The room temperature fluorescence of DNA
by Timothy Isamu Aoki

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

Abstract:
The fluorescence of native DNA was observed at room temperature in neutral aqueous solutions. The fluorescence appears to be mostly from the individual nucleotides, and is reduced in intensity by a factor of 2 relative to the emission of an equimolar mixture of nucleotides. A minor component of the emission appears at wavelengths above 400 ran. This component is thought to arise from the singlet manifold of excited state complexes (exciplexes), but other possibilities such as highly fluorescent impurities or minor components of the DNA itself are not rigorously excluded. Reliable quantitative results for the quantum yields, fluorescence spectra and fluorescence excitation spectra required careful purification of commercial DNA preparations. Changes in the fluorescence characteristics are noted with extremes of temperature and pH. The changes observed strongly support the authenticity of observations at neutral pH and ambient temperature. Excited state interaction between bases in the double helical polymer appear to be much less extensive under these conditions than at 80°K.

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ABSTRACT

The fluorescence of native DNA was observed at room temperature in neutral aqueous solutions. The fluorescence appears to be mostly from the individual nucleotides, and is reduced in intensity by a factor of 2 relative to the emission of an equimolar mixture of nucleotides. A minor component of the emission appears at wavelengths above 400 nm. This component is thought to arise from the singlet manifold of excited state complexes (exciplexes), but other possibilities such as highly flourescent impurities or minor components of the DNA itself are not rigorously excluded. Reliable quantitative results for the quantum yields, fluorescence spectra and fluorescence excitation spectra required careful purification of commercial DNA preparations. Changes in the fluorescence characteristics are noted with extremes of temperature and pH. The changes observed strongly support the authenticity of observations at neutral pH and ambient temperature. Excited state interaction between bases in the double helical polymer appear to be much less extensive under these conditions than at 80°K.

INTRODUCTION

Low Temperature

The luminescence of DNA and its components was first well characterized at 80°K by Eisinger and coworkers at Bell Labs in 1966 (1). At room temperature, the fluorescence from the nucleotides is 2 to 3 orders of magnitude less intense, and it was not until the early 1970's that it could be measured at all (2,3). The early reports contained significant errors that were later corrected (4). There is a large amount of data obtained from polymers and monomers at low temperature in alcohol/water glasses, and extremes of pH, but the applicability of the data to phenomena occurring under physiological conditions of temperature and solvent has often been questioned.

Skepticism appears to be justified. As more data is collected at room temperature, it is becoming clear that the two conditions of temperature often lead to quite dissimilar results. For example, the fluorescence (Φ_f) of nucleotides and DNA bases at low temperature is seen to decrease with excess excitation energy (5,6). At room temperature this occurs only for purine bases and not for the nucleotides and pyrimidine bases. This is because of the presence of highly fluorescent tautomers that occur only in the purines (7).

While there is detailed understanding of specific cases such as adenine, there are few meaningful generalizations that can be made regarding the relationship between the low and room temperature results. The photophysics of the monomeric components of DNA is an extensive and intriguing area in and of itself, but what follows here will refer only to aspects which appear to be of quite direct consequence to the observed luminescence of the biological polymer at room temperature.

The fluorescence of polymeric DNA at 80°K was seen to be somewhat broader, than that from an equimolar mixture of nucleotides, and the wavelength of the fluorescence maximum at about 360 nm was about 35 nm to the red of the nucleotides. The polymer fluorescence has been explained as originating in excited state complexes (exciplexes) as was first demonstrated by Forster and Kasper (8). In their study of the fluorescence of pyrene, they had observed the appearance of broad, unstructured and red shifted emission as the concentration of fluorophore was increased. Absorption and freezing point depression data showed that the fluorescing complex was formed in the collision of an excited pyrene molecule with one in its ground state. The approximately 5000 cm^{-1} stabilization of the excited complex is the result of electronic interactions which cause an energy minimum to be reached at a unique equilibrium intermolecular in distance in the

excited state.

