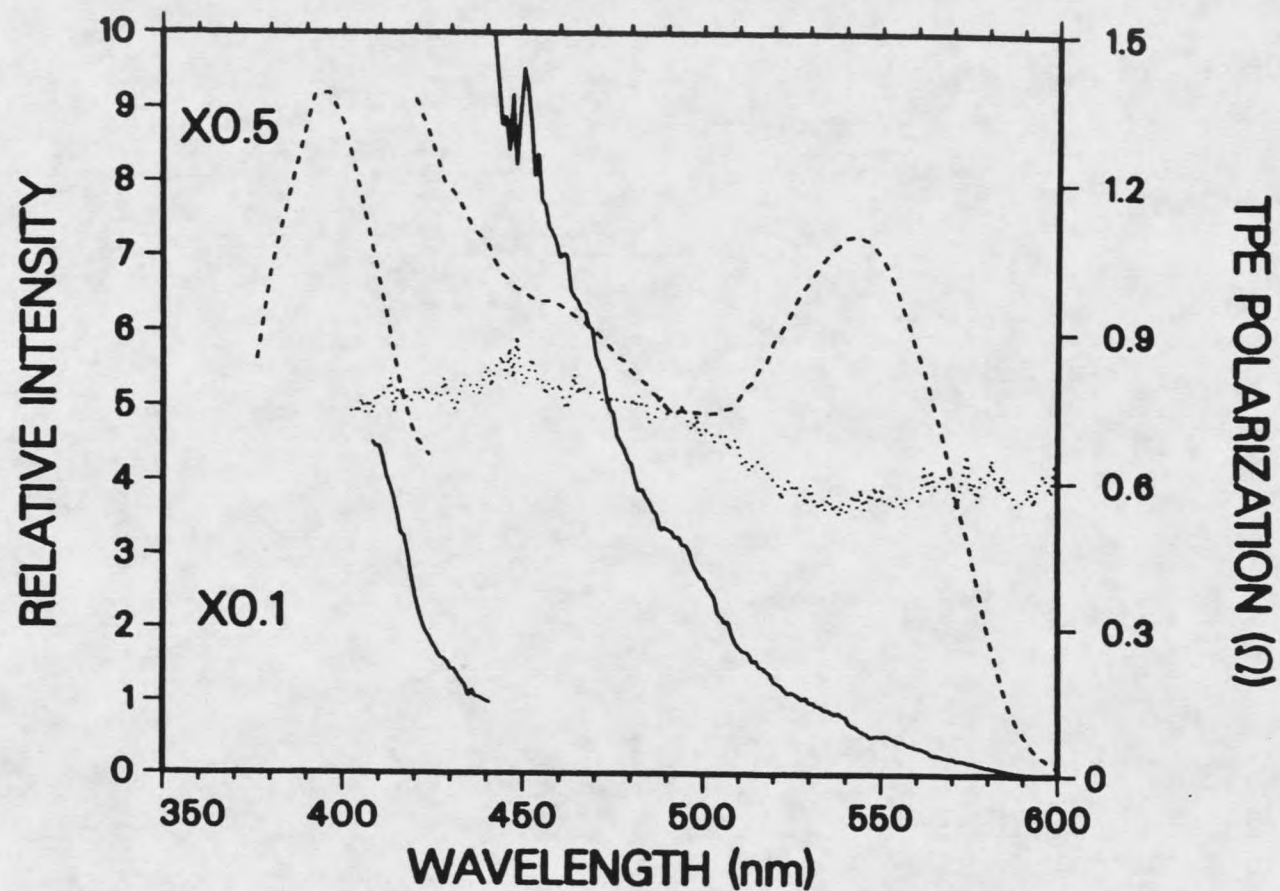


Figure 27. Two-photon fluorescence excitation spectrum and polarization of cytidine-5'-monophosphate in 0.05M phosphate buffer, pH7.0. Linear polarized two-photon profile (—); One-photon absorption profile (---); two-photon polarization (•••)

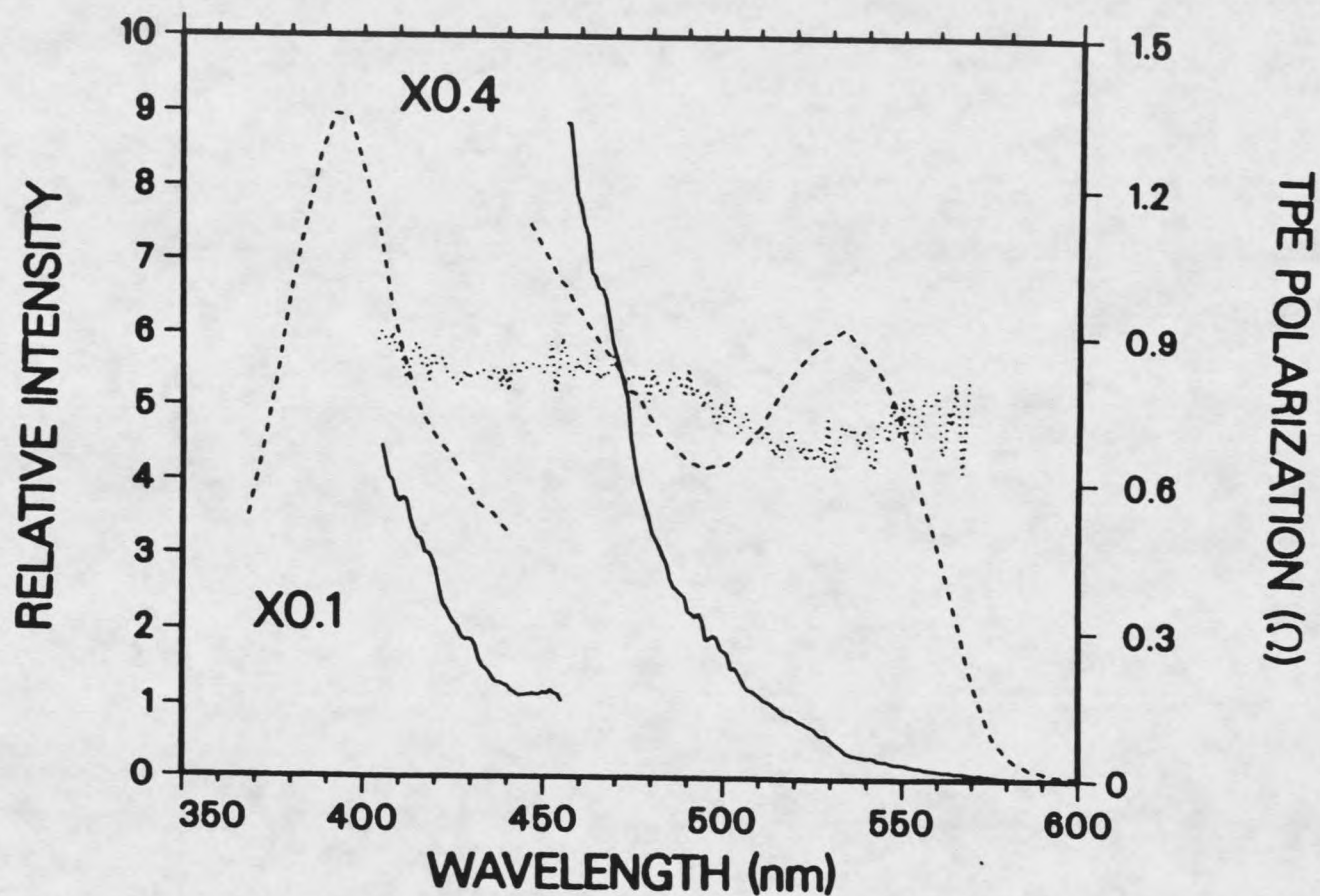


Figure 28. Two-photon fluorescence excitation spectrum and polarization of cytosine (Aldrich) in 0.05M phosphate buffer, pH7.0. Linear polarized two-photon profile (—); One-photon absorption profile (---); two-photon polarization (•••)

to the crystal spectra, and this is mirrored by an increase in Ω across 500nm (250nm) to 0.7. A third band (III), if present, would carry twice the absorption strength (again according to the crystal work) relative to band II at wavelengths beginning around 220nm. The two-photon polarization ratio begins to fall slightly just before 450nm (225nm) in agreement with this assignment. Although the fourth band peaks just beyond the low wavelength limit of these spectra, the observance of the three changes in Ω before the onset of the undisputed fourth band would further support the four electronic state composition of cytosine in the 195 to 300nm range. Unfortunately, the changes in Ω observed are too modest to forward a strong conclusion based on these spectra. A similar analysis could be applied to the spectra of cytosine, see Figure 28. However, there does not seem to be a difference in Ω for the wavelengths corresponding to band III. In addition, the rise in Ω at the far red edge (about 575nm) of both spectra could be the result of a receding two-photon signal relative to the solvent in this region.

The two-photon profile information, in both cases, did not provide any spectral features which would be beneficial to the analysis. A reversal of the relative strengths of

band I and II predicted by theory between one- and two-photon excitation could be the reason for the observed near monotonic increase in the relative two-photon cross section, see Table 6. The polarization trend also seems to follow along the same line as predicted by theory, but the values are most likely lowered by the same photoselection mechanism operating for thymine. Deviation from theory, as with thymine, was observed in the large relative δ calculated for the region corresponding to band III of cytosine. From INDO theory, the values of δ should decrease relative to band II with decreasing wavelength.

AMP

For the purine systems, the electronic structure increases in complexity relative to the pyrimidines. Consequently, there exists a propensity for the photochemical and radiative properties to increase in complexity as well. AMP in aqueous solvents offers no exception. The near degeneracy of the two $\pi\pi^*$ states (bands I and II, see Table 6) which reside under the first absorption band has proven to be sensitive to the solution environment, and therefore, the order of these states has always been the subject of debate. From theoretical INDO/S methods, these two states in AMP take on transition density

patterns comparable to the La (band II) and Lb (band I) states of benzene and indole (65). The integrated one-photon oscillator strength for the first absorption envelope has been calculated to be primarily characteristic of the La-like band II transition state. Recent polarized reflection work on 9-methyl adenine crystal suggest that this assignment by theory of the two nearly degenerate bands under the first absorption envelope is correct (97).

A third $\pi\pi^*$ state of weak oscillator strength has been suggested to peak at 230nm from CD and MCD experiments (9,10). Since there have been $n\pi^*$ transitions of comparable strength reported close to 230nm, care in analysis must be exercised in this spectral region (10,11,41). The absorption profile observed for the 195-210 region has been resolved into two nearly degenerate $\pi\pi^*$ states from both experimental and theoretical methods (9,10,65).

