



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

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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 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 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.

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 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 electron 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 transferred 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 excitation 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^{-1} 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 $3 \pm 1 \times 10^{-5}$, 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 $7-10 \times 10^{-5}$. 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 ≥ 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 ($\sim 15,000 \times g$) more easily and reproducibly accomplished the same purpose as had filtration. Additional centrifugation of the solution at high speed $45-50 \times 10^3$ rpm, SW 50L Rotor ($\sim 165,000 \times g$) sedimented the highly polymerized samples. The supernatant 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 ($5 \pm 1 \times 10^{-5}$), 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 apparent 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 $7-10 \times 10^{-5}$; whereas a deproteinized sample gave a $\Phi_F \sim 3 \times 10^{-5}$. 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 12×10^3 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 Soxhlet 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 $4-5 \times 10^{-5}$. 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 preparation 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 10³ 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% H₂SO₄, followed by rinses in boiling deionized water. The initial boiling in H₂SO₄ resulted in purple color in solution. Boiling in 20% H₂SO₄ 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 (λ_{ex} 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 width of 13.2 nm at half maximum. Condensing and collimating 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 XB0150, 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 $\pm 1^\circ\text{C}$ 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 an 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

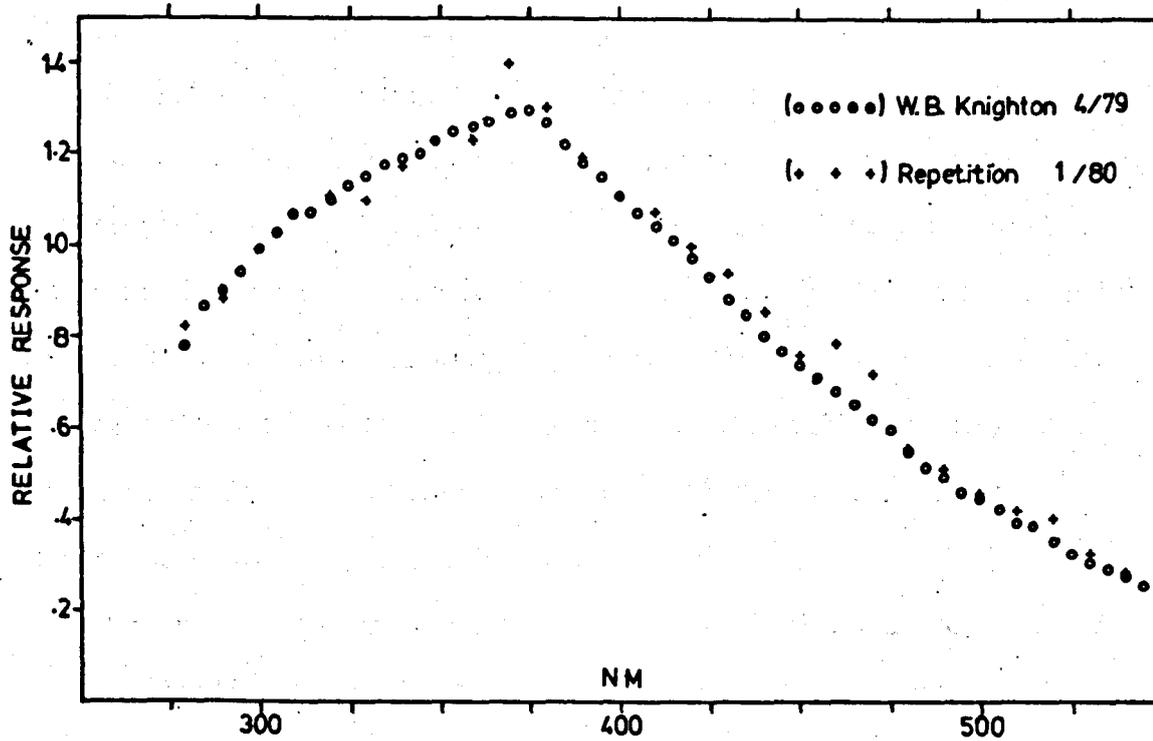


Figure 1. Relative response of photomultiplier tube. (o) circles are from W. B. Knighton, (+) present author, (o) are values used in correction of fluorescence spectra.

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 in 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

excitation monochromator is scanned through the absorption band of the sample. The fluorescence at the fixed wavelength is recorded as a function of excitation wavelength.

Under the conditions of the experiment, virtually all of the light is absorbed through most of the absorption band. Except at the edges, the variations of fluorescence signal due to the variations in the amount of light absorbed will be small compared to the total signal. Furthermore, the variations will not be linearly related to the amount of light absorbed. Therefore, the recorded data must be transcribed and corrected for sample absorption (see below in quantum yield calculation) as well as for the variation in excitation lamp intensity. The corrected data is then multiplied by the absorption spectrum of the sample. The resulting string of values are normalized to one at the wavelength of absorption maximum, and are the calculated excitation spectra.

Quantum Yields

A centrally important quantity to be determined in this research is the fluorescence quantum yield, or the ratio of the light emitted vs the light absorbed. As it is an extremely difficult task to determine this absolutely, the values reported here and generally in the literature are related to a standard with an established value. In this work, the standard used was L-tryptophan which has been

intensively studied. There is a wide variation of values reported; ranging from .13 to .20 (33-36). A value of .15 was used which is in agreement with the most reliable reports (32,36).

Because of the extremely low fluorescence signal produced by DNA it is necessary to use quite concentrated samples. Typical solutions had a maximum absorbance at 260 nm of 5 to 15 in a 1 cm cell. With the high absorbance of the samples the exponential decrease of light intensity along the path length is quite sharp. The fluorescence will not then be linearly related to the absorbance. This lead us to the use of a cell holder which was translatable along the excitation light path. In this way, the narrow region at the front of the cuvette, in which almost all the light is absorbed and from which almost all of the fluorescence occurs can be focused on the entrance slit of the detection monochromator. Once the position at which the fluorescence signal is maximum is found, the quantum yield can be calculated relative to a solution of the standard compound which has the same absorption.

In practice, it was desirable to be able to calculate quantum yields without preparing a standard of matching absorbance. In order to do this, Dr. Callis derived a mathematical expression which would predict the variation in fluorescence that would occur as a function of absorption. It straightforwardly accounts for the sharp decrease

in light transmitted along the path length in strongly absorbing solutions and the optics which focus emitted light on the entrance slit of the detector monochromator.

$$G = 2.3 A \int_0^a (1-x/a) e^{-2.3Ax} dx$$

In this expression, G is the predicted fluorescence relative to that at infinite absorbance from a solution with absorbance A at the excitation wavelength. The exponential term is the Beer's Law decrease of the light along the cell path length. The linear term approximately accounts for the optics which focus the fluorescence. It is assumed that the amount of light reaching the photomultiplier tube decreases linearly with distance (x) away from a sharp maximum to some point (a) where it becomes zero. The integral can be evaluated between 0 and a to give:

$$G = 1 - \left(\frac{1 - e^{-2.3 A \cdot a}}{2.3 A \cdot a} \right)$$

The point (a), where the light reaching the photomultiplier tube from the fluorescence cell is effectively zero is found experimentally by trying values of (a) which give the best fit of the expression to a series of values of fluorescence intensities from standard solutions having a wide range of absorbances. The best fit was found from a value $a = .21$ for tryptophan samples ranging in absorbance from $\sim .4$ to

40 at the absorption maximum.

The expression for (G), the predicted relative fluorescence at a measured absorption, using this value for (a) was incorporated into the calculation of the quantum yields

$$\Phi_f = \frac{F(\lambda_{fmax}) \cdot A_f}{X(\lambda_{ex}) \cdot G(\lambda_{ex}) \cdot K}$$

where F = the fluorescence signal intensity measured at the maximum

A_f = area under fluorescence curve 280-500 nm (see above)

X = lamp intensity measured from the Rhodamine B quantum counter at the excitation wavelength.

K = factor which scales the other quantities, in a calibration experiment, to give the quantum yield of L-tryptophan equal to .15.

The value K was taken as 6800 from the same data as used to derive the best value for a (in G). This was periodically checked by measuring the fluorescence from L-tryptophan. The precision of the quantity K was found to be $\pm 16\%$ over the period of 4 years.

Therefore, the values of the quantum yields reported must be taken as being no more reliable than this.

Polarization Ratio

Measurements of polarization ratios (I_v/I_h) were made with only one polarizer in order to maximize signal to noise ratio. The

intensity of emission was measured with the polarizer vertical and horizontal in lab coordinates. This ratio should vary between the limits of 2.0 and .75. The quantity of interest is the angle between the absorbing and emitting dipoles (θ) averaged over all possible orientation of the dipoles (37) is related to the measurement of I_v/I_h by the expression

$$\frac{I_v}{I_h} = \frac{S + 3}{4 - 2S}, \text{ where } S = \langle \cos^2 \theta \rangle.$$

The reliable measurement of the polarization was hampered by the weak emission of DNA and excitation light lost due to the low transmittance of polarizing film (~.14 at 260 nm) and optical filters used to prevent stray light scattering.