Formation of excited state dimers (excimers) of pyrene and other aromatic hydrocarbons depends on diffusion of the electronically excited molecule during its excited state and on the occurrence of the electronic interactions upon collision. Diffusion is not possible in rigid glasses at 80°K for the individual nucleotides, but for DNA, dinucleotides, and polynucleotides, the neighboring bases are held at sufficiently close distances for the exciplexes to form. The formation of exciplexes does not occur in all dinucleotides or polymers at 80°K (or at room temperature). Furthermore, the formation of excimers in rigid environments (as well as fluid) appears to be sensitive to the degree of stacking between neighboring base pairs and hydrogen bonding between complementary Watson and Crick bases. For example, the fluorescence of most dinucleotides containing adenosine is clearly from exciplexes at 80°K, whereas the fluorescence of the dinucleotide TpT shows both monomer and excimer-like fluorescence. Partially protonated CpC and poly C at pH 5 and poly d(AT) which form double stranded helical structures have fluorescence which are strongly red shifted (1). The difference between the fluorescence spectra of poly rA and poly dA, the latter showing a much larger red shift, indicates how subtle changes in the relative geometry of the neighboring bases can substantially alter the emission.

A second dramatic difference between the DNA polymer and mononucleotide luminescence noted at 80°K was the 10 fold reduction of intensity of the polymer spectrum relative to the monomer. Heat denaturation caused the DNA polymer emission to increase by a factor of 2 under these conditions. A later report indicated that the increase in intensity varied from a factor of 2 to 7 depending on concentration and method of denaturation (10).

The fluorescence (of DNA) at 77°K is thought to originate only from adenosines and thymidines. This was concluded on the basis of the similarity of the spectrum of DNA to that of poly AT, and the 10 fold decrease of fluorescence from poly G:C and poly C:G complexes relative to mixtures of the free mononucleotides. Presumably the decrease in the poly G and poly C complexes and in the native DNA is due to the formation of Watson and Crick type hydrogen bonds between G and C.

In another report from the Bell Labs group (10), the triplet state and phosphorescence of DNA was examined. As was found with the fluorescence, the phosphorescence was decreased by a factor of about 10 in the native polymer in comparison to a mixture of nucleotides. The decrease was much less in denatured samples. In part this was explained by the quenching of emission from guanosine-cytidine base pairs at the singlet level before intersystem crossing could occur.

The phosphorescence (as well as the fluorescence) was seen to resemble that from poly dAT. It was further concluded that thymidine anions were almost solely responsible for the phosphorescence. This was because the absence of election spin resonances (ESR) corresponding to adenosine in both poly dAT and DNA, and the disappearance of the adenosine ESR signal upon protonation. This suggested that the adenosine triplet is quenched by proton transfered from excited thymidine in the polymers. Also it was not possible to directly populate the triplet of neutral thymidine by UV light absorption.

The conclusion was later revised when it was found that the triplet state of thymidine monophosphate (TMP) could be populated by triplet energy transfer from acetone or acetophenone (11). The ESR of (TMP) sensitized in this way resembled the polymer's signals more closely than that from the (TMP) anion. The low intersystem crossing rate of neutral TMP and thymine prevents direct population of the triplet states in dilute solutions of the mononucleotide or base. Incorporation in the polymer apparently facilitates triplet transfer from neighboring purines which have higher intersystem crossing rates.

A somewhat conflicting report had been published previously by Bershon and Eisenberg (12). In it the phosphorescence of DNA had been attributed to emission from the purines. The purine mononucleotides phosphorescence yields were greater, and the spectra they observed

from DNA displayed some of the same vibronic structure seen in the monomeric purine spectra. They used a solvent 95:5, glycerol:water which is known to cause denaturation, and excitation light of shorter wavelengths than was used later by the Bell Labs group. Both factors would tend to enhance the emission from purines.

The conflict concerning the moieties responsible for phosphorescence was clarified later by Imakubo (13) studying the emission wavelength dependence of the 2 decay components seen in phosphorescence measurements. He found the long lived (~2.0 sec) component which is similar to the phosphorescence lifetime of the purines, comprises about 70% of the emission at 405 nm with wavelength of excitation 280 nm. The short lived component (~.3 sec) which is similar to the phosphorescence lifetime of TMP comprises about 70% of the emission at 465 nm, with exictation at 265 nm. Since the quantum yield for phosphorescence from thymine is an order of magnitude less than that for adenosine, this confirms the conclusion of the Bell Labs group that about 90% of the triplet state of DNA at 80°K resides in thymine. The structured emission seen by Bershon and Eisenberg is more correctly interpreted.