The two-photon polarization values measured for AMP, see Figure 29, harmonized with one-photon data on the assignment of band I as the lowest singlet transition state. As predicted by INDO/S theory, the second band was calculated to have a lower value in Ω relative to band I. Resolving the positions of the remaining transitions, bands III and IV, in the 400-500nm two-photon range would be

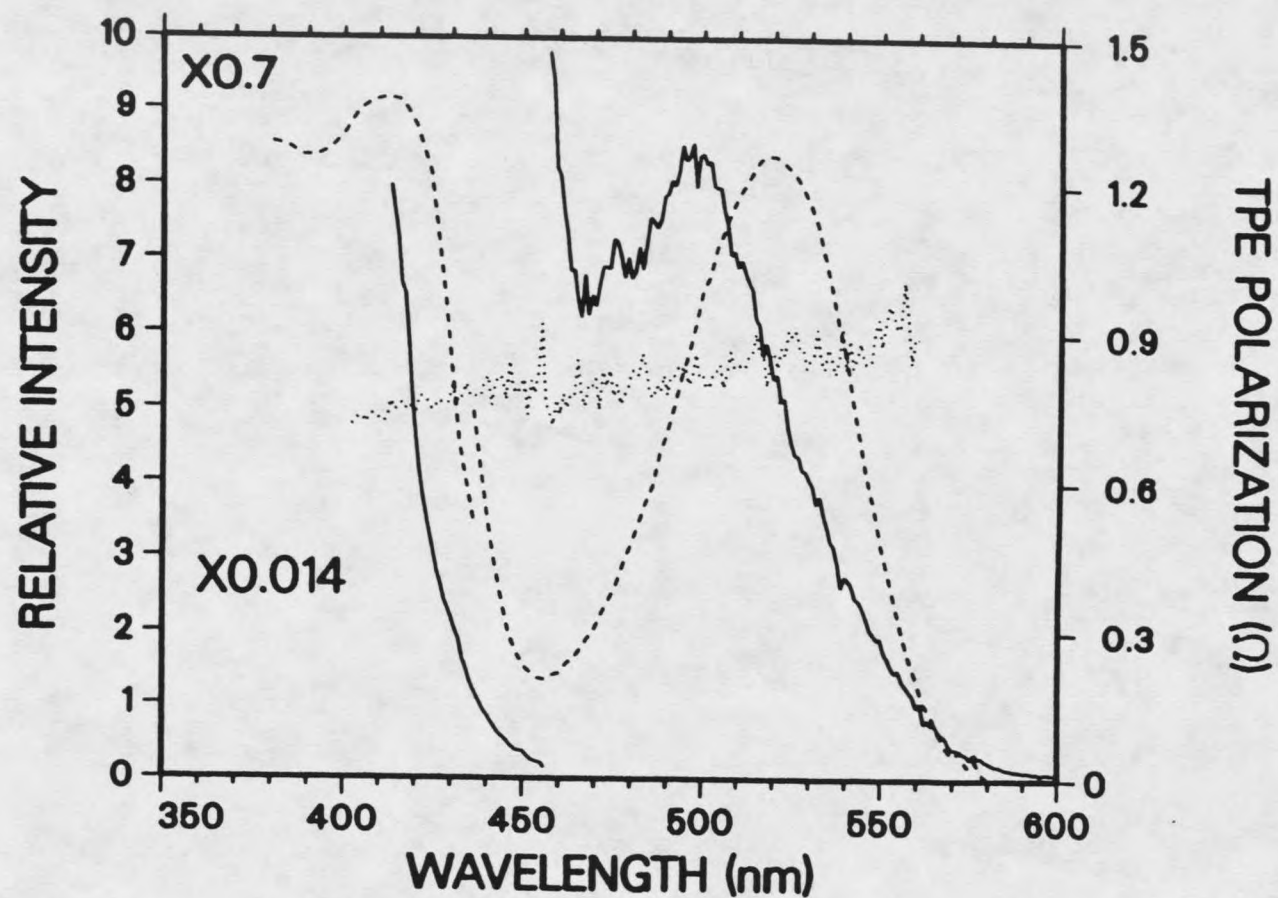


Figure 29. Two-photon fluorescence excitation spectrum and polarization of adenosine-5'-monophosphate in 0.05M phosphate buffer, pH7.0. Linear polarized two-photon profile (-); One-photon absorption profile (---); two-photon polarization (•••)

difficult since there was little variation in Ω observed throughout this region. Invariance in Ω for these two transitions also was expected from theoretical prediction (65).

There are several possible explanations which apply to the two-photon profile information illustrated in Figure 29. First, the two-photon transition resolved at 500nm may be evidence in support of the strong two-photon absorbing band III predicted unanimously in theory. However, for this band to display the predicted reversal in transition strength, the relative two-photon cross section would have to be several fold larger in comparison to the first two-photon band of TMP, for example. As shown in Figure 20, the relative cross sections are comparable in each case.

With equal two-photon absorbing strength, a second possible explanation would be that peak resolved for AMP corresponds to the first UV absorption band, but is shifted by 2000 cm^{-1} through the same mechanisms operating on TMP. If the first spectral feature of AMP corresponds to the first UV absorption band, then band III could be responsible for the seventy fold increase in absorption strength observed to the blue of 450nm, see Figure 29. Although once again a similar feature was observed for TMP, the increase

observed for AMP was three times larger. Perhaps the increase observed for AMP is derived from the same mechanism as TMP in the 400-450nm range, but that the addition of a strong second component created the difference between the two molecules.

Finally, the observed two-photon spectral features could be the result of the impurity detected in the sample by one-photon methods. This final possibility was minimized by the adjustment of the monochromator to select out the 315nm fluorescence originating from the AMP, and not the 380nm impurity. However, more accurate two-photon fluorescence emission studies would be required to eliminate this possibility.

GMP

The experimental effort for elucidating the electronic state composition of GMP has placed the assignments of this nucleotide on solid foundation. From the collective body of numerous experimental techniques, there is unanimous agreement on the assignment of a $\pi\pi^*$ state as the lowest excited singlet state under a variety of experimental conditions (9-11,29,43-45,98). The second transition can also be ascribed to a $\pi\pi^*$ state residing at approximately 255nm. This definite separation in states, which comprise

the first absorption envelope, explains the accuracy of assignment which could not be achieved with the near degeneracy of the AMP excited states.

Past theoretical efforts to predict the transition moment directions for this pair have been in very poor agreement with those deduced from polarized spectra of 9-ethylguanine crystal (11,98). For almost all theoretical parameter schemes and semiempirical methods, the order of the two transition moments would be reversed and given equally weighted oscillator strengths in direct contradiction to the experimental results. However, recent calculations in which the chromophore is immersed in a simulated crystal matrix give values conforming to the experimental picture (99). Such a result suggests that theory may be reliable when environmental effects are considered.

Two remaining transitions are resolved in the UV region of the spectrum. Polarized reflection measurements on 9-ethyl guanine crystal reveal a weakly absorbing $\pi\pi^*$ transition, labeled M band, at approximately 215nm (98). A fourth $\pi\pi^*$ state peaking at about 210nm rounds out the 200-300nm excited state composition of GMP (43,44,65,98).

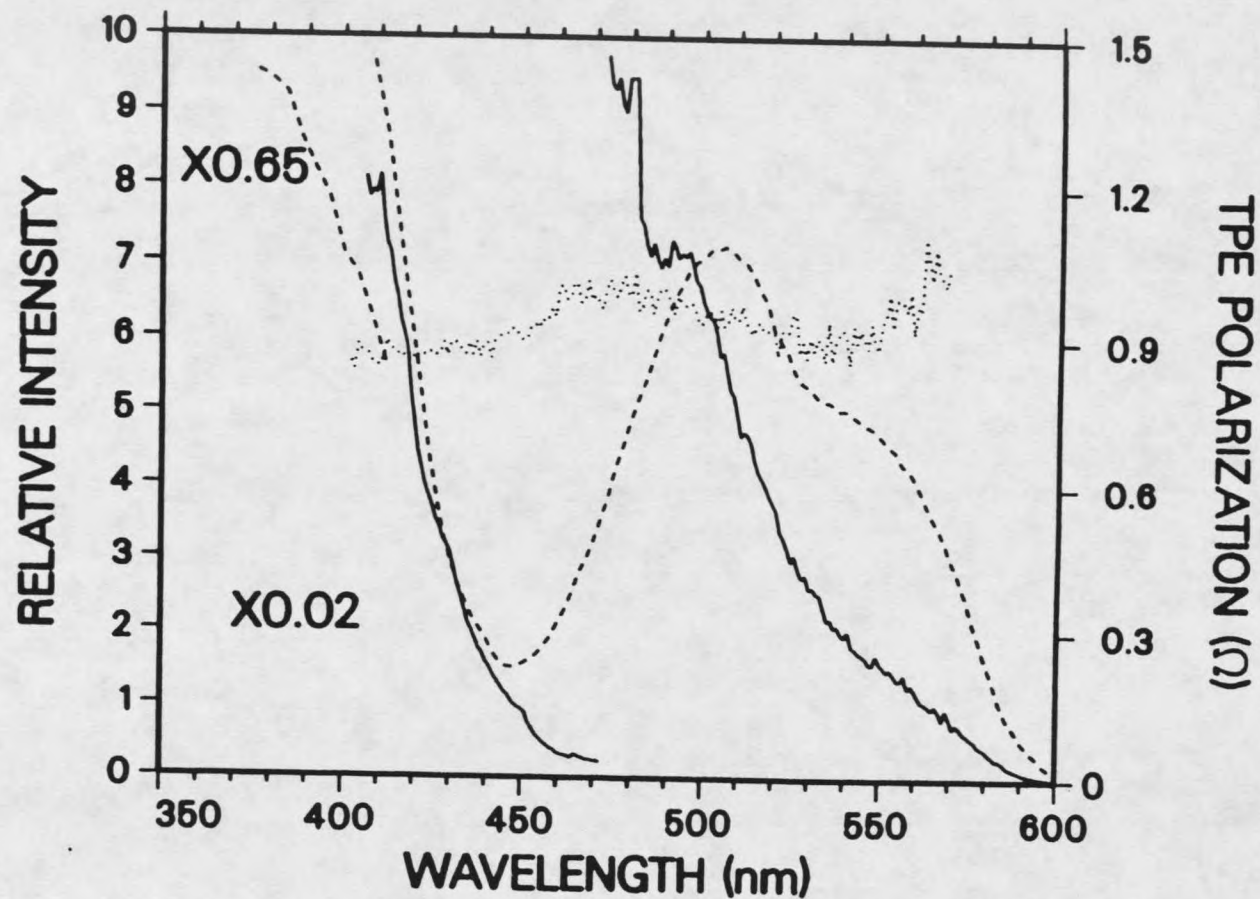


Figure 30. Two-photon fluorescence excitation spectrum and polarization of guanosine-5'-monophosphate in 0.05M phosphate buffer, pH7.0. Linear polarized two-photon profile (—); One-photon absorption profile (---); two-photon polarization (•••)

The assignment of a $n\pi^*$ transition moment within this UV range is weakly founded, yet theory suggests such a state mixed within the first two $\pi\pi^*$ transitions (32).

The INDO/S predictions of the two-photon properties of guanine (9H), and GMP by implication, are tabulated in Table 6. When compared to the two-photon polarization and spectral data for GMP in neutral aqueous solution (see Figure 30) general agreement is again observed. As shown, the predicted high ($\Omega=1.45$) to low ($\Omega=1.0$) trend for calculated values of Ω are mirrored by a similar experimental drop with decreasing wavelength. Therefore, the two- and one-photon assignments of bands I and II correspond. Experimental evidence for band III was found through a rise in polarization approaching $\Omega=1.0$ at 470nm. This was roughly 15nm more to the red than previously suggested by prior experimental treatments. The predicted spectral dominance of band III under two-photon excitation may be reason for the near monotonic increase in the two-photon cross section with decreasing wavelength. The magnitude of this increase in δ and the lack of a predicted recovery may suggest that this molecule is not excluded from the mechanisms operating on the other nucleotides. As illustrated in the one-photon profile of Figure 30, the

onset of band IV about 440nm was followed by a similar drop in Ω as predicted in theory.