Heat Denaturation

Fluorescence. Observation of the heat induced changes in DNA fluorescence were made at fixed wavelengths of excitation (260 nm) and fluorescence (325 nm). The time course of the experiment (typically ~1 hr) was followed on the x axis of the xy recorder, set usually to trace at 50 sec per inch. Samples were heated in a quartz cuvette which was tightly sealed with a teflon lid to prevent evaporation. The lid was held in place with a brace fashioned from brass shim stock, brass plates and screws. The capped cell was contained in a

blackened balsa wood box mounted on the movable stage of the detector monochromator.

Uniform heating of the solution was sought with constant stirring from a finned, round magnetic stir bar. Heating rate was generally on the order of 2 to 5 degrees per min. accomplished by a stream of compressed air heated by passage through a 500 water resistive heater. By adjusting the operating voltage to the heater and/or the air flow, the temperature could be controlled to $\pm 2^\circ$ for sufficient time to obtain the full scan of the fluorescence or fluorescence excitation spectra.

Absorption. Observation of the absorption changes at 260 nm as a function of temperature was accomplished using 1.0 mm path length quartz cells in the Carey-14 spectrophotometer. The 1 mm quartz cell was tightly capped and mounted in a second quartz cell of dimension 30 x 10 x 45 mm. The large cell was filled with distilled water. The temperature sensing diode and stirring bar were placed in the distilled water "bath." Heating was via the same resistive heater. Heated air was blown into the sample cavity of the Carey-14. A cell holder and stirring motor mount were constructed from plexiglass to fit into the Carey-14.

pH Experiments.

Titration were performed in the fluorescence cuvettes by

addition of approximately 1 molar NaOH, KOH or H_3PO_4 with a 10 microliter syringe or pipette. A magnetic stirring bar was used to minimize extremes of concentration while performing titrations. Five to ten minutes were allowed after additions of titrant for the polymer solutions to reach equilibrium.

RESULTS AND DISCUSSION

Introduction

Measurement of the fluorescence characteristics of DNA at room temperature neutral solutions is the central focus of this research. The major obstacle to accomplishing this goal has been the extreme weakness of the fluorescence from DNA under these conditions. The faintness of emission compounds problems of purification always connected with fluorescence measurements. The purification procedures are further complicated in the case of DNA because the fluorescence characteristics are sensitive to conformational state.

Fluorescence data has been obtained for DNA from a variety of biological sources and subjected to four increasingly stringent methods of purification. The results from the numerous experiments for quantum yield, spectral shape, and excitation spectra are recounted briefly with regard to each type of measurement along with the variations in each due to purification method or biological source. Comparison is made between earlier experiments and the results obtained later in the research when the problems of purification, and stray light artifacts were mostly solved. The later results are felt to most accurately characterize the fluorescence of pure native DNA in natural solution. The differences due to

purification procedure, and light scattering artifacts were usually about the same as the precision of the experiments and it was a slow and difficult process to decide which was which.

Quantum Yields

The lower limit of fluorescence quantum yields (Φ_f) observed has been $3.3 \pm .5 \times 10^{-5}$ and is considered to be the best estimate for native DNA at pH 7 and 25°C.

The value has been chosen out of the rather wide range observed (up to ca. 1×10^{-4}) because, successively more rigorous methods of purification (especially for removal of proteins) gave lower Φ_f 's. It is unlikely that a trace impurity would cause a decrease rather than increase in the already low level of fluorescence as seen from the mononucleotides ($.4$ to 1×10^{-4}) in this lab and elsewhere (31,4). The lifetimes of the monomer excited states at room temperature have been shown to be on the order of a picosecond (38,39) and it would require very high concentrations of quenching impurities to cause such an effect.

The decrease in Φ_f seen for the native polymer relative to equimolar mixtures of monomers at room temperature is much less than is seen at 80°K. It was suggested in (1) that the quenching at 80°K arose from hydrogen bonding between complementary base pairs. At

room temperature it is probable that the rate of quenching of emission in this pathway is largely overshadowed by the drastically increased rate of radiationless deactivation seen in the independent monomers.

The reason for the dramatic decrease in fluorescence of DNA components at room temperature is not well understood. It has been suggested by Eastman and Rosa, in the case of adenine (40), that the radiationless relaxation competing with fluorescence is dependent upon solute-solvent interaction. That is, the rearrangement of the solvent molecules surrounding the excited molecule facilitates internal conversion to the ground state. In viscous and/or low temperature solvents, this process is only slightly faster than the spontaneous emission rate, where as in less rigid media it is much faster. They speculate that the quenching at 80°K of fluorescence multi-stranded polynucleotides relative to the monomers occurs because the polymer is a less rigid "solvent" than the glycol water glass and would better promote radiationless deactivation.

The opposite situation is observed at room temperature. In single stranded synthetic polynucleotides at room temperature, the quantum yields are greater than the Φ_f for the monomers, whereas for the neutral double stranded hydrogen bonding polynucleotides the Φ_f are about the same as for the monomers (14). This is in accord with Eastman and Rosa's view as it is reasonable to expect the neighboring

molecules in the polymer to be less likely to reorient than the solvent and be less likely to promote internal conversion to the ground state. However, the hydrogen bonding double stranded polymers would seem even less likely to reorient. This indicates a separate process connected with hydrogen bonding that is causing an increase in the internal conversion or decrease in spontaneous emission.

Values at the lower limit of 3×10^{-5} were observed consistently only for DNA from *E. coli* which had been treated with proteinase K and phenol extracted. Such low Φ_f were observed in one instance from a sample of calf thymus DNA (Sigma, lot #99C-9540) which had been purified in the same manner, and one sample of hen erythrocyte DNA which had been treated only with purified SDS after the method of Kay et al. (25). Subsequent experiments on these batches gave Φ_f of $\sim 4 \times 10^{-5}$. This may be due either to decomposition of samples in storage or undetermined experimental errors. Higher values observed in other samples are believed to be due mostly to the presence of fluorescing impurities and less certainly to the conformational state of the macromolecule and/or the biological source.

Typical results are given in Table 1. Details of the variations in Φ_f with regard to purification procedures and/or biological source are given below.

Table 1. Fluorescence Quantum Yields of DNA

DNA	Purification	$\Phi_f \times 10^5$	λ_{ex}	optical filter
Calf thymus	Proteinase K, Phenol extraction	3.4 \pm .2	260	+
		4.1 \pm .2	280	+
Calf thymus	centrifuged 14x10 ³ rpm and 45x10 ³ rpm	5.1 \pm .1	260	-
		6.7 \pm .1	280	-
Calf thymus	centrifuged 14x10 ³ rpm only	5.4	260	-
		11.6	280	-
Hen Erythrocytes	SDS, Acetone rinse	4.2	260	-
		3.9	280	-
	centrifuged 14x10 ³ rpm and 45x10 ³ rpm	5.6 \pm .3	260	-
		5.9 \pm .2	280	-
E. coli bacteria	Proteinase K, Phenol extraction	3.2 \pm .2	260	+
		3.7 \pm .3	280	+
	centrifuged 14x10 ³ rpm and 45x10 ³ rpm	5.2 \pm .2	260	-
		5.5 \pm .2	280	-
	centrifuged 14x10 ³ rpm only	5.4	260	-
Thermophilic bacteria	SDS, Acetone rinse	3.5 \pm .1	260	-
		3.8 \pm .2	280	-

1) As received from vendors:

The fluorescence measurements from DNA samples used as received from vendors were highly irreproducible. At the concentrations used in experiments the solutions of the untreated samples were often visibly turbid and irreproducibility was attributed to light scattering from the visible debris, aggregates and fluorescing impurities.

2) Filtration and low speed centrifugation

Samples which were simply filtered, in the initial stages of research, or later, centrifuged at ca. $2 \times 10^4 \times g$ gave Φ_f between 6 and 10×10^{-5} . The Φ_f from these samples also showed a much greater dependence on the wavelength of excitation increasing in some instances by more than 2 fold in going from excitation at 250 nm to 280 nm.

This is strongly suspected to be due to protein impurities, simply because of the biological origins of the samples. The fluorescence of proteins is mostly due to tryptophan moieties whose absorption spectra is to the red of the DNA bases. Because of the much stronger Φ_f of tryptophan ($\Phi_f = .15$) as more of the excitation light is absorbed by tryptophans, the Φ_f should increase. A very crude demonstration of this was obtained from later fluorescence spectra from a sample which contained calf thymus DNA which had been

purified by high speed centrifugation and proteinase K in the ratio of 100:2, DNA:protein.

The quantum yields ranged from 35×10^{-5} ($\lambda_{\text{ex}} 280$) to 13×10^{-5} ($\lambda_{\text{ex}} 260$).