Room Temperature

Owing to the dramatic decrease in luminescence intensity in going from 80°K to 300°K, experimental data which is directly related to DNA

fluorescence at room temperature is scarce. The most relevant are the luminescence studies of dinucleotides and polynucleotides. The dimers and polymers have been broadly classified into 2 important groups by Vigny (14): ones which form excimers and those which do not. ApA, poly A, and partially protonated poly C, poly d(AT) and poly G-poly dC fall in the former grouping. Their spectra at room temperature are dominated by an intense peak at about 400 nm. Vigny and coworkers (4,14,15), who have done the most extensive studies, attribute the peak to excimer fluorescence. Off hand, this seems somewhat questionable. The shift in the wavelength maximum from 315 nm for AMP to about 400 nm in poly rA is about 6400 cm^{-1}. This is a 6 times greater lowering in energy than seen in the excimer fluorescences at 80°K for the same compounds. For poly C (pH 5) the room temperature excimer stabilization is about 5300 cm^{-1} and 2500 cm at 80°K. However, provided that the excimer is long lived, its energy could be significantly lowered by reorientation of the emitting bases or solvent molecules in the fluid media. Solvent relaxation has been invoked to explain the red shift of 4500 cm^{-1} in the fluorescence maximum of protonated adenine that occurs as the temperature is raised from 80°K to ~300°K (16).

Morgan and Daniels have speculated, that the origin of the broad, redshifted emission in poly A, ApA, poly C and CpC is due partly to

phosphorescence from both independent monomers and excimers (17a,b,c). The proposed phosphorescences arise from decomposing experimental curves into four components and assigning them to triplet and singlet states of monomers and excimers. A two state stacking model gives monomer-like emissions (fluorescence and phosphorescence) from unstacked bases and red shifted emissions from stacked ones. In this scheme phosphorescence is required from both the stacked and unstacked bases, the emission of the stacked bases being about 2 times that from the unstacked.

This does not seem unreasonable as phosphorescence at room temperature has been reported elsewhere. Kalyanasundaram et al. (18) reported phosphorescence spectra from aromatic hydrocarbons trapped in micelles which were identical to spectra observed at 80°K. Phosphorescence from intramolecular excimers of di-naphthyl alkanes was reported by Okajima, Subudhi and Lim (19). The excimer phosphorescence is red shifted about 1000 cm^{-1} to the red from the normal phosphorescence observed at 80°K. However, with closer inspection of the related studies and the known excited state parameters of the DNA monomers at room temperature, it becomes apparent that the scheme described by Daniels is difficult to support. The difficulty lies more in the details of the scheme than in the probability of excimer phosphorescence. A simple kinetic argument

concerning this will be presented in the discussion of the DNA fluorescence seen at wavelengths ≥400 nm.

DNA (At Room Temperature)

There have been three previous reports concerning the room temperature fluorescence of DNA (14,20,21). All reports agree (probably fortuitously) on the quantum yield (Φ_f) of the native polymer under these conditions being $3\pm1 \times 10^{-5}$, but significant differences are noted with regards to the fluorescence spectrum and its interpretation.

The report by Daniels (20) and the one by Anders (21) both give the fluorescence maximum at about 350 nm. The spectra are also quite broad being ca. 100 nm full width at half maximum (fwhm). The origin of the fluorescences in exciplexes similar to those seen at 80°K is implied as the spectra are strongly red shifted and even more broadened than those at low temperatures. The third report, by Vigny and Ballini (14), gives the fluorescence of DNA polymer as being like that from the individual nucleotides with a wavelength of the fluorescence maximum at ca. 330 nm and ca. 70 nm fwhm. Their excitation spectrum indicates emission from independent nucleotides.

The reasons for the disagreement are not clear as the reports indicating exciplex-like fluorescence appear quite incomplete. Both

are based on one type of DNA (calf thymus) which was apparently used as received from vendors. Though pH's were reported, no indication of buffering material or its concentration were noted. No excitation spectra were given. Besides the questionable data and experimental methods, their interpretation is clearly in error. The quantum yield for DNA is compared to the values of ca. 10^{-6} given for the nucleotides by Vigny in 1971 (2a,b). These were later corrected by Vigny 1976 (4) and are in the range of 10^{-5} - 10^{-4}. From this, Anders and Daniels were led to speculation about the enhancement of fluorescence in DNA relative to the mononucleotides at room temperature.

In contrast, the report by Vigny and Ballini (14) was done on DNA obtained from a variety of vendors and biological sources. The solutions were well buffered and of a slightly higher DNA concentration (10^{-3} M in nucleotides). The quantum yield of 3×10^{-3} was obtained only after careful purification of DNA isolated from mouse skin. Quantum yields from materials which were purified only by dialysis gave Φ_f of $7\text{-}10 \times 10^{-5}$. It would appear then, that the agreement on quantum yields reported is fortuitous.