Analysis of the Two-Photon Response

The two-photon spectral profiles for each of the nucleotides and bases displayed common trends which were not anticipated in theory. The two common properties observed in these spectra were the shift in the two photon maximum of AMP, TMP and thymine, as well as, the enhancement of the two-photon absorption strength with decreasing wavelength. Although such a finding, in and of itself, may suggest that the theory inadequately describes these systems, further experimental evidence must be advanced to authenticate the spectral information. Only after such experimental evidence is offered can useful discussion regarding the theory be addressed.

This is particularly important in systems which are studied for the first time under a new spectroscopic procedure. Investigations to provide such data are offered and discussed in the following sections.

Control Molecules

Molecules, which have been the subject of extensive theoretical and experimental investigation under multiphoton

methods, were run along with the nucleic acid systems as a check on the procedure. Benzene and pyrimidine represented the best two candidates to serve this purpose. The corrected and uncorrected spectral profile shown in Figure 31 for benzene exposes some questions.

Only one segment, coumarin 500 (C500), in the dye profile of benzene falls within the range capable of being corrected for deviations in the second order coherence. When this corrected segment was used to construct the profile, the spectral structure shown in panel A of Figure 31 was generated. As shown, both of the progressions predicted in theory, $v_{14}v_1$ and $v_{18}v_1$, are resolved (100). The relative wavelengths and intensities displayed were found to be in agreement with previously published two-photon results (71). Such a result suggests that the spectra observed for the nucleotides were not an artifact of the procedure. That is, the spectral shifts and the strong two-photon cross section are intrinsic to the DNA bases. An independent test was to compare the two-photon peak ratios with the one-photon absorption spectrum.

In theory, the combination of the v_{14} mode with the Franck-Condon ring breathing v_1 mode for benzene requires that the progression follow the one-photon spectra. Such a

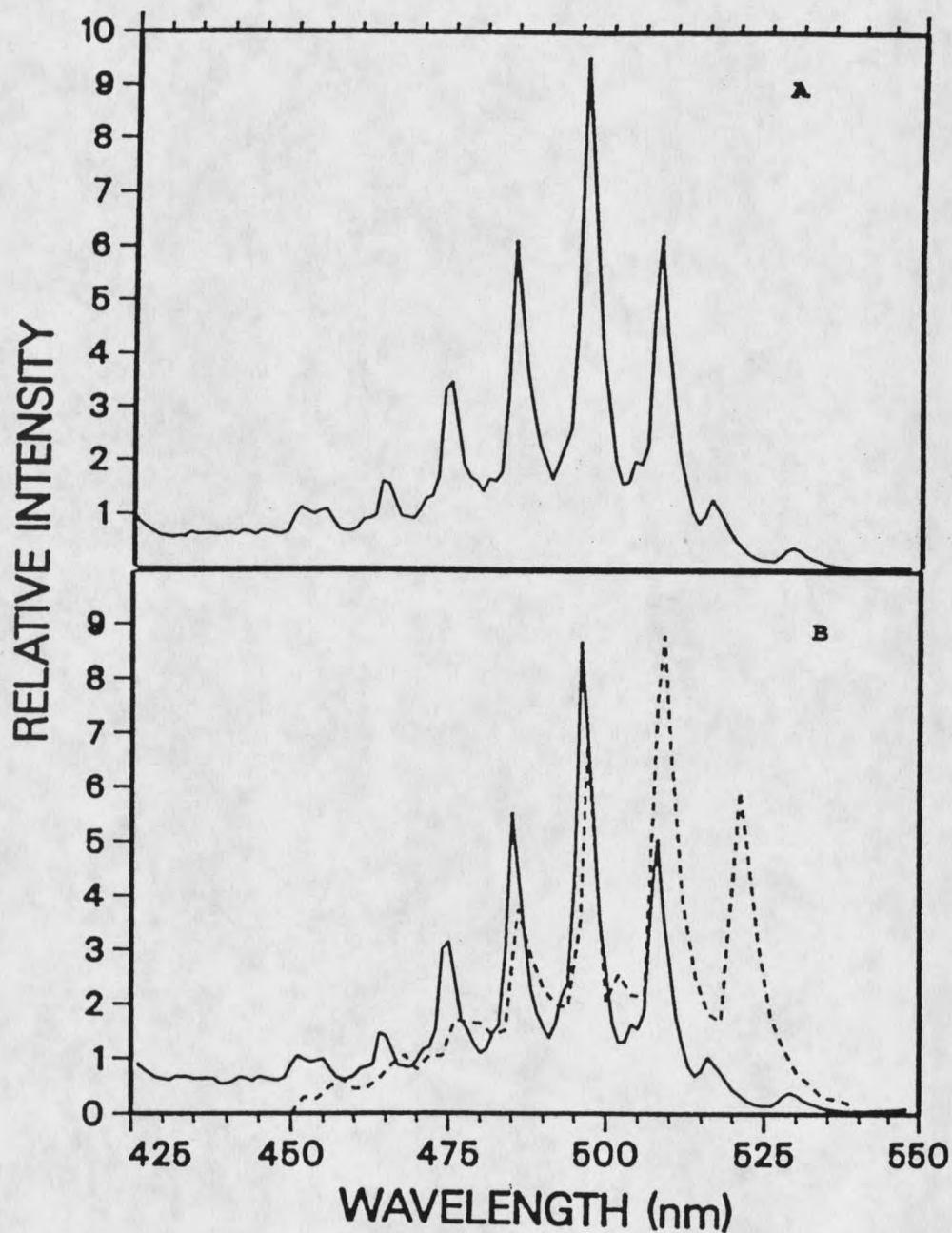


Figure 31. Two-photon fluorescence excitation spectra of benzene. Corrected, panel A; uncorrected, panel B; one-photon absorption profile (---)

requirement is extended to two-photon since the one-photon spectrum results from a similar progression involving a ν_6 bond stretching vibration. With the intensities being derived through similar vibronic mechanisms, the profile under both forms of excitation should be similar (101).

If the two-photon profile was created without using the C500 segment, spectral features were generated which corresponded nicely to the one-photon progression as shown in panel B of Figure 31. Recent three-photon experiments on benzene in solution agree with the one-photon progression in intensity (102). All four spectra, the one-photon and the three two-photon spectra, seem to converge at wavelengths shorter than 496nm (248nm, one photon). Only for the peak intensity associated with zero quanta of ν_1 was discontinuity observed. Provided that the two-photon result in panel B for benzene is correct, such a result should cast doubt on the previously published benzene two-photon fluorescence excitation spectrum (71). Unfortunately, the only region (490-570nm) in question coincides with the two-photon maxima observed for TMP and AMP. Although now the observed shift must be treated with caution and investigated further, the predicted lack of enhanced short wavelength two-photon strength for benzene observed by experiment

supports the two-photon result for the nucleotides and bases as an intrinsic molecular property.

Pyrimidine was also chosen not only for structural reasons, but because theory and the two-photon experiment have been previously reported (36). For pyrimidine in cyclohexane, Figure 32, there was nearly a one to one correspondence of the two-photon polarization values between this and the published result. Such correspondence disappears with regard to the two-photon profile. The pyrimidine result obtained in this work was independent of the concentrations used which varied from 0.7M to 0.1M (data not shown). The comparisons between the two studies would suggest that the mechanisms operating on the relative two-photon strength was the same regardless of polarization of the exciting laser light.

The pyrimidine two-photon profile of this work seems to resemble closely the result obtained for pyrimidine in pH 7.0 buffer, see Figure 33. As shown, the polarization values increased to $\Omega=1.2$ at about 400nm in response to a blue shift of the second $n\pi^*$ state residing at shorter wavelengths than the vibronically shifted $\pi\pi^*$ state (36). The increased purity of the $\pi\pi^*$ transition at about 450nm was demonstrated by a further decrease in Ω compared to the

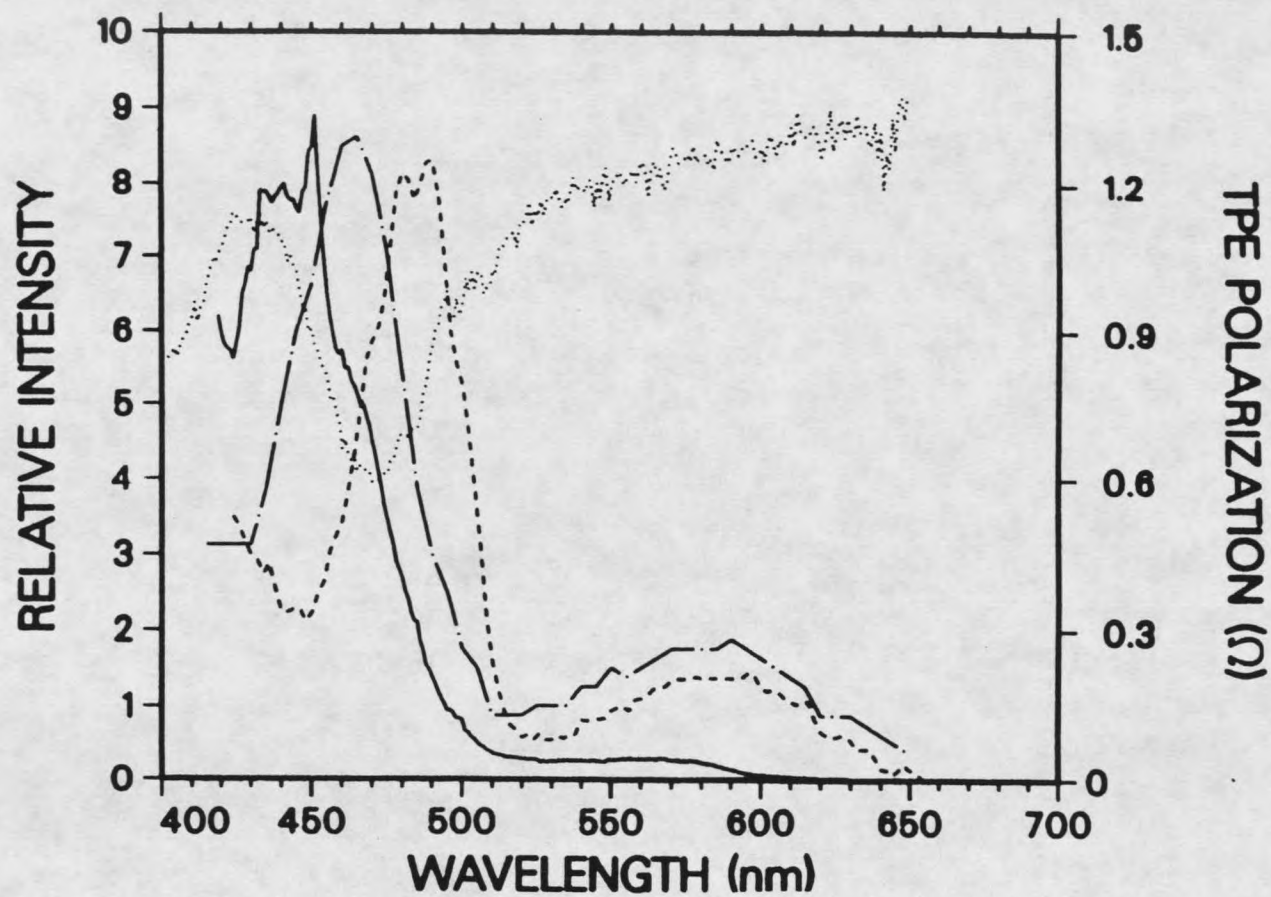


Figure 32. Two-photon fluorescence excitation spectrum and polarization of pyrimidine in cyclohexane. Linear polarized two-photon profile (-); Linear polarized two-photon profile from ref. 36 by permission; One-photon absorption profile (---); two-photon polarization (•••)