3) Low and high speed centrifugation

Fluorescence quantum yields from $5-7 \times 10^{-5}$ were observed from DNA from *E. coli* bacteria, calf thymus and hen erythrocytes which had been purified in this manner. The lower limit of quantum yields from DNA's purified in this manner indicated (in light of later deproteinization procedures) the presence of impurities which are not removed by centrifugation alone. The higher values in the range observed may have resulted from the introduction of fluorescent artifacts at some point in the manipulations, or the presence of such artifacts in materials as received from vendors. The DNA samples from the different biological sources gave similar range quantum yields under this purification procedure, but the average Φ_f from different lots of calf thymus were slightly higher than those from hen erythrocytes or *E. coli* bacteria. The calf thymus DNA Φ_f also showed a somewhat greater dependence on wavelength of excitation.

4) Proteinase K and phenol extracted samples

The deproteinized DNA's observed were mostly from *E. coli* and calf thymus. An experiment was performed on DNA from extreme

thermophilic bacteria similar to Thermus aquaticus which gave somewhat anomalous fluorescence spectra.

As mentioned before, the *E. coli* DNA consistently gave the lowest quantum yields observed and the purified material appeared to be the least degraded. The quantum yields from calf thymus DNA varied from 3 to 5×10^{-5} . With calf thymus and *E. coli* samples which gave the lowest Φ_f , almost the entire sample could be spooled in a fibrous mass on a glass stirring rod after addition of cold ethanol. With the samples giving the higher quantum yields, a portion of the sample could not be spooled on the glass rod and precipitated from solution as flocculated particles. While this may be merely a coincidence, it does suggest that alteration of the polymer conformation in purification and isolation procedures can be another source of small but significant changes in the fluorescence.

A more likely explanation for the range in quantum yield between 3 and 5×10^{-5} is that for the eukaryotic DNA samples (calf thymus, and hen erythrocytes) some fluorescent contaminant was not thoroughly removed by the deproteinization procedures in some cases. (See below heat, denaturation results).

Fluorescence Spectra

The spectra obtained from the most reliably purified samples and

using optical filters in the excitation light path did not vary drastically from spectra of less knowingly prepared samples. This is to say that the fluorescence spectral shape has not been a very sensitive measurement of sample purity more than to say the materials examined were all essentially the same. While it could be said the commercial preparation were almost pure DNA as received, real though small differences between samples of varying purity were more evident by comparison of quantum yields and excitation spectra. The best quantitative estimate of pure native DNA is shown in Figure 2. It is the averaged spectra from two samples each of calf thymus and *E. coli* DNA which gave Φ_f $3-4 \times 10^{-5}$. A spectrum of an equimolar mixture of the mononucleotides is shown for comparison. (Adenosine was used instead of AMP as no fluorescently clean samples of the latter could be obtained.) The monomer curve is reduced by a factor of 2 for comparison of the spectral shapes. (Wavelengths of excitation is 260 nm in both.) There are two general features that should be noted. First is that the fluorescence maxima both occur at 325 nm and the peaks centered here are of very similar width. Secondly, in the polymer spectrum above 370 nm there is a component which is not seen in the monomer mixture. It comprises about 25% of the area under the fluorescence curve.

The principal maximum of the polymer fluorescence at 325 nm and

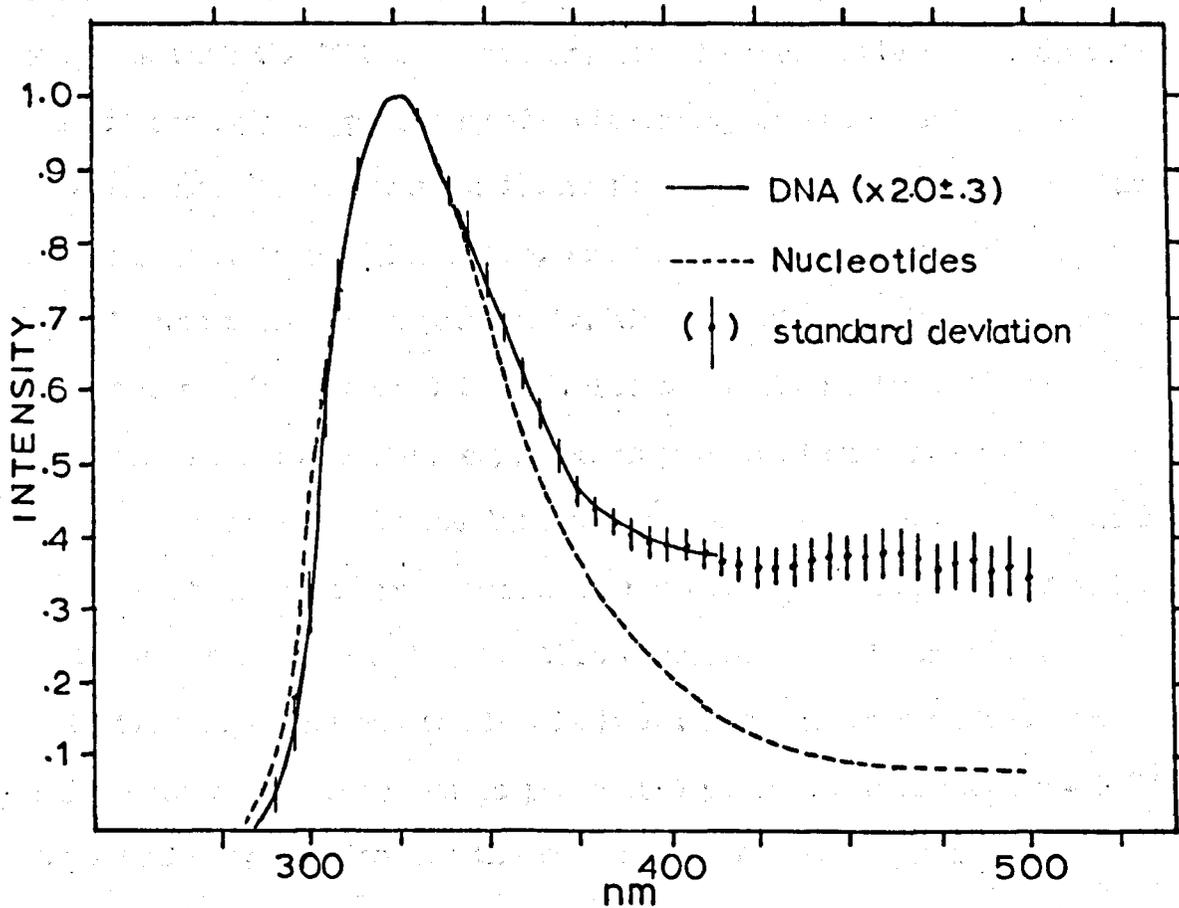


Figure 2. Fluorescence spectrum of two samples each *E. coli* and calf thymus DNA, pH 7.3 and 25°C. Excitation is at 260 nm using optical filters. Dashed line is fluorescence spectrum of an equimolar mixture of nucleotides GMP, CMP, TMP and Adenosine as no pure AMP available. Fluorescences are corrected for photomultiplier sensitivity.

the peak shape are almost identical to the spectrum from the nucleotide mixture. The intensity from the polymer is about half that of the nucleotides. Its similarity to the monomer spectrum strongly suggests that the DNA bases are emitting independently of one another. This is strongly supported by the similarity of the excitation spectrum for fluorescence at 325 nm from the polymer to that from the nucleotide mixture. (See below, excitation spectra section).

As was pointed out previously, these results are in disagreement with two previous reports (20,21), but are still believed to be correct. In the dozens of experiments performed on various DNA samples, there was only one instance when a broadened spectrum with a maximum at about 350 nm, similar to the one reported by Daniels and Anders has been observed here. This occurred when the sample was acidified to pH 3.3 and the DNA is known to begin to denature. At this point emission is due to protonated purines and the Φ_f is $\sim 10^{-4}$. A possible explanation for the spectrum by Anders might be sought in photochemical effect due to the high intensity light source. Fluorescence from samples of DNA excited by the beam of a scanning electron microscope (20 kV accelerating voltage) had a maximum around 400 nm which shifted to longer wavelengths and with exposure time (41).

None of the three previously published reports discusses the

fluorescence seen at wavelengths more than 400 nm. The position of the maximum inferred from subtracting away the monomer like portion of the fluorescence, (~420 nm) is similar to that observed at room temperature from some synthetic polynucleotides and dinucleotides which are known to form excited state complexes (4,14,15). As was discussed in the introduction this is most easily rationalized in the polynucleotides as being emission from long lived singlet exciplexes. The similarity of wavelength maxima and the temperature and conformation dependence (see below in heat denaturation section) also suggest a similar interpretation here. There is no direct evidence that I am aware of that this is or is not so in either the case of the synthetic poly nucleotides or in DNA from living sources. However, the series of papers by Morgan and Daniels (17a,b,c) put forward speculation that triplet state emissions are responsible for the long wavelength emission in polynucleotides. Owing to the observed similarities between the emission of the synthetic polynucleotides and the long wavelength component seen in the DNA spectrum, a detailed discussion of the work of Morgan and Daniels is in order at this point.