The report by Vigny and Ballini (14) appeared in a book that was brought to my attention as this work to be reported here was in progress. In the pages that will follow, the fluorescence spectrum in neutral solution, excitation spectrum for fluorescence at 325 nm, and

quantum yield for the most purified samples of DNA are in agreement with their report. Discussion of emission at wavelength $\geq$400 nm was not included in the report by Vigny and Ballini and will be discussed in some detail here for the first time. Also, examination of changes in the fluorescence with heat denaturation and titration to extremes of pH are presented for the first time here and should extend our knowledge of the room temperature fluorescence of DNA.

In general, the result of this work has been to show that the fluorescence at room temperature has two discernible parts. The first, though decreased in intensity by a factor of 2 is very similar to the spectrum of the nucleotides. The other weaker component, found at longer wavelengths shows considerable resemblance to the extremely red shifted luminescence seen from certain polynucleotides at room temperature. Neither part seems to resemble the DNA spectrum at 80°K. These results have been observed with minor variations from DNA samples from a variety of biological sources and subjected to a range of purification procedures. The polymer spectra are compared to those from mixtures of nucleotides to prevent the reporting of instrumental artifacts.

MATERIALS AND PURIFICATION

Materials

In the Appendix is a list of the materials used. The DNA preparations were purified as described below. The nucleotides and other compounds were used as received from vendor.

Purification

Purification of the DNA was a continual problem in the research. Four different methods were used during the course of the project. Successively more rigorous methods were used as it became clear that such measures were necessary.

Filtration. Initially, debris visible in commercial samples as received from vendors were removed by filtration through an ultra-fine sintered glass Buchner funnel, under moderate N_2 pressure. Difficulties in adhesion of the DNA to the sintered glass frit and in reliably cleaning the funnel caused abandonment of this approach.

Centrifugation. Use of the Beckman L-2 ultra centrifuge at low speed 1.2×10^4 rpm, SW-27.1 Rotor (~15,000 x g) more easily and reproducibly accomplished the same purpose as had filtration. Additional centrifugation of the solution at high speed $45\text{-}50 \times 10^3$ rpm, SW 50L Rotor (~165,000 x g) sedimented the highly polymerized samples. The supernantant solution was decanted, and artifacts from

small molecular weight impurities were substantially reduced. The DNA was isolated at the bottom of the centrifuge tube as a viscous gel. The gel was either allowed to resuspend in the centrifuge tube into fresh buffer over 2-3 days under refrigeration or else gently stirred in fresh buffer after transfer to a small glass stoppered flask. Reproducible quantum yields ($5\pm1 \times 10^{-5}$), fluorescence, and fluorescence excitation spectra were obtained from samples treated in this manner provided that clean teflon and exhaustively rinsed nitrocellulose centrifuge tubes were used in the low and high speed cycles, respectively. Polyallomer, polyethylene and polycarbonate tubes used at any point caused significant artifacts to be produced.

Deproteinization. The extensive measures to remove protein from commercial DNA samples were finally undertaken out of necessity. At first this was avoided because of the probability of introducing artifacts from the purifying reagents themselves, e.g., phenol has a $\Phi_f \sim .2$ in water (22) and is a commonly used reagent for this purpose. With perusal of literature of protein fluorescence, it became appparent that elimination of the possibility of protein artifact was necessary. The fluorescence of proteins containing tryptophan is shown very often to have a wavelength maximum in the region around 330 nm (23,24). It was not expected that centrifugation procedures would

remove high molecular weight proteins especially if they were tightly associated with the DNA.

Procedures to remove protein were finally begun when another group Vigny and Ballini (14) had shown that significantly lower quantum yields could be obtained from carefully deproteinized DNA samples. They reported that a variety of commercial preparations treated only by dialysis gave quantum yields of 7-10 x 10^{-5}; whereas a deproteinized sample gave a Φ_f ~3 x 10^{-5}. The procedure they used was after the method of Kay, Simmons and Dounce (25). The method relies on the denaturing effect of sodium dodecyl sulfate (SDS) on proteins and is widely used commercially.