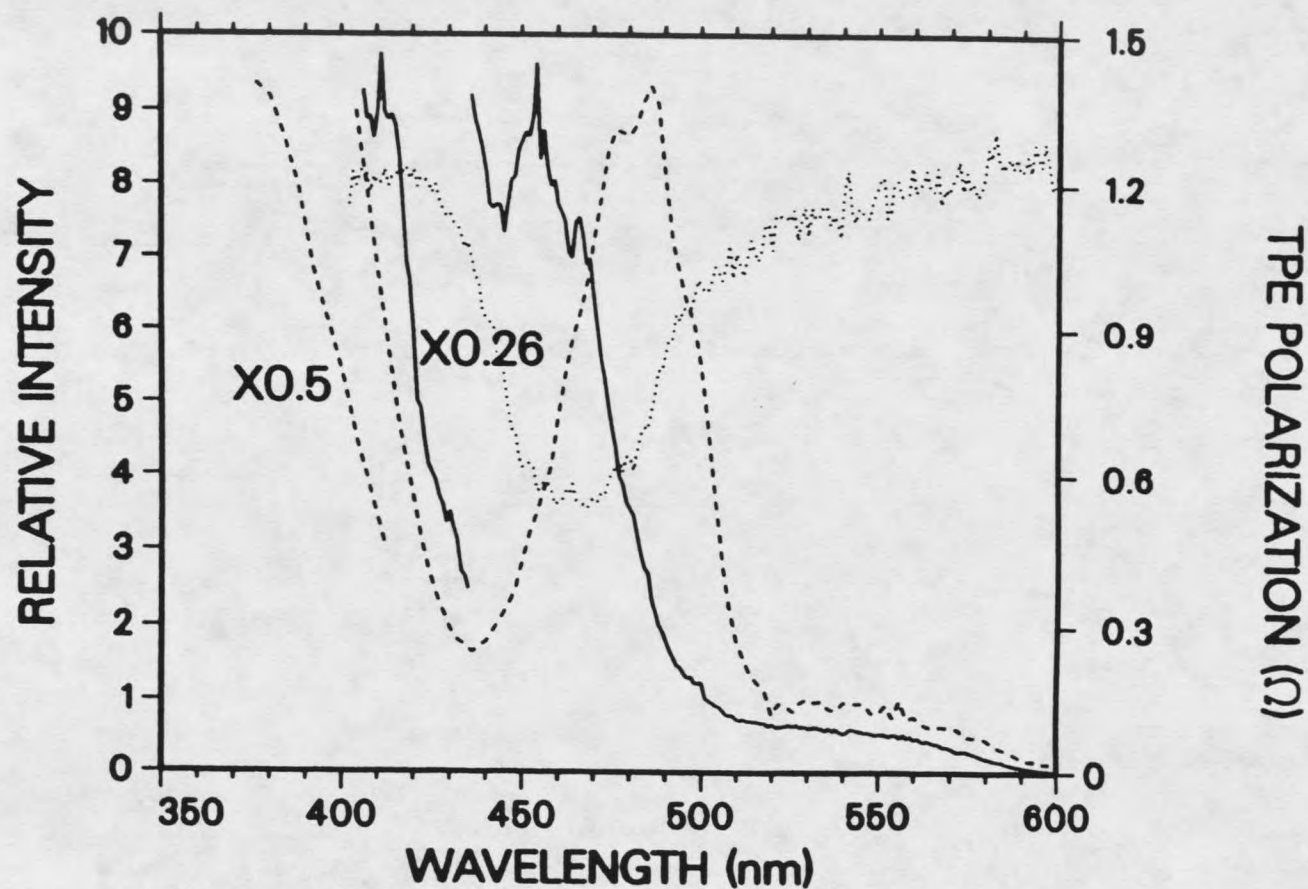


Figure 33. Two-photon fluorescence excitation spectrum and polarization of pyrimidine in 0.05M phosphate buffer, pH7.0. Linear polarized two-photon profile (-); One-photon absorption profile (---); two-photon polarization (•••)

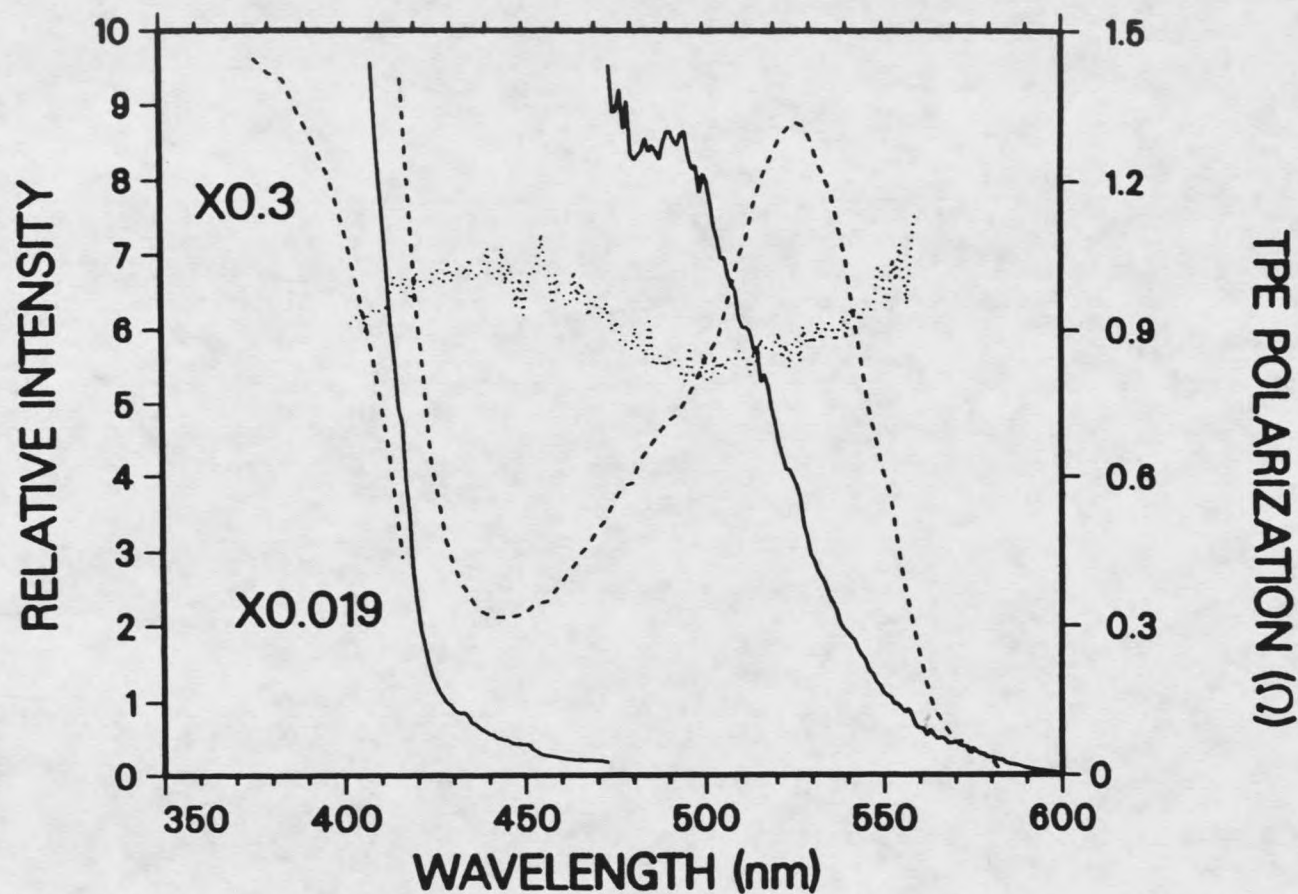


Figure 34. Two-photon fluorescence excitation spectrum and polarization of purine in 0.05M phosphate buffer, pH7.0. Linear polarized two-photon profile (-); One-photon absorption profile (---); two-photon polarization (•••)

Ω in non-polar environments. Although the shift of the $\pi\pi^*$ transition suggested by this work is larger than can be explained through the enhancement of intensity by vibration of a skeletal mode, the one- and two-photon spectra in Figure 33 do agree on the location of the first $n\pi^*$ transition centered at 560nm (280nm) in polar solvents.

There was considerable latitude in producing the only $\pi\pi^*$ band in both pyrimidine spectra generated in this work due to a severe curling of the dye segments in this range. Both results could be reevaluated as containing the lowest $n\pi^*$ state, and then, subsequently increase in a monotonic fashion to 400nm. Because there is some indication that the overall spectral characteristics of pyrimidine are relatively insensitive to the solvent environment (103), the coincidence of the two profiles generated by the author may be a plausible result. On the other hand, theoretical evidence in support of the pyrimidine electronic structure would favor the published results over those presented herein (36). Consequently, the two-photon properties revealed by the spectral profile of this work may be influenced by instrumental artifacts or solution impurities not detected under one-photon methods.

The uncorrected spectra under both experimental conditions gave the same result as shown in Figures 32 and 33 using the SHG procedure. Since there was good agreement between theory and the experiments with respect to the polarization values, some confidence can be placed on the information gained. The lack of agreement with the profile data cannot be reconciled since there was some reason to believe aspects surrounding both the new and published results. For example, the two-photon spectra of benzene and pyrimidine in this work were completed at the same time. Since the benzene two-photon spectrum closely matched the one-photon profile, the two-photon spectrum of pyrimidine of this work may be the correct result as opposed to the published work in which benzene and pyrimidine were run independently (36). However, until resolved experimentally, this molecule cannot be used as a control.

Purine in aqueous solvent was also run alongside the nucleotides and free bases. As shown in Figure 34, the two-photon spectral properties of purine are similar to those obtained for GMP, for example. The polarization values of Ω responded to the changes in state composition with wavelength as predicted by previous theory and experiments (34,35,38,39,104,105). The two-photon spectral profile of

purine was found to mismatch the one-photon spectra in much the same fashion, and presumably for the same reasons, as GMP.

Intrinsic Molecular Properties

From the two-photon properties displayed by benzene in Figure 31, the huge increase in two photon absorption strength toward the blue end of the spectrum may be an intrinsic property of the nucleotides. One plausible explanation for this increase, that protects the integrity of the theory, is that the intensity was being derived from a Rydberg transition residing in the second absorption envelopes of these systems. Such a state is predicted in the near-VUV for pyrimidine (34). A Rydberg transition centered at about 200nm has been experimentally observed in the two-photon fluorescence excitation spectrum of benzene in condensed media (106). Therefore, with all these systems retaining some form of the pyrimidine structure, the perturbations placed on each may be sufficient to shift the Rydberg character into the far-UV. This would explain why the valence π theoretical methods were unable to account for this strong absorption effect.

The alternate explanation regarding the enhanced δ for the nucleotides would simply be that semiempirical methods

are inadequate in describing these systems. Perhaps, the higher energy valence states are experimentally stronger by several fold. Regardless of whether these two possibilities hold, the presence of one (or several) strong two-photon transition would be sufficient to overwhelm the spectral features of the weaker lower states. The shift in the two-photon profile for TMP and AMP, for example, could be the result of averaging the weaker excited state bands with the tail of a dominate transition.