In poly A, ApA, poly C, CpC, as discussed by Morgan and Daniels, and in the long wavelength component of the DNA spectra a large lowering of the excited states of the polymers relative to those of

the free nucleotides is required. Explaining this lowering of the polymer excited state energy strictly in terms of singlet state exciplexes could entail long lived exciplexes, large electronic interaction in the exciplex, and solvent reorientation or internal reorientation of the exciplex. Even in fluid media at room temperature lowering the exciplex energy 5000 to 7000 cm^{-1} below the free nucleotides places extreme demands on these processes. Still these demands appear more plausible than the drastic changes in the excited state behavior of the DNA bases which is required by the explanation of Morgan and Daniels.

The formation of exciplexes and emission from excimer singlet states appears more plausible especially in view of the unusually high rates of radiationless relaxation seen in the DNA bases at room temperature. The spin forbidden nature of phosphorescent transitions makes the triplet radiative rates quite low generally. For the DNA bases, the presence of $n \rightarrow \pi^*$ transitions increases considerably the probability of triplet states and emission from them. Further increases in triplet radiative rates would appear to obscure any distinction between spin allowed and spin forbidden processes. Barring such increases in triplet radiative rates, the observation of room temperature phosphorescence in Daniels scheme requires extreme and sometimes unreasonable changes in excited state parameters of the

nucleotides.

A simple kinetic analysis based on the known properties of the nucleotides at room temperature will demonstrate this. The phosphorescence quantum yield (Φ_p) can be written as follows:

$$\Phi_p = \frac{k_{ISC}}{k_r + k_{nr}} \times \frac{{}^3k_r}{{}^3k_r + {}^3k_{nr}} = \frac{1}{\tau} \times k_{ISC} \times \frac{{}^3\tau}{{}^1\tau + {}^3\tau} \times \frac{{}^3k_r}{{}^3k_r + {}^3k_{nr}}$$

This can be applied to monomer and excimer phosphorescence in Daniels scheme because it is assumed that the excimer emission occurs only from excitation of bases that are stacked in the ground state. Monomer emission comes only from excitation of unstacked bases and there is no exchange between the two types of excited states.

The superscripts indicate the state multiplicity and subscripts r , nr and ISC denote radiative, non-radiative and intersystem crossing rate constants, respectively. The life times (τ) are the reciprocal of the sum of deactivation rates from singlet (${}^1\tau$) and triplet states (${}^3\tau$).

The excited state parameters of the nucleotides at room temperature were first measured by Eisinger and Lamola (38). The nucleotides were used to sensitize the emission of Eu^{3+} . Their elegant study indicated singlet lifetimes (${}^1\tau$) to be of the order of 10^{-12} sec and triplet lifetimes (${}^3\tau$) to be $\sim 10^{-6}$ in non deoxygenated

solutions. The intersystem crossing rates (k_{ISC}) were estimated to be on the order of 10^8 sec^{-1} . The $^1\tau$'s are in agreement with values calculated from fluorescence quantum yields (3,4) and polarization measurements by Knighton and Callis (42). The triplet data for pyrimidines is in agreement with photochemical measurements by Johns and coworkers (43-45) and transient absorption measurements by Salet et al. (46).

The phosphorescence quantum yields proposed by Daniels were $\sim 10^{-5}$ (poly C) and $\sim 10^{-4}$ (poly A) the yields of excimers being about twice that from the monomers. If the product $^1\tau \cdot k_{ISC} \cdot ^3\tau \cdot \dots$ is formed from the values measured for the monomers at room temperature, and 3k_r is taken as its value at 80°K then the increase required is about 10^5 - 10^6 for the product of the 4 parameters in the polymer.

It is reasonable to expect the radiative rate 3k_r not to change in an independent monomer with temperature as it is an electronic property of the molecule. However, when a large perturbation such as the formation of an excimer occurs the results are difficult to predict. In this regard the work of Okajima et al. (16) suggest that a large $\sim 10^3$ - 10^4 fold increase in the 3k_r for naphthalene excimers. The normal or monomer phosphorescence was not detectable with a 100 fold increase in sensitivity, even though transient absorption on measurements showed that the monomer triplets far outnumbered excimer

triplets. The triplet lifetimes of the monomer and excimers were seen to be the same from observation of delayed fluorescence. The absence of excimer fluorescence indicated that in most cases excimer triplets formed from monomer triplets not from intersystem crossing from the excimer singlet.

In both the naphthalene and the nucleotides at room temperature the singlet radiative rates and intersystem crossing rates are competitive or of the same order of magnitude. The quantum yields for both processes Φ_f and Φ_{ISC} are near unity for naphthalene and on the order of 10^{-4} for the nucleotides because of the greatly enhanced non-radiative relaxation from the singlet at room temperature. It is not possible to rule out room temperature phosphorescence from the nucleotides a priori, but the triplet radiative rates from the nucleotides are already ~ 500 greater in the nucleotides than in naphthalene at 80°K . Further increasing the triplet radiative rates of nucleotides in polymers by even as much as 10^2 to 10^3 would require their oscillator strength to become comparable to a weakly allowed singlet.

Using the values of the triplet radiative rates at 80°K for both naphthalene monomers and nucleotides (0.03 and ~ 1.0 , respectively), Φ_{ISC} .7 (for naphthalene) (72) and 10^{-4} (for nucleotides), and an upper limiting value of 10^{-6} sec for the τ^3 for both naphthalene and nucleotides in non-deoxygenated solution phosphorescence quantum

yields can be estimated. $\Phi_p = \Phi_{ISC}^3 \tau^3 k_r$. A value of 10^{-8} is predicted for naphthalene and 10^{-10} for the nucleotides. Observing phosphorescence at room temperature appears to be about 100 times less likely from the nucleotides. As phosphorescence has been observed from neither naphthalene nor the nucleotides at room temperature, speculation about its occurrence in polynucleotides require that the polymeric conformation induce dramatic increases in some or all of the excited state parameters $^1\tau$, K_{ISC} , $^3\tau$, or 3k_r . The analogy with the naphthalene excimer phosphorescence might justify Daniels scheme except that it requires almost equal large increases in monomer and excimer excited state parameters.

To observe increases of five or more orders of magnitude in the phosphorescence of mononucleotides as proposed by Daniels is unlikely. If the polymeric environment should lead to a large increase in the singlet lifetime, ($^1\tau$), Daniels' model would require that the 1k_r be reduced proportionately (all other parameters being the same values as for the monomers) in order to maintain the fluorescence to phosphorescence ratios proposed. I am not aware of any sound reason for this to occur.

Since the data used by Daniels was not from reportedly deoxygenated solutions it is doubtful that the $^3\tau$ could be expected to be much more than 10^{-6} for unstacked bases. The quenching rate

constant for pyrimidine triplets by O_2 was found by Johns (43) to be $3 \times 10^9 \text{ moles}^{-1} \text{ sec}^{-1}$, near the diffusion controlled limit.

Kalyanasundaram et al. (19) reported millisecond lifetimes for aromatic hydrocarbons trapped in micelles. Such lifetimes could be measured only after baking glassware at 1000°C and purging samples with ultra-pure N_2 . Purging with impure N_2 caused a four-fold decrease in ${}^3\tau$. It is conceivable that the formation of a triplet excimer could decrease the quenching rate of O_2 and thereby increase ${}^3\tau$ for the excimer. Maintaining the ratio of 2 between the quantum yields of the excimers and unstacked nucleotides would require the same decrease in the quenching rate of the unstacked bases or a gratuitous increase in one or all of the other parameters ${}^1\tau$, k_{ISC} or 3k_r of the otherwise independent bases.

Similarly, it is conceivable that the interaction of excimers might produce a shifting in the relative energies of $(n - \pi^*)$ and $(\pi - \pi^*)$ excited states which would result in greatly enhanced intersystem crossing and/or radiative rates. The intersystem crossing rates (k_{ISC}) are already about the same as the singlet radiative rates in the monomers at room temperature. Large increases in k_{ISC} don't seem likely, and would require proportional increases in 1k_r to account for fluorescences. Again, the requirement that the unstacked or monomer-like Φ_f 's being with a factor of two is difficult to

rationalize.

In summary to this digression, the possibility of excimer phosphorescence can't clearly be ruled out. However, it is unlikely that in the scheme proposed by Daniels it is the correct explanation for the emission seen at ca. 400 nm in the polynucleotides. The wavelength maxima for these emissions is in the same region as the phosphorescence observed at 80°K. Phosphorescence from excimers would probably be red shifted. Daniels interpretation requires almost equal contribution from monomer phosphorescence, which is highly unlikely. Because of this, his scheme seems unduly contrived and unsupportable. Until time resolved data giving indications to the contrary is obtained, the extremely red shifted luminescence in these synthetic polymers and DNA should not be treated in terms of such complicated composites. There are other explanations for the long wavelengths emission that more plausible than phosphorescence.