Briefly, the method used by Vigny and Ballini is as follows. The detergent is mixed into the DNA solution (or cell lysate) at 0.5% concentration. The solution is made 1 M in NaCl .01 M EDTA. Centrifugation at 12 x 10^3 g and 5°C precipitates most of the detergent and denatured protein. This is followed by ethanol precipitation and acetone rinsing of precipitated DNA. The DNA is then redissolved and dialyzed.

Attempts to repeat the procedure here met with limited success as problems were encountered with fluorescent impurities in the SDS and dialysis tubing. SDS as received from vendors (Baker and Fluka) contained strongly fluorescing impurities. Treatment of the DNA with

such material produced obvious contamination of the DNA. The SDS was made fluorescently clean by extraction in a Soxhelet extractor with successive batches of purified diethyl ether (see below) for 7 days. The criterion for purity was that the fluorescence of a 5% solution showed no fluorescence above the buffer background with excitation 230 to 330 nm. When the SDS had been purified, DNA samples treated with it showed quantum yields between 4-5 x 10^{-5}. However, persistent impurities in dialysis tubing used in the process made the results somewhat suspect.

In addition, a second type of treatment using an enzyme, proteinase K, followed by phenol extractions was undertaken. The data reported for the most reliably deproteinized samples is from material treated in this manner. The method is taken from Hilz et al. (26).

The enzyme proteinase K is isolated from a fungus Triticachium album and has been used previously in the isolation of high molecular weight nucleic acids. It was shown that the action of the enzyme on protein is enhanced by the presence of the detergent sodium dodecyl sulfate (SDS), but that its action on small oligo-peptides is inhibited by SDS. In this same paper it was shown that SDS effectively inhibits the action of nuclease enzymes. There was some concern that the prepartion of proteinase K might have contained some trace nucleases, so SDS in conc. (0.25 to 0.50%) was always included

in deproteinizing procedures to prevent artifacts arising from the nucleases. The procedure used is as follows:

The dry DNA sodium salt as received from vendors was dissolved in pH 7.3 phosphate buffer (.05 M) at a concentration of 1mg/ml by stirring in a cold room (4°C) usually overnight. The dissolving solutions were contained in glass stoppered Erlenmyer flasks using teflon or glass coated magnetic stirring bars. When the DNA was in solution, SDS (0.25–0.50% w/w) and proteinase K (50 g/ml) were added and the solution was gently shaken for 5 hr in a 37°C constant temperature bath. The solution was then extracted twice with an approximately equal volume of buffered saturated phenol which had either been freshly distilled or else stored frozen (~–5°C) after distillation. The phenol buffer was .5 M tris, pH 8, 0.1 M EDTA. The phenol layers were removed after 10 min centrifugation at 10-12 x 10^3 rpm (SW 27.1, teflon tubes).

Care had to be taken to remove phenol or some derivative of it from the teflon tubes after each experiment. The removal was accomplished by repeated boiling in 20% H_2SO_4, followed by rinses in boiling deionized water. The initial boiling in H_2SO_4 resulted in purple color in solution. Boiling in 20% H_2SO_4 was repeated until solutions remained clear. If this was not done, subsequent batches of DNA which had been contained in contaminated centrifuge tubes showed

an intense yellow tint which was of course unacceptable. Most of the residual phenol was removed by extraction with diethyl ether (5-10 times). Remaining traces of phenol were removed by repeated (2-4 times) precipitation of the DNA with an approximately equal volume of purified ethanol at 0°C. Solutions were made .5 to 2.0 M in NaCl before additions of ethanol. Each precipitation was followed by several rinses of ethanol. The phenol was considered to have been removed when the fluorescence spectrum of the solution remaining from the ethanol precipitation (after removal of the DNA) contained no discernible fluorescence peak at 295 nm (λex 265) above the level of phosphate buffers. Acetone rinses were sometimes used after precipitation from ethanol but did not appear to affect fluorescence characteristics of DNA.

In order for the last criterion for the removal of phenol to be used, it was necessary to obtain fluorescently clean ethanol. This was done by refluxing technical grade 95% ethanol over NaOH or KOH, followed by distillation and filtration over freshly heated and cooled charcoal (24). The period of refluxing was from 8 to 24 hrs. The purified product was used for DNA precipitation only when fluorescing impurities were less than or equal to those observed in buffers used to dissolve the DNA.

The ether used in the SDS purification and in initial removal of

phenol from DNA solutions was freed of fluorescing impurities by refluxing and distillation over 1% KOH and 0.3% K_2MnO_4.

