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

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 (δcir/δlin) was low and constant for the 26Onm region of thymine and TMP, but exhibited a maximum at 220nm, therefore, confirming the singular nature of the lowest ππ* band and the composite nature of the 205nm band. The data for CMP is consistent with either three or four ππ* 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 (ΔR□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 (pKa=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.

POLARIZED ONE- AND TWO-PHOTON FLUORESCENCE EXCITATION SPECTROSCOPY ON SELECTED NUCLEIC ACID BASES

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A thesis submitted in partial fulfillment
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APPROVAL

of a thesis submitted by

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.

November 2, 1989
Date

Chairperson, Graduate Committee

Approved for the Major Department

November 1, 1989
Date

Head, Major Department

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Signature Scott D. Williams

Date 8/8/89

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

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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.

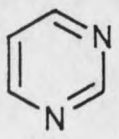
For the first time, two-photon fluorescence excitation spectroscopy has been applied to the monomeric bases and nucleotides of deoxyribonucleic acid (DNA). A new and detailed one-photon photoselection study of thymine and uracil is also part of this work. With the complete elucidation of the electronic 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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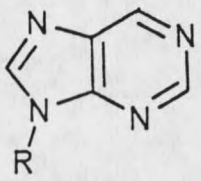
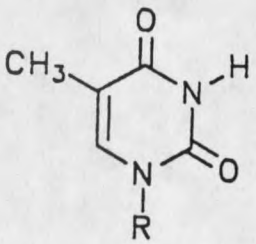


II

R= H, DEOXYRIBOSE



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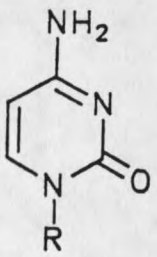

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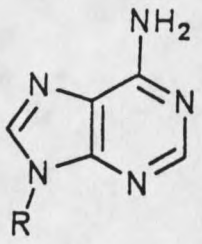

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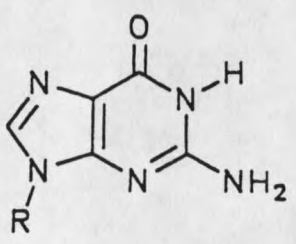

VI

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 guanidine (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 quadrapole) 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 $M^{-1}cm^{-1}$ (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, $\tau_f \cong 1$ 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	Φ_f (x10^4)[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, pH≅5 (19). From a high and constant fluorescence anisotropy (R≅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 pKa 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 ($\Phi_f \cong$ 1.0) were used when monitoring with a fluorescence detection scheme. In fact, as late as 1979, the two-photon absorption spectrum of benzene (Φ_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 ($\Phi_f \cong 0.003$) 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, semiemperical 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} cm^4 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),

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

where N_x is the number of photons, C is the concentration in units of molecules cm^3, I_0 is the intensity (photons sec^{-1} 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 steridians. 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 Hammamatsu 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 $3X10^{-8}$ coulombs. If this charge is collected over 0.2 milliseconds with an integration time constant of 100μs, 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 halflife (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

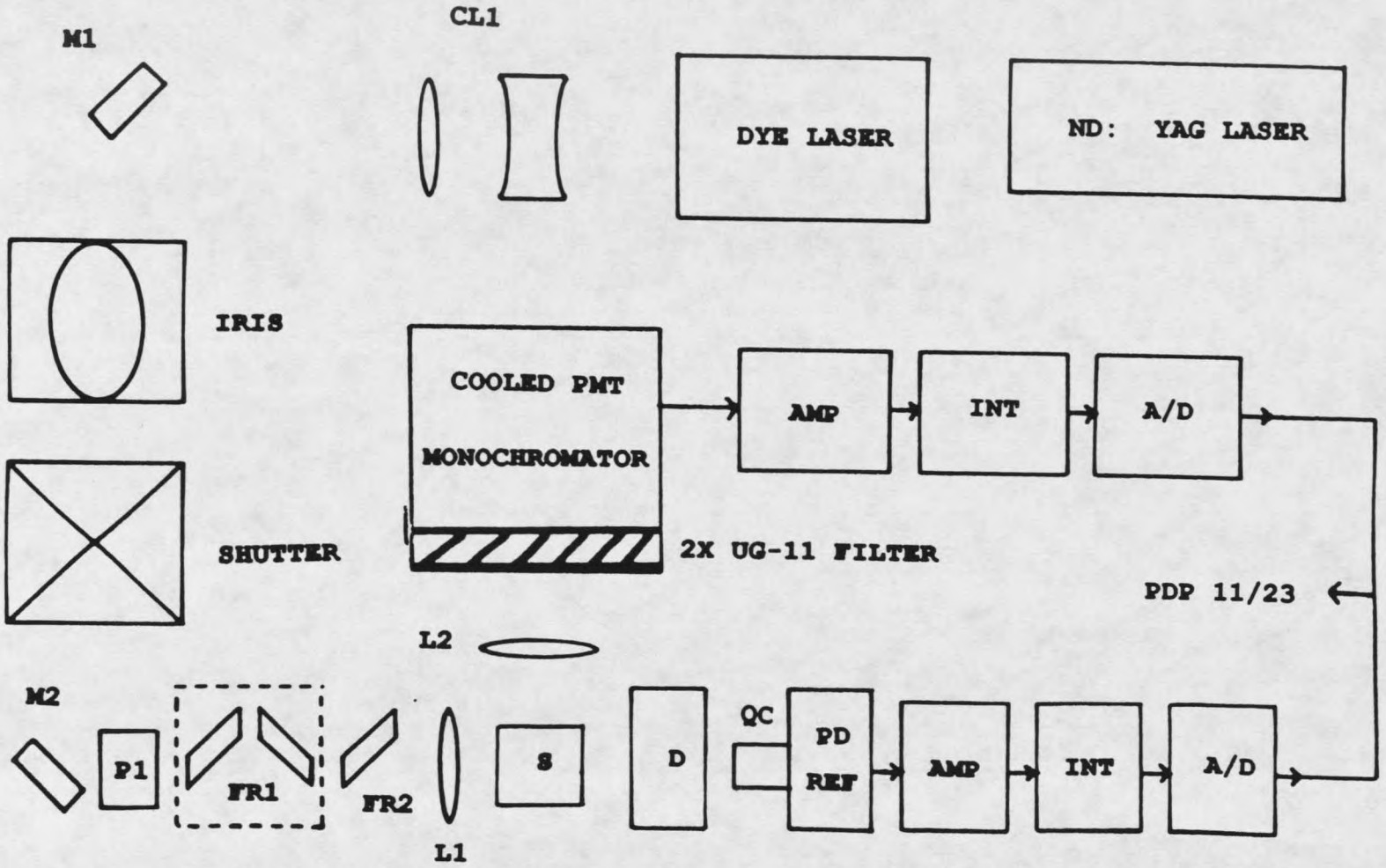

Figure 1. Polarized two photon fluorescence excitation apparatus