Because of the extremely short lifetimes for the nucleotides indicated by quantum yields and polarization measurements $\leq 10^{-12}$ sec, it might be possible that the long wavelength emission occurs from vibrationally unrelaxed exciplexes. That is, the excited state lifetime is of about the same magnitude that would be expected for a single intermolecular vibration involved in forming the exciplex. Emission could conceivably occur between vibrationally excited

exciplex states and highly repulsive exciplex ground state giving the very large red shift.

The possibility of a impurity introduced during preparation by vendors or in the subsequent purification performed here, can't be ruled out, but the consistency of the results from all samples tested argues strongly against this. More optimistically, the "impurity" might be something besides one of the four major nucleotides which is tightly attached to the DNA and significant in some aspect of its biological function. Relatively highly fluorescent rare bases are known to occur in transfer RNA's and their emission characteristics have been well documented in some cases (e.g., Y base (47-49) and 4-thio-uridine (50,51)). Others, N-4 acetyl-cytosine and 7 Me Guanosine (52) don't fluoresce as strongly in t-RNA as they do free in solution and have been less extensively studied. The absorbance and emission of the Y base and 4-thio-uridine are shifted to longer wavelengths than that from the 4 common bases. The presence of similar highly fluorescent moieties in DNA may have gone undetected by conventional means and become apparent in the fluorescence spectra because of the low intrinsic ϕ_f of DNA. Absorption measurements by Sutherland and Griffin (53) from very concentrated and carefully purified DNA preparations showed absorption above 300 nm.

Also suggestive of this possibility (or some other "impurity") is

that the fluorescence at wavelengths ≥ 400 nm can be excited by light at ≥ 300 nm. The absorption was not measured reliably in 1 cm cells at the concentrations used in the fluorescence experiments reported here so the quantum yields can not be estimated. However, the intensity in purified samples of DNA was seen to be consistent within an order of magnitude. Similar spectra with fluorescence above 400 nm were observed by Vigny et al. (54) with excitation at 305 nm.

The main features of spectral shape, the monomer like principal peak, and the component at wavelengths ≥ 400 nm were seen consistently in all polymer samples. Only small differences in the relative intensity of the two components were observed with different purification procedures, biological sources and without the use of filters for the excitation light. The differences between samples were always much less than the difference between the polymer spectra and spectra from monomer mixtures.

In the spectra from these more reliable samples pictured in Figure 2, the relative intensity of the long wavelength component is greater relative to the main peak at 325 nm than was observed in spectra using less purified samples and/or unfiltered excitation light. This can be reasonably explained both by the removal of protein and scattered stray light. Both artifacts appear to be more intense at 330 nm than at 400 (see below section on stray light). As

was mentioned before, the fluorescence of proteins often occurs with a maximum about 330 nm and peak width comparable to the DNA fluorescence. Stray light scattered from the excitation monochromator appears to be more intense at shorter wavelengths (see below).

Consistent minor differences in shape of the fluorescence curve were seen in the case of the DNA from hen erythrocytes and that from an extreme thermophile obtained from Dr. Gordon Julian. The relative intensity of the long wavelength fluorescence of the DNA from hen erythrocytes was less than for *E. coli* or calf thymus DNA when all samples had been treated by high speed ultracentrifugation. The deproteinized DNA from the thermophilic bacteria was the opposite, its long wavelength fluorescence being relatively greater when compared to similarly purified samples from *E. coli*. The source of the differences is not known. Deproteinized samples of hen erythrocyte DNA were not soluble in buffers of a wide range of ionic strengths so reliable spectra of deproteinized samples were not obtained. It is possible to speculate that the spectral shape is due to protein contaminants in centrifuged samples. Further studies on the thermophilic DNA were not pursued either even though the results from it appear reliable. The possible connection between the organisms' ability to live at elevated temperature and the somewhat unusual fluorescence spectrum of its DNA is an intriguing question but it was

thought to lie outside the scope of this project. The G-C content of the related species Thermus aquaticus has been given as ~67% (55) suggesting that higher G-C content may enhance the relative intensity of the long wavelength fluorescence. Other source of DNA with documented high G-C content were not available.

Excitation Spectra

Figure 3 shows the average of 4 fluorescence excitation spectra from deproteinized samples of DNA from *E. coli* and calf thymus. The fluorescence is viewed at 330 nm and the excitation light is passed through optical filters which are described below. Being from the most carefully purified samples and light sources it is considered the most reliable estimate of the excitation spectrum of the fluorescence from DNA in its native state at room temperature. The most striking feature is the approximately 30% increase in fluorescence intensity in going from 240 to 280 nm excitation. The spectrum bears a remarkable resemblance to the spectrum in Figure 4 obtained from an equimolar mixture of the nucleotides. This figure shows the absorption of the monomer mixture, the fluorescence excitation spectrum and excitation spectrum predicted from the individual absorptions and fluorescence intensities of the components. The excitation is expected to exceed the absorption spectrum above 260 nm because of the higher quantum yield and relative absorption of pyrimidines in this region.

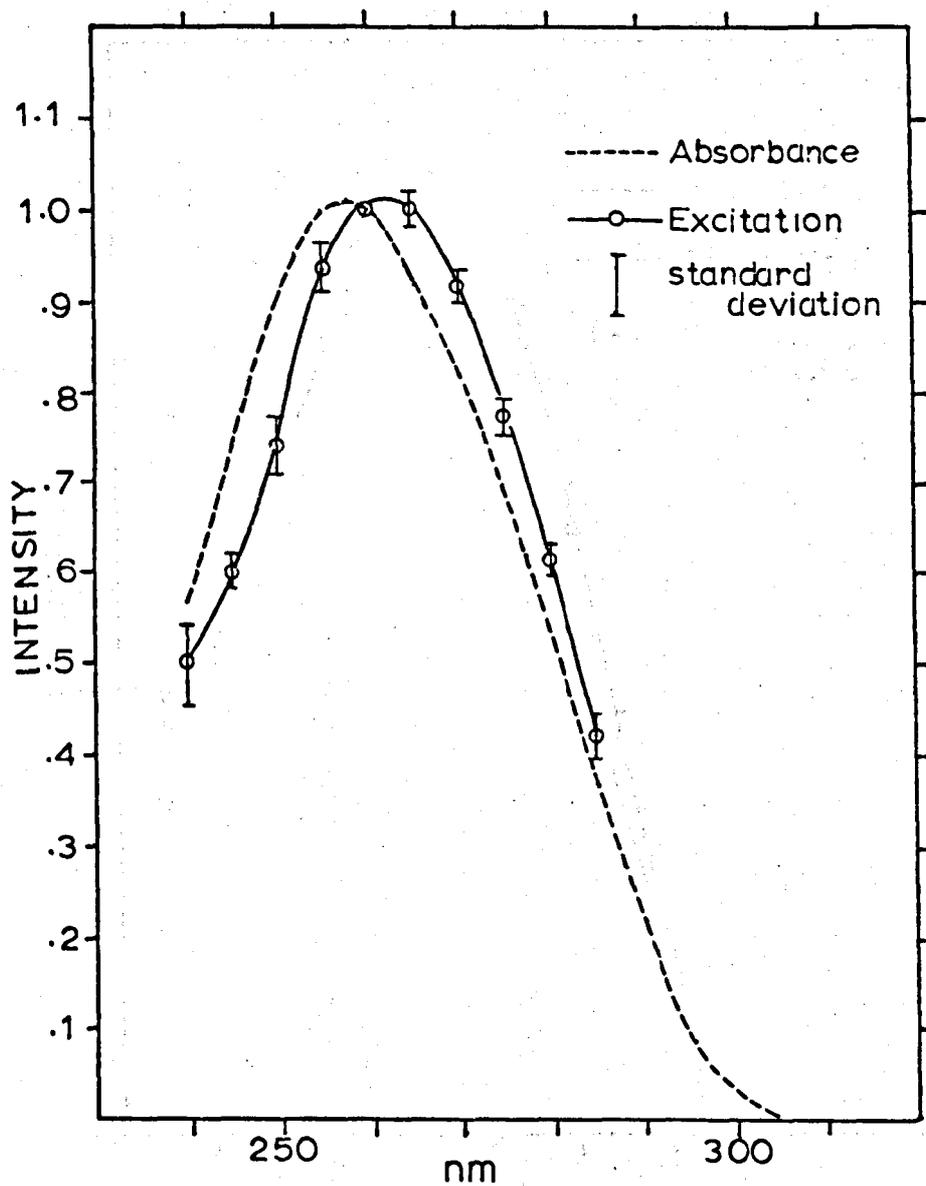


Figure 3. Averaged absorption and excitation spectra of fluorescence at 330 nm from 4 samples deproteinized DNA (same as Figure 2). Optical filter in excitation beam.