Water used in the preparation of buffers for the final solution of DNA was prepared from tap deionized water or tap deionized water distilled from 1% KOH, .3% K_2MnO_4 solution. Before use, all buffer solutions were checked for fluorescence background. The background levels in buffers used were always less than 1 part in 15 of the strong Raman line for water at ca. 3500 cm^{-1}.

EXPERIMENTAL

Instrumentation

Spectrofluorometer

The instrument had been assembled from components by Dr. Callis and previous students. It was operated in essentially the same configuration for the room temperature experiments as was described previously in the Ph.D. theses of Morgan (27) and Wilson (28). Emission is viewed at 90° from the excitation light path.

Monochromators. Bausch and Lomb .5 meter, f/5 grating monochromators were used both to disperse the excitation light source and the observed emission. Reciprocal linear dispersion is 6.6 nm/mm Usually both slits on both monochromators were adjusted to 2.0 mm. Giving band with of 13.2 nm at half maximum. Condensing and colliminating lenses supplied by the manufacturer were used to focus the excitation source on the entrance slit of its monochromator and a narrow, approximately 2 mm, portion of the fluorescence cuvette on the entrance slit of the detection monochromator.

The sample cuvette is placed on a stage at the entrance of the detection monochromator. The stage is translatable both along the detection and excitation light paths. In practice, the distance along the detection light path was fixed, and the position along the

excitation path was adjusted for each experiment. This was a crucial factor in making fluorescence measurements from concentrated samples (See below in calculation of fluorescence quantum yields.)

Excitation source. The excitation source was an Osram XBO150, 150 watt, high pressure Xenon lamp. It was contained in an aluminum housing built previously here. The lamp and housing were mounted on a freely movable aluminum plate with tripod legs adjustable for height. The intensity of excitation light incident on the sample cuvette was sensitive to position of the lamp. This was frequently adjusted by maximizing either the 3500 cm^{-1} Raman transition of water or the fluorescence of Rhodamine B at 640 nm with excitation wavelength at 260 nm.

The lamp was powered by an Oriel optics Universal Power Supply No. C-72-20 operating at 18 to 19 volts and 8 to 9 amps DC.

After leaving the excitation monochromator, the light was focused by a 51 mm diameter, 60 mm focal length plano-convex quartz lens. A line from the entrance slit of the detector monochromator intersected the exciting light path ca. 20 mm beyond the focal point of the lens so that the detector would always "see" a slightly defocused excitation source. This arrangement was arrived at by trial and error in maximizing the fluorescence signal from strongly absorbing samples. (See section on calculation of quantum yields.)

Photomultiplier tube and fluorescence data recording. The photomultiplier tube was an EMI 9558QC operated at -1150 volts DC. The operating voltage and amplification of the output was provided by a Pacific Photometric Model 11 amplifier. The amplified signal is fed into the Y coordinate of a Model 7030A Hewlett-Packard (Moseley) XY recorder. The X scale of the recorder was coupled to either the excitation or detection monochromator via 10 turn, 20 k Ω variable resistors which varied the voltage of a 1.5 volt dry cell battery.

Absorption Spectrometer

Carey-14 spectrometer was used in all absorption measurements. Initially the absorbance of concentrated solutions was obtained by dilutions. Later in the research, 1 mm path length cells were made available to me by Dr. Kenneth Emerson. The wavelength accuracy of the Carey-14 was checked by the absorption of benzene in hexane and comparison with published standards (29).

Digital Thermometer

A digital thermometer used was constructed by Bruce Anderson following a design by Dr. Richard Geer. The solid state components were mounted on a "bread board." The bread board variable resistors, digital panel meter, diode inputs were mounted on a plexiglass (later an aluminum frame). The instrument was prone to failures from various

causes, but when calibrated and operating, gave reasonable results ±1°C relative to a Hg thermometer in the same medium. Calibrations were performed by setting zero with the probe immersed in a water-ice slush. A high point was set as the boiling point of water (corrected for ambient pressure) with the probe immersed in a flask of boiling water.

pH Meter and Electrodes

Sargent-Welch Model NX digital pH meter and a Sargent-Welch Model S-30070.10 electrode were used in all experiments. Calibrations over pH range of 3 pH units were performed before each experiment using commercial standard buffers.