Another possibility for the shifts in the two-photon profile would be that the intensity is being "borrowed" via vibronic coupling much like with benzene. The 1500cm^{-1} displacement from the one-photon spectrum for TMP represents a typical frequency observed for a skeletal vibration. Since the relative two-photon strengths of all the DNA bases are at least an order of magnitude larger than benzene, a vibronic mechanism seems unlikely. In addition, for the full skeletal displacement frequency to be displayed, nearly 100% of the intensity would have to be borrowed from the coupled state(s). To check this reasoning, INDO/S calculations were performed on several ring breathing displacements for uracil and found not to greatly affect the two-photon absorption (data not shown).

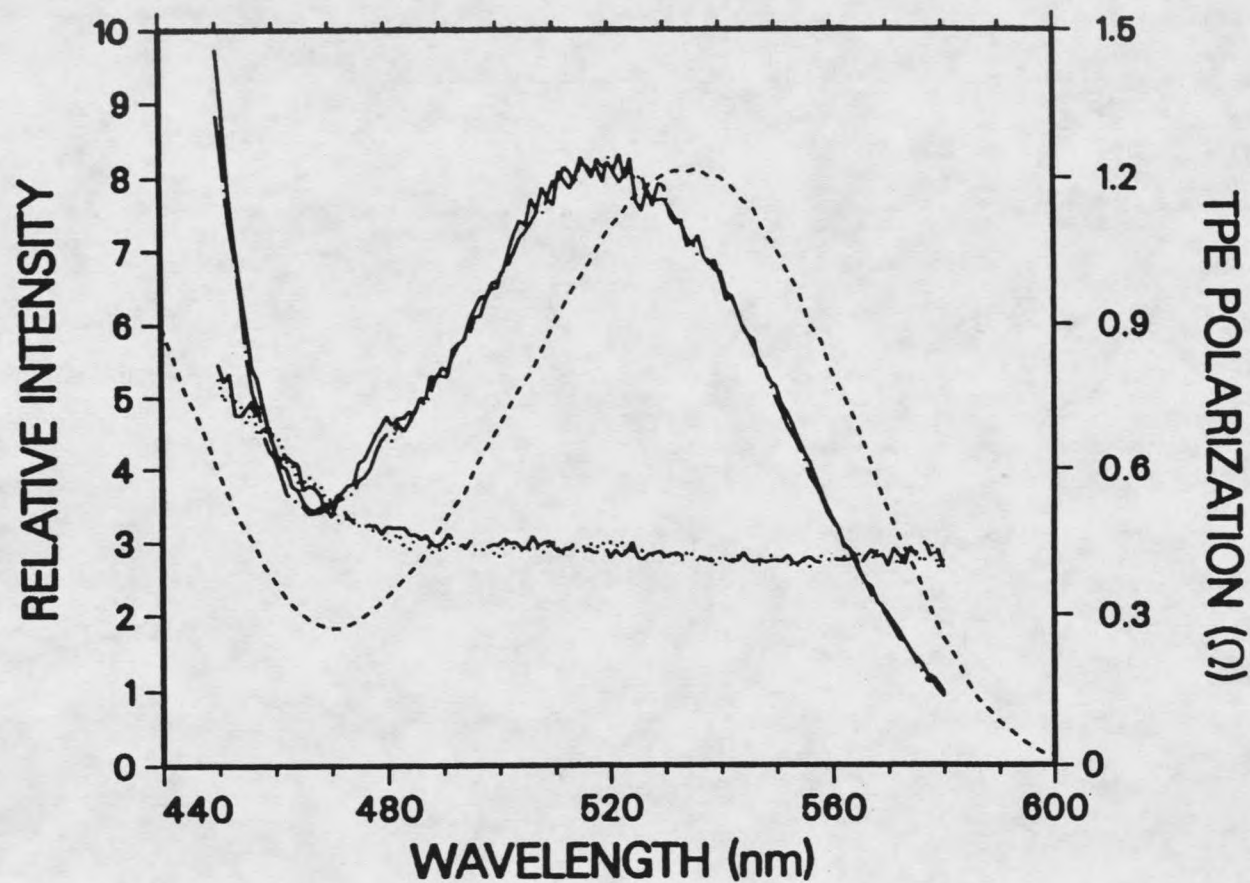


Figure 35. Two-photon fluorescence excitation spectrum and polarization of TMP in 0.05M phosphate buffer, pH7.0 and water. Linear polarized two-photon profile in pH7.0 buffer(—) and water (---); One-photon absorption profile (•••); two-photon polarization in pH7.0 buffer (-•-) and water (---)

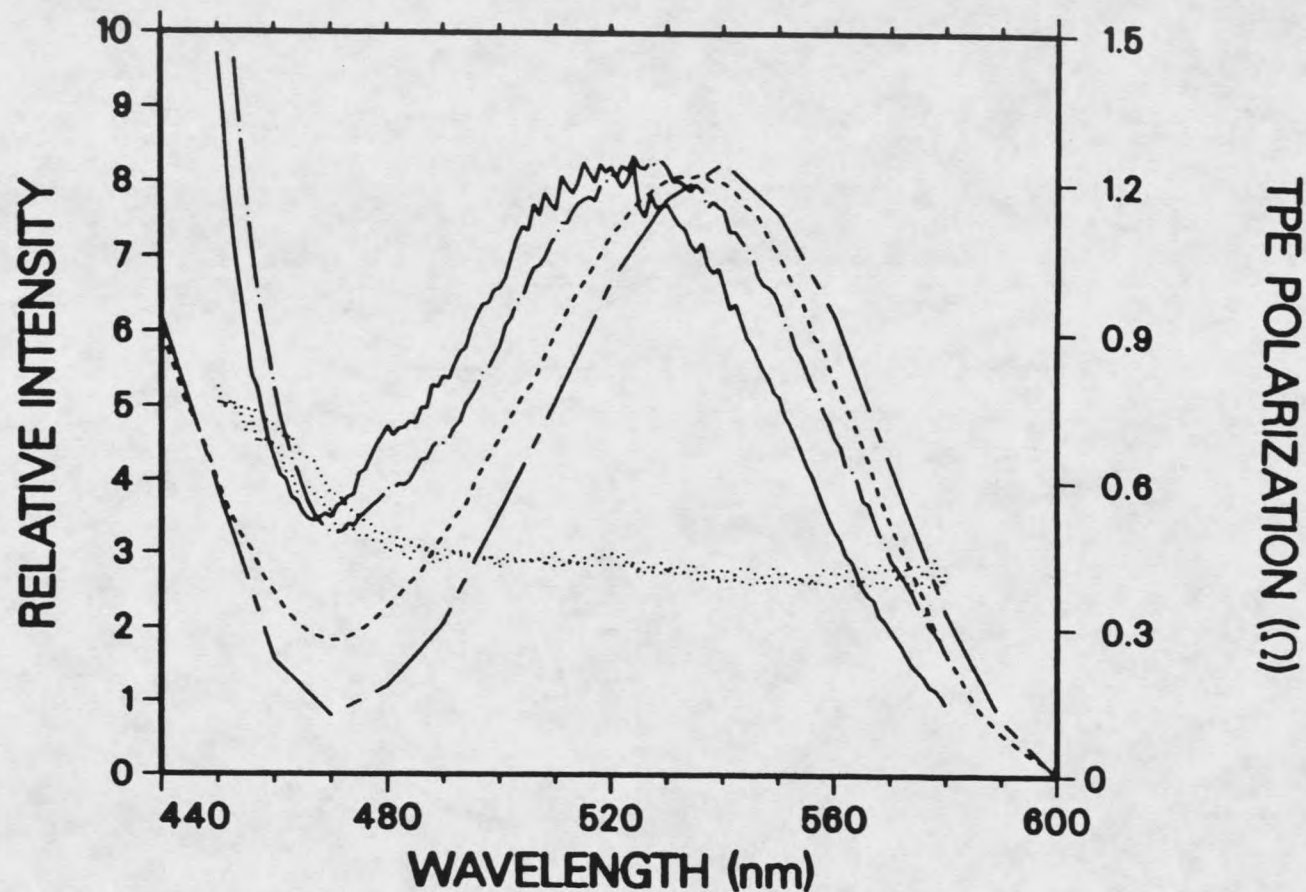


Figure 36. Two-photon fluorescence excitation spectrum and polarization of TMP in 0.05M phosphate buffer, pH7.0 and glycerol. Linear polarized two-photon profile in pH7.0 buffer (—) and glycerol (-·-); One-photon absorption profile in buffer (---) and glycerol (---); two-photon polarization in pH7.0 buffer and glycerol (···)

The effect of the solvent environment on the two-photon signal was pursued as a plausible profile shifting mechanism. To address this concern, several solvent conditions were tested with TMP. Since there is some evidence of fluorescence quenching by anions such as SO_4^{-2} (107), the two-photon spectrum of TMP in water was conducted in conjunction with a buffered sample. As shown in Figure 35, there was direct correspondence between the two conditions in both the profile and the polarization. For TMP in glycerol, Figure 36, a similar blue shift of the two-photon result relative to the one photon absorption spectrum was observed. Therefore, the two-photon profile characteristic of TMP was obtained independent of the type of polar solvent. Similar two-photon spectra were displayed for TMP in cold (5°C) glycerol and ethylene glycol (data not shown). Since the two-photon characteristics intrinsic to the DNA base sample were not necessarily responsible for the observed discrepancies, extrinsic avenues were explored.

Extrinsic Experimental Properties

For a collimated laser beam with a gaussian transverse distribution (TEM_{00}), the focal spot diameter at some distance f from the focussing lens varies directly with wavelength (72). With the focussing lens held at a constant

length from the sample throughout the 400 to 650nm range, the irradiation volume will decrease with decreasing wavelength. Such a focussing mechanism could explain the monotonic increase in δ which would not be compensated by the reference detection assembly. An experiment was designed to test the dependence with and without the focussing lens on the two-photon profile. From measurements on TMP, see Figure 37, three important findings were demonstrated.