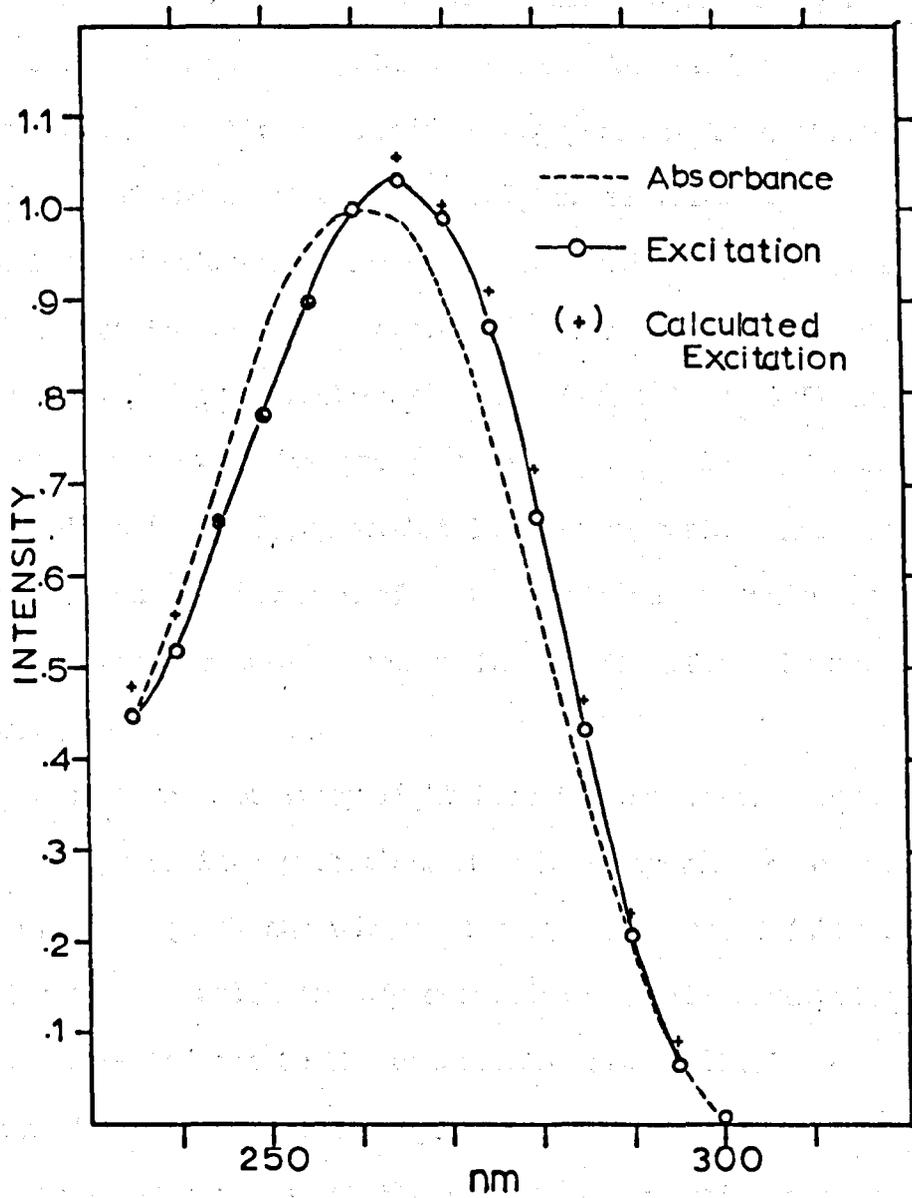


Figure 4. Excitation spectrum for fluorescence at 330 nm from equimolar mixture of nucleotides. (+) calculated from quantum yields of individual monomers multiplied by fractional absorption at λ_{ex}

In both figures, the excitation spectrum is similarly red shifted from the corresponding absorption spectrum. The similarity of the two suggests strongly that the pyrimidine and purines are emitting independently of one another in the polymer as well.

The limited polarization measurements made can only serve to place limits on the degree of energy transfer (see below). The moderate amount of polarization observed (λ_{ex} 260, λ_f 325) indicated that there is not extensive energy transfer occurring. The data of Rahn and Sellin (56) offers similar limited support. Measurements of the photoinduced deiodination of 5' iodocytidine in denatured DNA indicated energy was transferred no further than from the nearest neighboring base.

The elimination of stray light from the excitation source was crucial to such an interpretation. Previous experiments on similarly deproteinized samples, but without the use of chemical filters gave excitation spectra which closely coincided with the absorption spectrum. This led one to the conclusion that excitation energy was being transferred from excited pyrimidines in DNA.

In the earlier stages of the research, excitation spectra similar to the ones estimated to be the best were obtained from samples which had been purified by low and high speed cycles of centrifugation.

Excitation light was not filtered. In view of the results with the deproteinized samples with and without the use of filtered excitation source and the somewhat higher quantum yields, the increase in fluorescence excitation at wavelengths more than 260 nm can be attributed to protein or other contaminants in samples. This notion is reinforced by excitation spectra from calf thymus samples which had only been clarified by filtration or low speed centrifugation. In these samples an almost two-fold increase in fluorescence excitation was observed over the same wavelength region, (240-280 nm).

Excitation spectra for fluorescence at 400 nm from deproteinized samples of *E. coli* and calf thymus DNA are shown in Figure 5. The spectra were obtained using chemical filters for the excitation light source. In contrast to the excitation spectrum for fluorescence at 330 nm, the excitation closely follows the absorbance. This was seen in another fashion by comparison of the fluorescence spectra produced by different wavelengths of excitation. The relative intensity of the fluorescence at wavelengths more than 400 nm appeared to increase with decreasing wavelength of excitation. However, when the fluorescence intensity is normalized to equal absorption and excitation intensity the fluorescence at these longer wavelengths remains essentially constant.

The close coincidence of the excitation spectrum of fluorescence

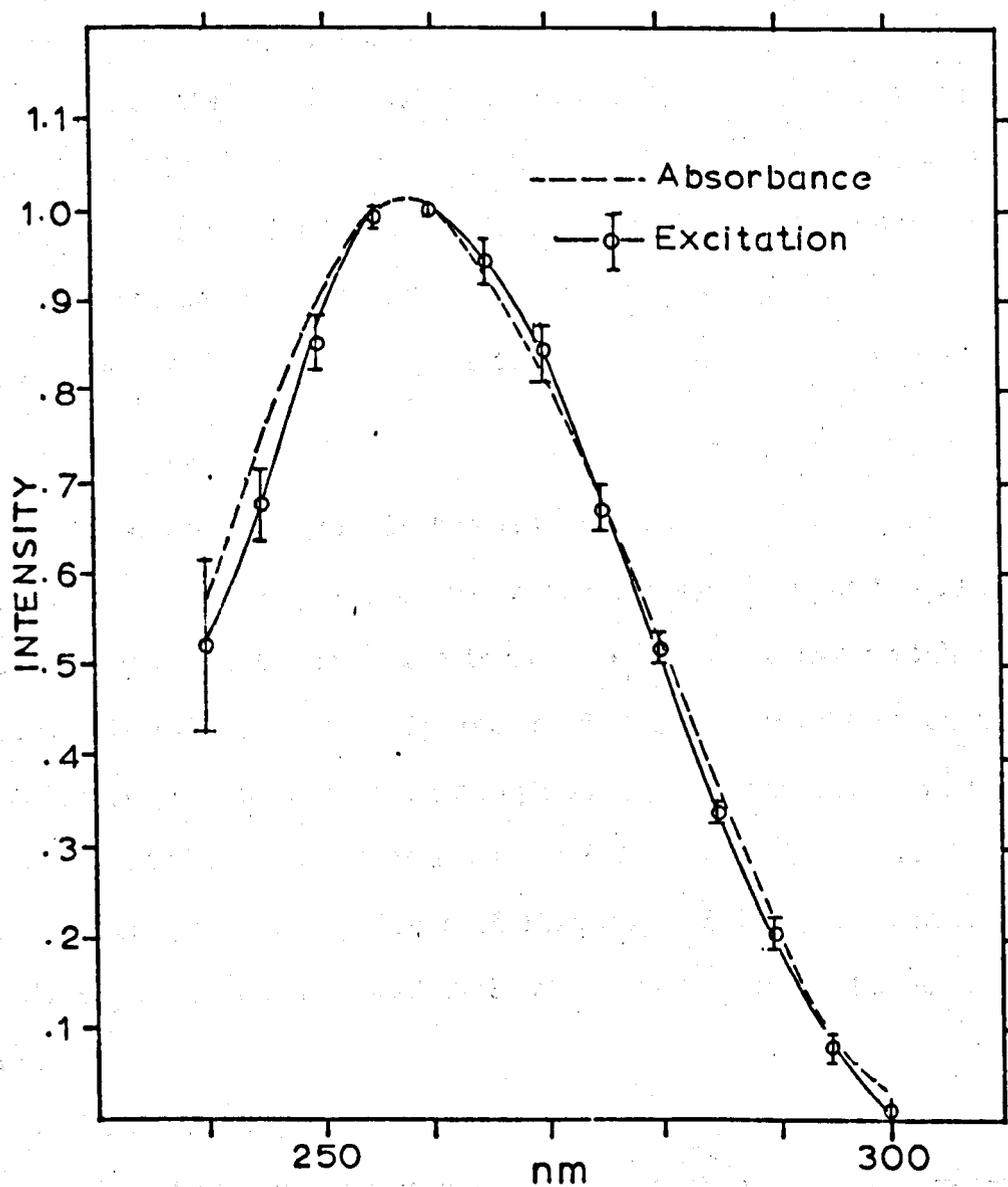


Figure 5. Excitation spectrum for fluorescence at 400 nm from deproteinized calf thymus and *E. coli* DNA. Optical filter used in excitation light path.

at 400 nm with the absorption spectrum might be expected as the fluorescence of the pyrimidine is of about the same intensity as the purines at this wavelength. The excitation of the monomers follows the absorption quite closely also. Not much can be concluded about the nature of the long wavelength component of the fluorescence from the excitation spectrum. Measurement of polarization of emission was not possible due to low signal to noise ratio at long wavelengths.