Fluorescence Cuvettes

The Suprasil cells used were purchased from Markson Company. The cells had either 5 mm or 10 mm path length and a 1.25 mm wall thickness. None of them gave evidence of significant fluorescence from the cell walls in the instrumental geometry employed. After each use, the cells were rinsed in distilled H_2O and concentrated nitric acid. Throughout most of the research, they were stored under concentrated nitric acid with the aim of preventing the accumulation of heavy metal deposits on the quartz surfaces. Before each experiment the cell and buffers used were tested for fluorescent

impurities.

Polarizer

For the polarization studies only one polarizer was used. It was a Polacoat PL-40 polarizing film 5 cm in diameter on quartz. The polarizer was held in a threaded mount at the exit slit of the excitation monochromator. Quartz wedge depolarizers were placed at the exit slits of the excitation monochromator and entrance of the detection monochromator to eliminate the anisotropy of grating transmittance.

Rhodamine B Quantum Counter

The intensity of the excitation source was monitored with the use of a Rhodamine B quantum counter (28). It consisted of a concentrated solution (3 mg/ml) of Rhodamine B in ethylene glycol contained in a stoppered quartz cuvette. The fluorescence signal from the Rhodamine B solution was measured at 640 nm at some time during the course of each fluorescence experiment. It was assumed that the fluctuations of the lamp intensity were small over this time span.

Calibration of Monochromator

The detection monochromator was periodically calibrated in the following manner. The slits were closed to .1 mm. A MgO screen (made

by holding a piece of quartz directly above a piece of burning magnesium ribbon) was placed in the cell holder with the face to be viewed at about a 45° angle to the monochromators. The spectral lines from a low pressure Hg lamp were recorded. The wavelength selector drum on the detection monochromator was adjusted until the wavelength read from the drum at the maximum coincided within .5 nm with the known values for the line emission wavelength (29).

The excitation monochromator was calibrated relative to the detection monochromator using the MgO screen as before. The excitation monochromator was set at a arbitrary wavelength, and the reflected band recorded. The wavelength selector drum on the excitation monochromator was adjusted until the readings on the two selector drums coincided to ±1 nm in the wavelength range from 240 to 300 nm.

During the fluorescence experiments all the monochromator slits were kept at 2 mm width. This gave a band pass at half maximum height of 13.2 nm in the region of DNA absorbance and fluorescence (240-300 nm).

Calibration of Photomultiplier Response

Calibration of the photomultiplier tube for the variation of its response with wavelength of impinging light has been performed previously by others in this lab (27,28,31).

The procedure used was to position an MgO screen as in the wavelength calibration of the monochromators. At 5 nm intervals in the wavelength region from 280 to 500 nm, the excitation monochromator is fixed and a scan is made of the band of light reflected from the MgO screen. To account for the variation of excitation lamp intensity over this long wavelength range, the fluorescence of the Rhodamine B quantum counter at 640 nm is monitored as a function of excitation wavelength. The signal obtained from the MgO screen is divided by the Rhodamine B signal at the same excitation wavelength. Care was taken to account for variations of the absorption of Rhodamine B which are expected to affect the intensity of the fluorescence signal from the quantum counter. See below in calculation of quantum yield.

The result obtained by previous students was repeated to about 5% precision over the wavelength range 280-500 nm. The values used to correct fluorescence spectra for the photomultiplier response are shown as circles (o) in Figure 1. They were obtained by W. B. Knighton and are in very close agreement with the values reported by Morgan (26) and Wilson (27). The plus signs (+) are values obtained by the author of the present work. Some disagreement can be seen, particularly at wavelength about 460 nm. The discrepancies were neglected as they are small. Agreement throughout the rest of the spectrum is much closer and previous measurements (27,28,31) did not