The first direct result of the overlap of the two-photon profiles with and without the focussing lens in place suggest that a wavelength dependent focussing mechanism was not responsible for the observed shift relative to the UV absorption band. Second, the correspondence of spectra generated with a one millimeter versus a ten millimeter spot size supported the idea that the incidence of high electric field strengths was not creating spectral distortions. These distortions, in response to high electric field strengths, are usually the result of the fields ability to stabilize ionic over covalent states (108). For laser light of 520nm, for example, the electric field strength felt by the sample under the experimental conditions used for this study would be on the order of 10^5 V/cm in relation to a

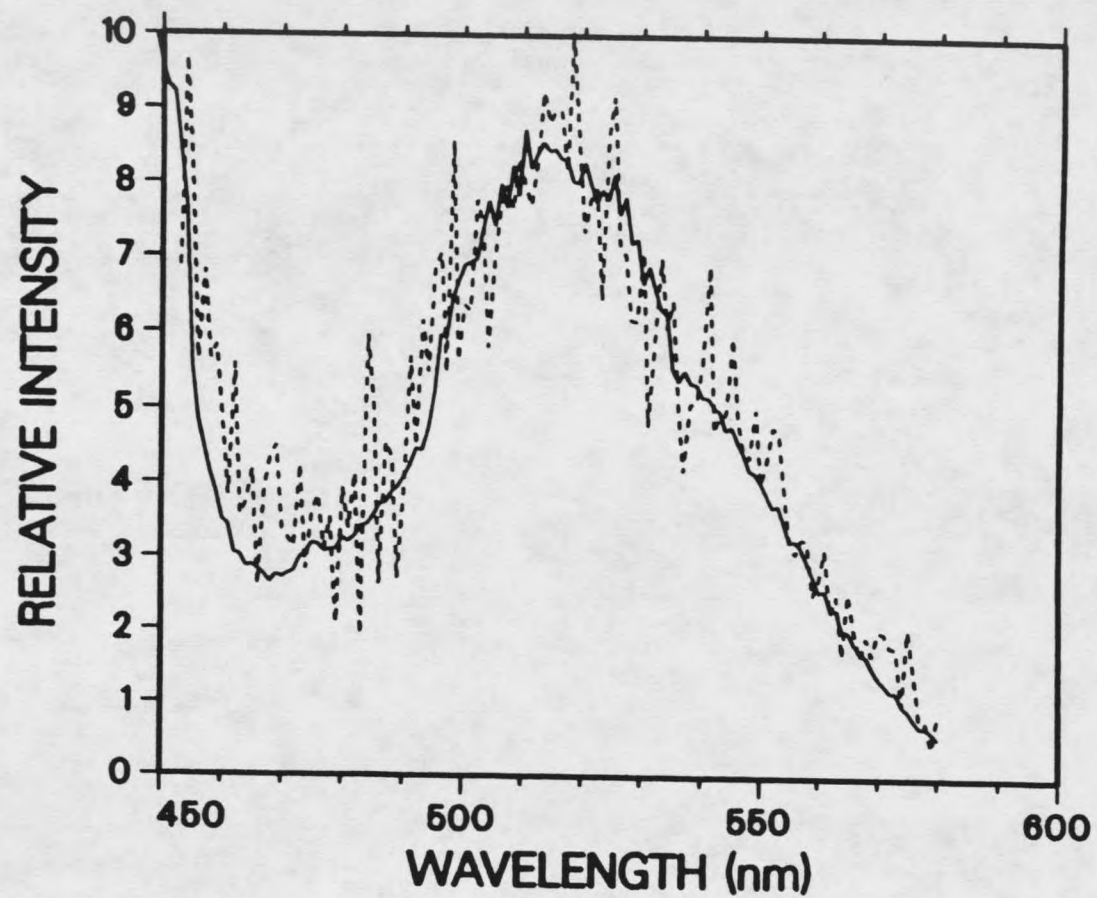


Figure 37. Two-photon fluorescence excitation spectra of TMP with (—) and without (---) a focussing lens

unfocused field strength of about 10^4 V/cm. These electric field strengths are roughly three orders of magnitude less than the field strength required to effect state ordering in linear polyene systems (108). The final result extracted from the focussing lens experiment was that the same two-photon profile and the quadratic behavior to the incident light intensity was retained throughout a range of focal spot sizes for reasons previously addressed in the experimental section.

The reference detection apparatus was also scrutinized since any wavelength dependence in the reference signal could result in the type of distortion observed for each sample. The integrating sphere incorporates magnesium oxide (MgO) as a reflective coating and such coatings may develop wavelength dependent reflection efficiency with age. To test this optical device, two procedures were conducted. First, the integrating sphere was recoated with fresh MgO. Second, the integrating sphere was replaced with a set of Lambertian diffusing disks. As a result, identical profiles were generated in both cases to those previously obtained. Qualitative checks on the integrity of the Rhodamine B quantum counter solution (3g/l) also suggest there did not seem to be a reason to suspect the reference detection

apparatus. Further support for the elimination of the reference detection apparatus as a source of systematic error came in the generation of an identical two-photon shift of TMP by a separate power squared detection procedure outlined in the final section.

The two-photon studies of the DNA bases also presented a new challenge in the construction of a profile which is void of outstanding structural features. For such a broad and diffuse band, the amount of "play" in fitting the segments together increases the risk of introducing errors. Since the profile data seemed to consistently mismatch in the same fashion, the condition that the segments must be joined with equal overlap was questioned. Three samples, TMP, pyrimidine (in cyclohexane) and benzene, were all used in the same two-photon excitation experiment where the profile would be generated with identical instrumental settings. That is, the detection gain and the beam spot size remained constant within each dye segment. In addition, the laser was tuned such that the energies at the dye ends were equivalent. Such a measured precaution was taken to minimize the introduction of a distortion due to a heavily power weighted segment end. Since there is considerable structure in the benzene two-photon spectrum, the correct

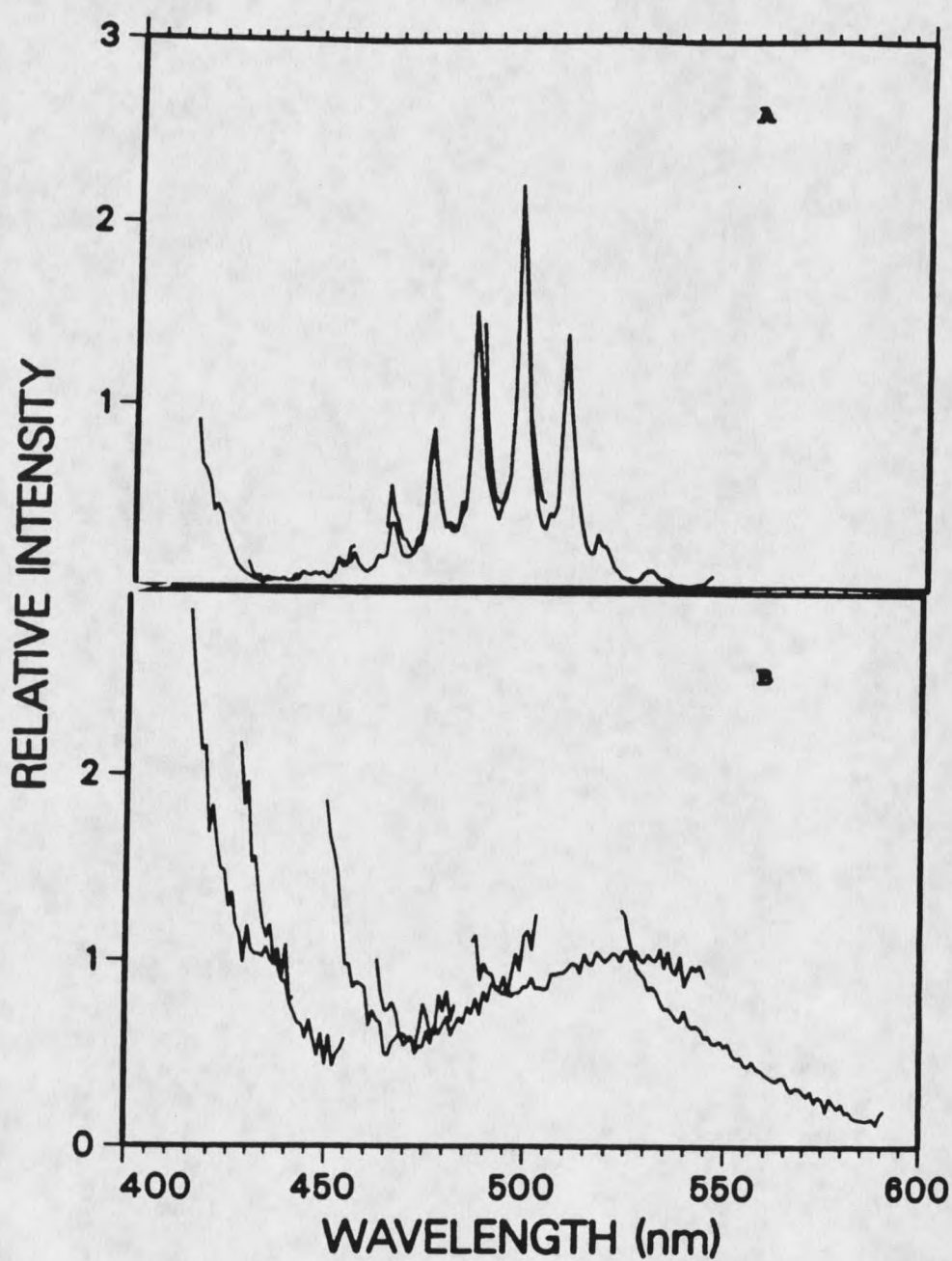


Figure 38. Dye segment overlap procedure.
Panel A, benzene; Panel B, TMP

overlap of these segments would be easier to determine.

With the gain of the detection system held constant, the overlap factors which were used to generate the benzene profile should be identical to those for TMP and pyrimidine. As shown in Figure 38 panel B, the factors used for benzene (panel A) were applied to TMP with the result supporting the previous method of segment overlap for wavelengths longer than 450nm. In panel B, the two-photon profile of TMP displayed the same shift relative to the UV spectra and the absorption cross section remained high with shorter wavelengths. The spectrum of pyrimidine when constructed with the same set of factors revealed a profile similar to the one illustrated in Figure 32. Loss of overlap for wavelengths shorter than 450nm would suggest that perhaps there was a short wavelength weighted distortion inherent in these dyes.

Second Order Coherence. The effects of laser multimode phenomena on the two-photon spectral characteristics are well documented (72,78,109). In fact, the beam mode quality with laser tuning can be followed by monitoring the changes in two-photon intensity (72). The weighted distortions of the two-photon profiles for the DNA bases, if real, may be the result of such multimode effects. Short of buying a

ring or grazing incidence dye laser to operate in single mode, a SHG based power square sensor (78) or a standard such as MSB (109) can be used to correct for the variation in the beam mode quality.

Even in the absence of such correction procedures, a two photon profile can still be constructed with certainty by overlapping the dye segments provided that the second order coherence, a measure of the beam mode quality given by $\langle I^2 \rangle / \langle I \rangle^2$, is wavelength independent. The only requirement to satisfy this condition is that mode quality remain constant within a single dye and not necessarily between dyes. A weighted distortion could be the result of a common trend operating in several segments where there is a higher second order coherence associated with the short wavelength end, for example, in comparison with the other dye end. A SHG power square sensor was used to monitor and correct for changes in the second order coherence.