Photochemistry

It was noted early in the research that performing the fluorescence experiments caused a gradual increase in the fluorescence intensity above 400 nm. The increase was most evident with excitation at wavelengths ≥ 270 nm. The observed changes are almost certainly an indication of the formation of cytosine photoproducts as was demonstrated by Hauswirth and Wang (57,58) and Vigny and Favre (59). These workers found the rate of formation of the photoproducts and calf thymus DNA and showed their absorption maximum to be around 330 nm.

Using the experimental rate data (57) and estimates of the light fluxes used in experiments here, it was estimated that a two-fold increase in the fluorescence due to the cytosine photoadducts would take about 10 hr. This was qualitatively confirmed by experiment. Since the formation is slow on the time scale of obtaining a

fluorescence spectrum and the absorption of photoproducts is weak in the region less than 300 nm it was found that stirring the solution before the spectrum is recorded is sufficient to produce the same spectrum (at a given wavelength of excitation less than 300 nm) over a 4-5 hr period. It was not possible to reproduce excitation spectra at wavelengths over 300 nm over the same time span, but spectra of this sort recorded at the very beginning of the experiment session avoided the problem.

Polarization Ratios

Only limited reliable polarization data was obtained from neutral samples. Polarization ratios of 1.3 ± 1 ($\lambda_f = 325$, $\lambda_x = 260$) were obtained and correspond to θ values of $48^\circ \pm 5$. Although some energy transfer might be inferred from the apparent angle between absorbing and emitting dipoles it does not appear to be over much more than one base. The result is consistent with other studies of energy transfer (56). On the other hand the polarization of GMP is seen to decrease with excitation into the second absorption band (60). This might be observed in a situation where very little energy transfer is occurring. Any conclusions on the limited data available would be hastily drawn at this point.

Stray Light Scattering

Both the detection and excitation monochromators will transmit small amounts of light of wavelengths other than those in the nominal band pass. Rayleigh scattering of this "stray light" in the excitation beam by the macromolecular samples and debris in solution produced artifacts in the fluorescence and fluorescence excitation spectra. The artifacts could almost completely be eliminated by the use of optical filters placed in the excitation light path. Comparison of spectra obtained with and without the use of the optical filters gave estimates of the magnitude of the stray light contribution in earlier spectra obtained without the optical filters.

In the initial stages of the research, sample solutions prepared from DNA as received from vendors often contained large amounts of debris which were removed by filtration through ultra fine sintered glass. Later, debris were removed by ultra centrifugation. Centrifugation produced consistent results which appeared to be relatively free of artifacts due to scattered stray light. In the later stages, using DNA preparations which had been extensively treated to remove proteins, it became apparent that light scattering artifacts were no longer negligible because of the smaller quantum yields observed, and debris introduced into samples in the process of removing proteins.

It is not known to what extent the scattered stray light is due to debris and/or to the macromolecular sample. Centrifugation at low speed was effective in reducing levels of debris but stray light scattering could still be observed in samples treated in this manner. Rigorous filtration procedures were not attempted as the total effect is shown to be small.

Accounting for the small contribution in the analysis below gives reasonable assurance that the spectra for the native samples at ca. 25°C are correct. However, the quantitative accuracy of heat and alkali denatured spectra, some of which were obtained without the use of optical filters, are somewhat less reliable. It has been shown for heat denatured DNA that the scattering intensity at 90° to the incident beam is increased about 4-fold over that from the native polymer (61). The study was done at higher ionic strength .1 and about 1/10 the DNA concentration used in the fluorescence experiments. In the instances of heat and alkali denaturation a 2 and 5 fold increase in fluorescence intensity respectively was observed. If all the stray light scattered were known to be from the macromolecule, (typically $\leq 10\%$ of total intensity at 325 nm) this would require that the actual increase in the fluorescence intensity from denatured samples be about 20% to 30% less than that observed. It is likely that debris unaltered by the denaturation contribute a significant

portion of the estimated scattered stray light. In any event the problem should not affect the conclusions drawn from the experiments.

The scattered stray light present in fluorescence spectra can be classified into 2 types:

1) Light that is of wavelengths different from the nominal excitation band pass and appears in the fluorescence spectrum at those wavelengths.

2) Light that is of the wavelength of the excitation band pass but which appears at different wavelengths in the fluorescence spectrum.

Artifacts in fluorescence spectra are due mainly to the first type of stray light. Stray light of the second type has been shown to virtually be eliminated by the strongly absorbing solutions used. This was demonstrated by placing a thymidine sample ($A_{260} \sim 2$) in the excitation light path and scanning the fluorescence of a DNA sample. The fluorescence of the DNA cannot be excited as about 99% of the excitation light is absorbed by the thymidine. The resulting trace is barely distinguishable from the buffer background. When the optical filters are placed in the excitation light path, along with the thymidine, the resulting trace is completely indistinguishable from the buffer background except for a small peak at ~ 350 nm where the absorbance of the filters is weakest. The strongly absorbing samples

($A_{260} = 5-20$) used in fluorescence experiments are assumed to reduce the stray light of the second type to negligible levels.

The filters used to reduce the stray light at wavelengths between 300-500 nm were prepared from Cation X (2,7 dimethyl-diaza-(3,6)-cycloheptadiene-perchlorate) (Calbiochem lot #620322), NiSO_4 and CoSO_4 . The absorption of the combined filters, see Figure 6, had maxima in the region of interest at 323, 395 and 500 nm and minima at 255, 350 and 440 nm.

Lack of large variation in the shapes of the fluorescence curves obtained with and without the use of the filter indicated that the contribution of stray light to the fluorescence spectra was small, but not completely negligible, see Figure 7.

The contribution of stray light of the first type was estimated by comparison of fluorescence spectra obtained with and without the filters. At a given wavelength of excitation, it is assumed that the fluorescence signal without the use of the filter, ($S_n(\lambda_f)$) consists of stray light ($L(\lambda_f)$) and the actual fluorescence from the DNA, ($F(\lambda_f)$)

$$(1) \quad S_n(\lambda_f) = F(\lambda_f) + L(\lambda_f)$$

When the filter is in the excitation light path, the fluorescence signal (S_f) is reduced in two ways. The actual fluorescence is reduced by loss of excitation intensity to the absorption of the