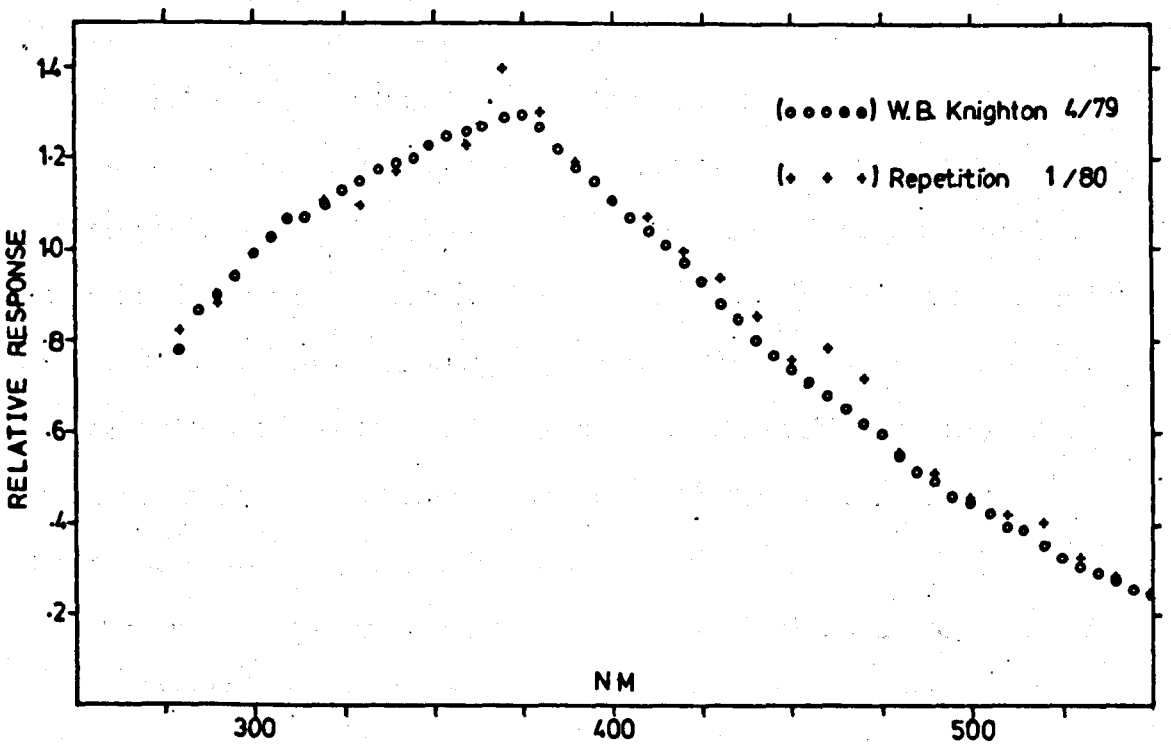

Figure 1. Relative response of photomultiplier tube. (o) circles are from W. B. Knighton, (+) present author, (o) are values used in correction of fluorescence spectra.

indicate the features there. They occur in the region of a minimum of Rhodamine B absorbance making it more likely that the features are artifacts.

Recording of Fluorescence Spectra

With the excitation monochromator set at the absorption maximum (260 nm), and detection monochromator set at the fluorescence maximum the sample was translated along the excitation light path until a maximum signal was obtained. The fluorescence spectrum was recorded from 280 to 500 nm. For a typical sample the fluorescence spectrum was recorded at 250, 260, 270, 280 and when possible 290 nm, as a small variation is spectral shape occurred with excitation at different wavelengths (see below).

The recorded data was corrected for variations in response of the photomultiplier tube. This was done in the following manner. The height of the fluorescence curve was transcribed at 5 nm intervals into notebooks. These numbers were divided by the relative response of the photomultiplier tube at the appropriate wavelength. The resulting values were normalized to 1.00 at the maximum so that spectra from concentrated samples could be compared for spectral shape with those obtained from more dilute solutions.

For calculations of the fluorescence quantum yield (Φ_f), the values of the corrected and normalized fluorescence spectrum were

multiplied by the wavelength interval (5 nm) and summed. This closely approximates the area under the fluorescence curve. This area is multiplied by the measured photocurrent at the maximum and is directly proportional to the total emission.

The contribution of fluorescing impurities in the buffers was estimated from the spectrum of the buffer at the same position of the fluorescence cell as was used in recording the sample spectra. As the fluorescence from any species will be in proportion to the light it absorbs, the buffer spectrum at a given excitation wavelength was scaled down by the ratio of the ~3500 cm^{-1} Raman transition of H_2O in the sample to that in the buffer. The scaled buffer spectrum was subtracted from the sample fluorescence spectrum before the spectra was transcribed into notebooks. The unscaled buffer spectrum never exceeded 20% of the total signal and was usually $\leq$10%. In strongly absorbing solutions scaling was not possible and the buffer background was ignored.

Recording of Excitation Spectra

As in the recording of fluorescence data, the fluorescence signal is maximized by adjusting the cuvette position in the optical path of the detector monochromator. The wavelength of the detector monochromator is fixed (usually at the fluorescence maximum) and the