As shown in Figure 39, the corrected profile for TMP is compared to the spectra generated through a simple overlap procedure. Evidence provided by the direct relationship between the two profiles suggest that the shift was not produced through changes in the mode quality of the laser beam. Any dispersion in the second order coherence creating

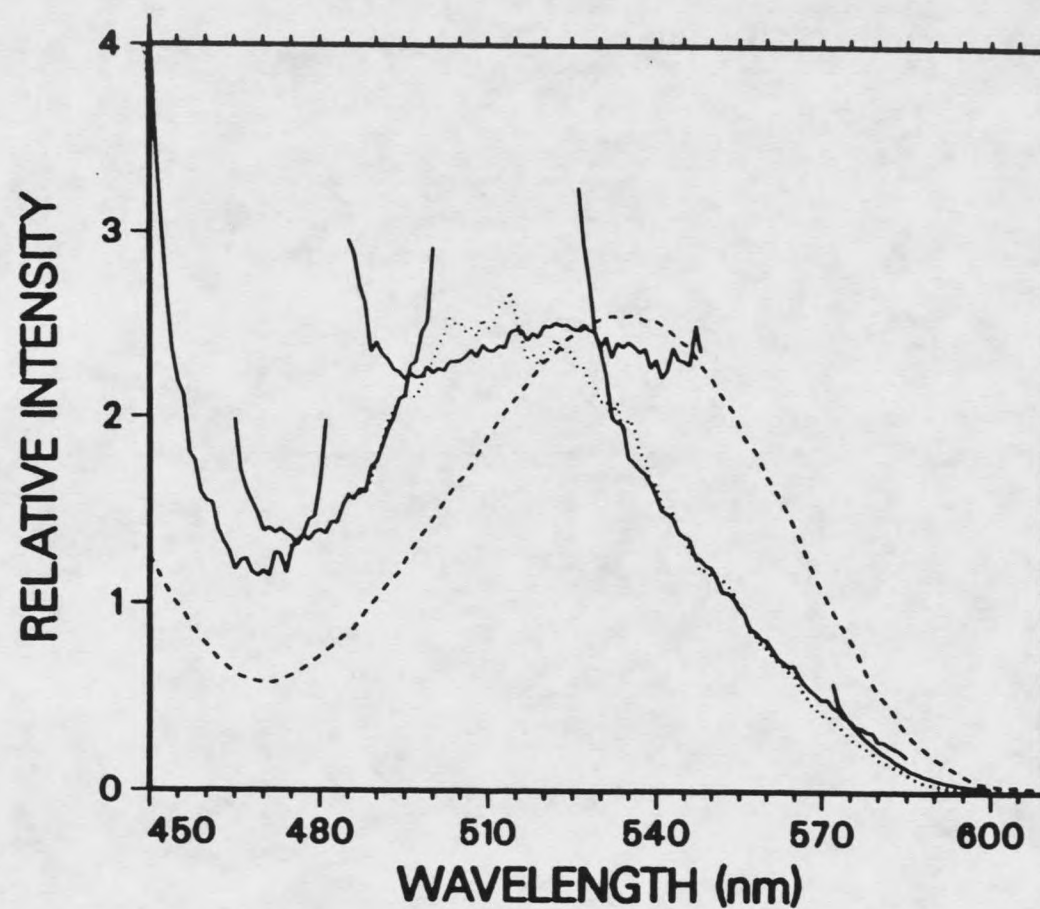


Figure 39. Overlap versus SHG corrected segments for TMP. Overlap of segments (-); corrected SHG segments (•••); One-photon absorption profile (---)

a change in two-photon response should be similarly compensated by the response in SHG since both depend on the square of the laser intensity. This result was further supported by the reproducibility of the two-photon displacement with time and laser realignment. To create the same laser conditions with a cooperative pair of multimode lasers would be improbable given the freedoms associated with such systems.

Since the SHG technique was modified and applied for the first time to correct the two-photon spectra of the DNA bases and selected indoles (110), the temporal and spatial properties of the pulses were measured directly as described in the experimental section. Temporal pulse information obtained using a boxcar integration technique were compared to the pulse profile generated by a single pulse digitizer. The values for the pulse widths and numerically integrated second order coherence were tabulated in Table 4.

Both the boxcar and pulse digitizer values for the second order coherence suggested that there was little variation in the beam mode quality with wavelength. However, this was not the case when the FWHM of each peak was analyzed. The change in the second order coherence can be shown to be inversely proportional to the measure of the

FWHM of each pulse. The smaller the pulse width the larger the value characteristic of the second order coherence. The discrepancy of the FWHM with the second order coherence can be rationalized by some observations made during the data analysis. First, the boxcar operating theory dictates that the pulse profile generated is actually a composite of several pulses. Any transient fluctuations in the second order coherence will be averaged out when applying this technique. In addition, the temporal pulse profiles appeared as though they were conditioned since they tended to bleed off with time at the tail of the curve. This was most likely the result of using a 100 foot coax delay line which contains some internal capacitance. Using the pulse digitizer, the structure and mode dynamics observed were well resolved for each pulse; however, the photographs revealed that each pulse contained a second component with a lifetime much longer than the main component. This second component was probably the detection of amplified spontaneous emission which was not proportionately decreased when attenuating the stimulated component with a polarizer. The presence of this second broad band (in wavelength and time) was evident upon inspecting the spatial photographs. The addition of broad component to the temporal profile will

create a false value of the second order coherence.

Since both measures of the second order coherence were unable to correct the two-photon segments generated for TMP, the FWHM of each pulse obtained by the digitized pulse analyzer was used. As shown in Figure 40, the curling of the two-photon dye segments for TMP were corrected such that direct overlap between segments was achieved. Evidently, the FWHM of each pulse adequately described the second order coherence properties within each dye segment as expected. The large increase in the relative two-photon cross section at shorter wavelengths was also reproduced when using the FWHM correction values (data not shown).

Although there was correspondence demonstrated in Figure 40 between the SHG generated profile and the profile built through correction with the temporal measurements, the slight red shift relative to the SHG result may suggest that the spatial properties of the beam are important in completing the picture surrounding the multimode beam quality. Unfortunately, the photographs obtained through spatial imaging were not sufficiently detailed to account for changes in the spatial distribution. Such information most likely will need to be resolved with the use of a photodiode array with digitization capabilities.

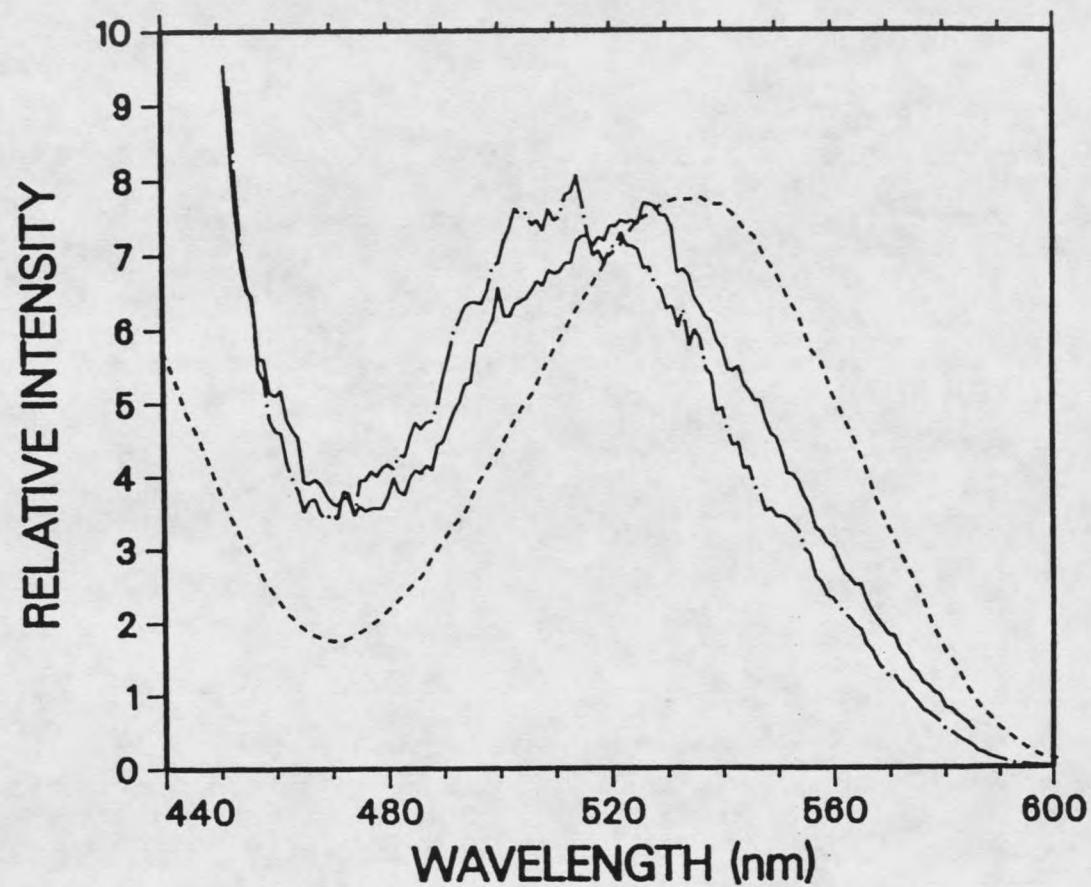


Figure 40. Two-photon fluorescence excitation spectra of TMP corrected using temporal measurements (-) and SHG measurements (-·-); One-photon absorption spectrum of TMP (---)

Presumably, spatial changes in beam quality would have to undergo spectacular changes in distribution to shift the spectra underneath the UV absorption spectrum, but slight changes may correct the temporally generated TMP two-photon spectrum underneath that obtained using a SHG technique.

SUMMARY

Thymine, cytosine and the four nucleotides composing the structure of DNA were studied for the first time using a polarized two-photon fluorescence excitation technique in aqueous solution. The accomplishment of obtaining these spectra destroys the conventional wisdom that only molecules with large fluorescence quantum yields can be investigated using this method. Consequently, this work on molecules with Φ_f equal to 10^{-4} may encourage the study of other molecules which were previously believed to be unsuitable for study. In addition, the foundation for studying the DNA macromolecule has been established for both the two-photon fluorescence excitation and emission spectroscopies.

As predicted by theory, all of the DNA bases were found to have at least an order of magnitude larger two-photon cross section than benzene. In addition, the polarization ratios that were obtained followed a pattern which represented the signature of the particular nucleotide under investigation. Although the trends in polarization followed the theoretically predicted values, the two-photon profile

information did not yield as much information as would have been hoped. With each nucleotide, the presence of a large increase in the two-photon cross section with shorter wavelength seems to dominate the spectra. Such a domination could be the result of a Rydberg state(s) which would not be predicted with the use of valence π semiempirical methods. Alternatively, the theory may fail to predict a strong two-photon cross section for transitions residing at these wavelengths. Only for the purines, did the theory suggest a cause for the increase in two-photon absorption strength. Investigations into finding systematic errors suggest that the two-photon result reflects intrinsic properties of the molecule at this stage. Some questions concerning the previously published two-photon spectrum of benzene have also been raised as a result of this work.

The relative trends in two-photon polarization for each base followed relatively close to the theoretically predicted changes; however, the experimental values were consistently lower. The low polarization in the case of TMP can be shown to be a result of photoselection where the value of Ω depends on both the tensor and the fluorescence transition moment direction. Since they all are characterized by anisotropic fluorescence emission, this

mechanism should be examined for the other bases.

Semiempirical methods provides calculated values of Ω assuming that the molecule is an isotropic emitter.

Consequently, the discrepancy between theory and experiment presumably arises in part from the anisotropic emission of the nucleic acid bases.

The two-photon polarization across the first absorption band of TMP harmonized with previous results in revealing only one transition. The two-photon polarization measurements were also instrumental in resolving the controversial third (220nm) band in TMP which remains hidden using one-photon methods. Two-photon polarization values of CMP also supported the four excited state picture of that nucleotide in direct contradiction with theory.

In accord with the two-photon results, the one-photon fluorescence anisotropy results of this work for thymine and uracil have confirmed the single state picture for these molecules in the region corresponding to the first absorption band. The dependency of the fluorescence anisotropy with wavelength has been shown to be pH dependent. By calculating the contribution due to the small average equilibrium concentration of the thymine anion at neutral pH, the drop in fluorescence anisotropy with

wavelength can be attributed to the presence of this impurity and not by the presence of another state.

Although these molecules do not possess the quantum yields desirable when using fluorescence as a detection means, the short radiative lifetimes more than make up the difference since such properties allow for the use of photoselection methods as a probe under conditions which are biologically meaningful. The use of new techniques, such as the two-photon work presented herein, can take advantage of these and other properties to clarify the electronic picture surrounding the molecule containing the genetic code.

LITERATURE CITED

1. Adams, R.L.P., et. al., "The Biochemistry of the Nucleic Acids", Champion and Hall, NY, (1981).
2. Watson, J., and Crick, F.H.C., Nature, 171, 737, (1953).
3. Stryer, L., "Biochemistry," 2nd ed., W.H. Freeman and Co., San Francisco, CA, (1981).
4. Zhen, W., et. al., Photochem. Photobiol., 48:4, 477, (1988).
5. Tyrell, R.M., Mutation Res., 129, 103, (1984).
6. Green, A.E.S. and Chai, S.T., Photochem. Photobiol., 48:4, 477, (1988).
7. Setlow, R.B., Proc. Nat. Acad. Sci., 71:9, 3363, (1974).
8. Miles, D.W. et. al., Proc. Nat. Acad. Sci., 62, 22, (1969).
9. Voelter, W. et. al., J. Amer. Chem. Soc., 90:22, 6163, (1968).
10. Sprecher, C.A. and Johnson, W.C., Biopolymers, 16, 2243, (1977).
11. Callis, P.R., Ann. Rev. Phys. Chem., 34, 329, (1983).
12. Brown, I.H. and Johns, H.E., Photochem. Photobiol., 8, 273, (1969).
13. Rhoades, D.F. and Wang, S.Y., Biochemistry, 9:22, 4416, (1970).

14. Brosh, D.E. and Haseltine, W.A., *Nature*, 298, 189, (1982).
15. Innes, K.K., Ross, I.G. and Byrne, J.P., *J. Molec. Spect.*, 22, 125, (1967).
16. Hug, W. and Tinoco, I., *J. Amer. Chem. Soc.*, 95:9, 2803, (1973).
17. Daniels, M., *Proc. Nat. Acad. Sci.*, 69:9, 2488, (1972).
18. Hauswirth, W. and Daniels, M., *Photochem. Photobiol.*, 13, 157, (1971).
19. Callis, P.R., *Chem. Phys. Lett.*, 61:3, 563, (1979).
20. Vigny, P. and Ballini, J.P. In "Excited States of in Organic Chemistry and Biochemistry", Pullman, B. and Goldblum, N., Eds.; Dordrecht and Holland, 1977.
21. Daniels, M. and Hauswirth, W., *Science*, 171, 675, (1971).
22. Eisinger, J., *Photochem. Photobiol.*, 7, 597, (1969).
23. Longworth, J.W., Rahn, R.O., and Shulman, R.G., *J. Chem. Phys.*, 45:8, 2930, (1966).
24. Sober, H.A., Ed. "Handbook of Biochemistry"; CRC: Cleveland, 1970.
25. Strickler, S.J. and Berg, R.A., *J. Chem. Phys.*, 37:4, 814, (1962).
26. Oraevsky, A.A., et. al., *Chem. Phys. Lett.*, 83:2, 276, (1981).
27. McClain, W.M. and Harris, R.A. In "Excited States", E.C. Lim, Ed.; Academic Press, New York, 1977.
28. Williams, S.A., Renn, C.N. and Callis, P.R., *J. Phys. Chem.*, 91, 2730, (1987).
29. Wilson, R.W. and Callis, P.R., *J. Phys. Chem.*, 80, 2280, (1976).

30. Becker, R.S. and Kogan, G., Photochem. Photobiol., 31, 5, (1980).
31. Brody, B.B., et. al., Chem. Phys. Lett., 147, 538, (1988).
32. Danilov, V.I. and Pechenaya, V.I., Int. J. Quant. Chem., 17, 307, (1980).
33. Snyder, L.C., et. al., J. Chem. Phys., 53:1, 256, (1970).
34. Robey, M.J. and Ross, I.G., Photochem. Photobiol., 21, 363, (1975).
35. Chen, H.H. and Clark, L.B., J. Chem. Phys., 51:5, 1862, (1969).
36. Callis, P.R., Scott, T.W. and Albrecht, A.C., J. Chem. Phys., 75:12, 5640, (1981).
37. Sundbom, M., Acta. Chemica. Scand., 25, 487, (1971).
38. Drobnik, J., Kleinwachter, V. and Augenstein, L., Photochem. Photobiol., 6, 147, (1967).
39. Smith, J.J., Spectrochem. Acta., 33A, 135, (1977).
40. Stewart, R. and Davidson, N., J. Chem. Phys., 39:2, 255, (1963).
41. Ingwall, J.S., J. Amer. Chem. Soc., 94:15, 5487, (1972).
42. Clark, L.B., J. Amer. Chem. Soc., 99:12, 3934, (1977).
43. Matsuoka, Y. and Norden, B., J. Phys. Chem., 86, 1378, (1982).
44. Fucaloro, A.F. and Forester, L.S., J. Amer. Chem. Soc., 93:24, 6443, (1971).
45. Callis, P.R., Rosa, E.J. and Simpson, W.T., J. Amer. Chem. Soc., 86, 2292, (1964).
46. Eaton, W.A. and Lewis, T.P., J. Chem. Phys., 53:6, 2146, (1970).

47. Novros, J.S. and Clark, L.B., J. Phys. Chem., 90, 5666, (1986).
48. Morgan, J.P. and Daniels, M., Chem. Phys. Lett., 67, 533, (1979).
49. Amex, B.G., et. al., J. Phys. Chem., 79:24, 2636, 2639, (1975).
50. Zielinski, T.J. and Rein, R., Int. J. Quant. Chem., 14, 851, (1978).
51. Czerminski, R., et. al., Int. J. Quant. Chem., 16, 605, (1979).
52. Scanlan, M.J. and Hillier, I.H., Chem. Phys. Lett., 98:6, 545, (1983).
53. Zielinski, T.J., Int. J. Quant. Chem., 22, 639, (1982).
54. Kwiatkowski, J.S., Person, W.B. and Szczepan, K., Acta Biochemica Polonica, 34:2, 165, (1987).
55. Johnson, W.C., Biopolymers, 10, 923, (1977).
56. Eastman, J., Ber. Bunsenges Phys. Chem., 73, 407, (1969).
57. Dreyfus, M., et. al., J. Amer. Chem. Soc., 97:9, 2369, (1975).
58. Anderson, B. and Callis P., Photochem. Photobiol., 32, 1, (1980).
59. Knighton, W.B., Giskaas, G.O. and Callis, P.R., J. Phys. Chem., 86, 49, (1982).
60. Knighton, W.B., M.S. Thesis, Montana State University, (1980).
61. Seibold, K. and Labhart, H., Biopolymers, 10, 2063, (1971).
62. Turpin, P. and Peticolas, W., J. Phys. Chem., 89:24, 5156, (1985).

63. Morgan, J. and Daniels, M., J. Phys. Chem., 86, 4004, (1982).
64. Fujii, M., et. al., Chem. Phys. Lett., 126:6, 583, (1986).
65. Callis, P.R., Photochem. Photobiol., 44:3, 315, (1986).
66. Mishra, P.C., Ind. J. Biochem. Biophys., 14, 214, (1977).
67. Gill, J.E., J. Molec. Spect., 27, 539, (1968).
68. Goppert-Mayer, M., Ann. Phys. (Leipzig), 9, 273, (1931).
69. Tam, A.C. and Patel, C.K.N., Nature, 280, 304, (1979).
70. Twarowski, A.J. and Klinger, D.S., Chem. Phys., 20, 259, (1977).
71. Scott, T.W., Callis, P.R. and Albrecht, A.C., Chem. Phys. Lett., 93, 111, (1982).
72. Birge, R.R. In "Ultrasensitive Laser Spectroscopy", Lim, E.C., Ed., Academic Press, Inc., 109, (1983).
73. Koch, T.H. and Von Trebra, R.J., U.S. Patent 4,428,859 (1984).
74. Koch, T.H., personal communication, (1987).
75. Dick, B., Gonska, H. and Hohlneicher, G., Ber. Bernsenges. Phys. Chem., 85, 746, (1984).
76. Hecht, E. abd Zajac, A., Optics, Reading, Mass., Addison-Wesley Publishing, Co., (1974).
77. Kuntz, S.K. and Perry, T.T., J. Appl. Phys., 39, 3798, (1968).
78. Jones, R. and Callis P., J. Appl. Phys., 64, 4301, (1988).

79. Halbout, J., et. al., IEEE J. Quant. Elect., QE-15:10, 1176, (1979).
80. Weber, G. and Teale, F., Trans. Faraday Soc., 54, 640, (1957).
81. Anderson, B., Ph.D. Thesis, Montana State University, (1983).
82. Birks, J.B., Photophysics of Aromatic Molecules, New York, Wiley-Interscience, (1970).
83. El-Sayed, M.A., J. Chem. Phys., 38:12, 2834, (1963).
84. Hercules, D.M., Fluorescence and Phosphorescence Analysis: Principles and Application, New York, Wiley-Interscience, (1966).
85. Jablonski, A., Bull. DE L'Academie, Pol. Des. Sci., 8, 259, (1960).
86. Peticolas, W., Ann. Rev. Phys. Chem., 18, 223, (1967).
87. McClain, W.M., J. Chem. Phys., 55, 2789, (1971).
88. McClain, W.M., J. Chem. Phys., 57, 2264, (1972).
89. Dick, B. and Hohlneicher, G., J. Chem. Phys., 70, 5427, (1979).
90. Wilson, R.W., Morgan, J.P. and Callis, P.R., Chem. Phys. Lett., 36, 618, (1975).
91. Kakitani, T. and Kikitani, H., J. Phys. Soc. Jpn., 42, 1287, (1977).
92. Morgan, J.P. and Daniels, M., Photochem. Photobiol., 27, 73, (1978).
93. Matos, J.M. and Roos, B.O., J. Amer. Chem. Soc., 110, 7664, (1988).
94. Raksanyi, K., Biopolymers, 17, 887, (1978).
95. Kuprievich, V.A., Int. J. Quant. Chem., 16, 833, (1979).

96. Zaloudek, F., et. al., J. Amer. Chem. Soc., 107:25, 7344, (1985).
97. Clark, L.B., J. Phys. Chem., in press, (1989).
98. Clark, L.B., J. Amer. Chem. Soc., 99:12, 3934, (1977).
99. Callis, P.R., personal communication, 1989.
100. Goodman, L. and Rava, R., Accts. Chem. Res., 17, 250, (1984).
101. Goodman, L. and Rava R. In Advances in Chemical Physics, vol. 54, Prigogine I. and Rice S.A., Eds., John Wiley and Sons, Inc., 1983.
102. Cable, J.R. and Albrecht, A.C., J. Chem. Phys., 85, 3155, (1986).
103. Baba, H., Goodman, L. and Valenti, P., J. Amer. Chem. Soc., 88:23, 5410, (1966).
104. Cohen, B.J. and Goodman, L., J. Amer. Chem. Soc., 87, 5487, (1965).
105. Clark, L.B. and Tinoco, I., J. Amer. Chem. Soc., 87, 11, (1965).
106. Scott, T.W. and Albrecht, A.C., J. Chem. Phys., 74, 3807, (1981).
107. Borresen, H.C., Acta. Chim. Scand., 17, 921, (1963).
108. Birge, R.R. and Pierce, B.M., J. Chem. Phys., 70, 165, (1979).
109. Johnson, M.C. and Lytle, F.E., J. Appl. Phys., 51, 2445, (1980).
110. Rehms, A.A., personal communication, 1989.

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