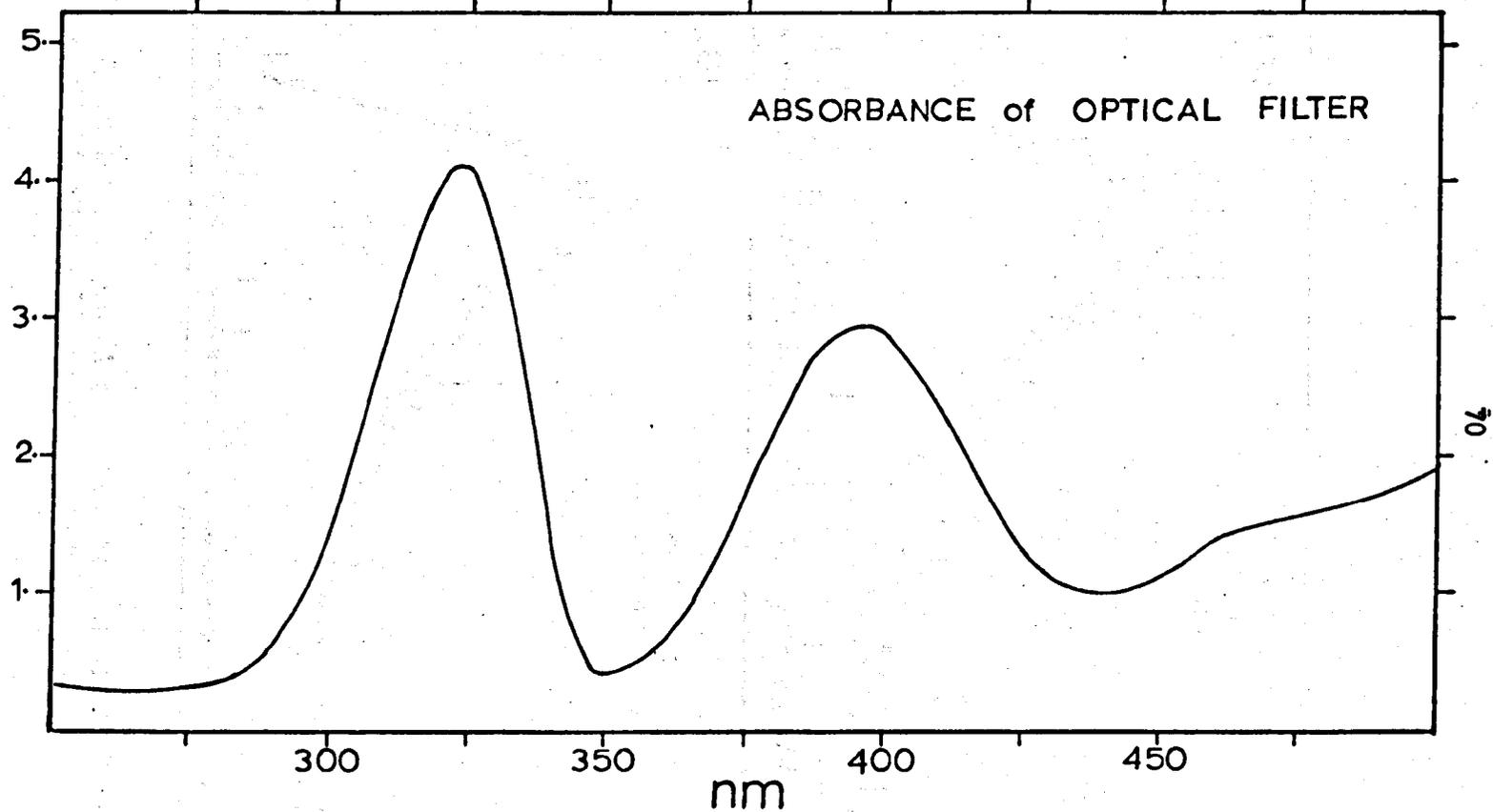


Figure 6. Absorption of optical filter made of Cation X (E_{\max} 323nm), NiSO_4 (E_{\max} 395), CoSO_4 (E_{\max} ~ 530 nm). The Cation X was contained in a quartz cell 5 cm path length. The NiSO_4 and CoSO_4 were mixed in a 1 cm pathlength cell. Mixing of the NiSO_4 and Cation X resulted in decrease of Cation X absorbance.

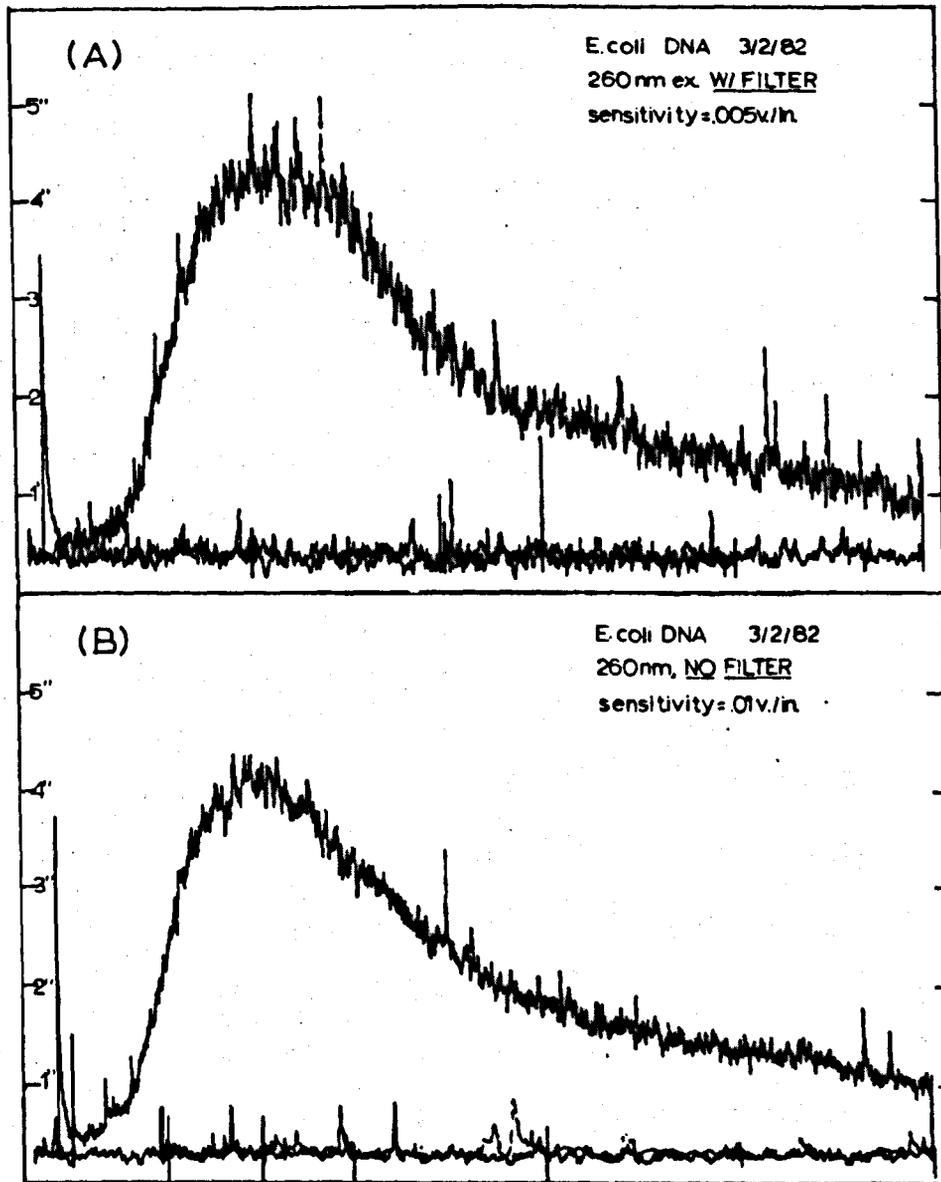


Figure 7. Typical experimental spectra obtained with (A) optical filter in excitation beam (B) without optical filter.

filter and reflectance from filter cuvette surfaces at the wavelength of excitation ($T(\lambda_{ex})$) stray light of the first type will be reduced at a given wavelength to a level proportional to the transmittance of the optical filter at that wavelength ($T(\lambda_f)$). So that at a given excitation wavelength:

$$(2) \quad S_f(\lambda_f) = F(\lambda_f) \cdot T(\lambda_x) + L(\lambda_f) \cdot T(\lambda_f)$$

It is assumed that the fluorescence signal is otherwise unaltered by the presence of the optical filter in the excitation light path.

Since the fluorescence signal and filter transmittances can be readily measured, Equations 1 and 2 can be solved for the scattered stray light contribution.

$$(3) \quad L(\lambda_f) = \frac{S_f(\lambda_f) - S_n(\lambda_f) \cdot T(\lambda_x)}{T(\lambda_f) - T(\lambda_x)}$$

The analysis was carried out at 5 nm intervals between 280 and 500 nm, using simple APL programs. Data used was from two neutral deproteinized DNA samples (*E. coli* and calf thymus). Both samples were centrifuged at low speed. It was found (as expected) that the intensity of the exciting light (250-280 nm) was reduced by the filter by a factor within $\pm 3\%$ of the transmittance of the filter in this wavelength range.

In both cases (with excitation at 260 nm), it was found that the ratio of the fluorescence intensity at the maximum obtained with the

optical filter over the obtained without the filter was 7-10% less than the transmittance of the filter. This is to say that the fluorescence spectrum obtained without the optical filter contained about 7-10% stray light at the fluorescence maximum.

As the control, a parallel experiment was done on a sample of GMP of similar absorption. The ratios of the fluorescence intensities, with and without the optical filter were the same as the transmittance of the filters from 250 to 280 nm within experimental error, $\pm 3\%$. The result is expected and indicates that there is not significant scattering of stray light from solutions of mononucleotides or artifacts produced from the experimental geometry.

Figure 8 summarizes the important details and limitations of the stray light scattering analysis on the samples of calf thymus and *E. coli* DNA, respectively. The figure contains three fluorescence spectra of similar shape which are scaled relative to the spectrum obtained without the optical filter (+). The spectrum obtained with the filter is shown at its intensity relative to the unfiltered (about half as large) (Δ). The third is the calculated fluorescence which would be observed without the optical filter if the stray light calculated were not present (o). The (\square) represent the calculated stray light.

In this figure, the similarity of the calculated fluorescence to

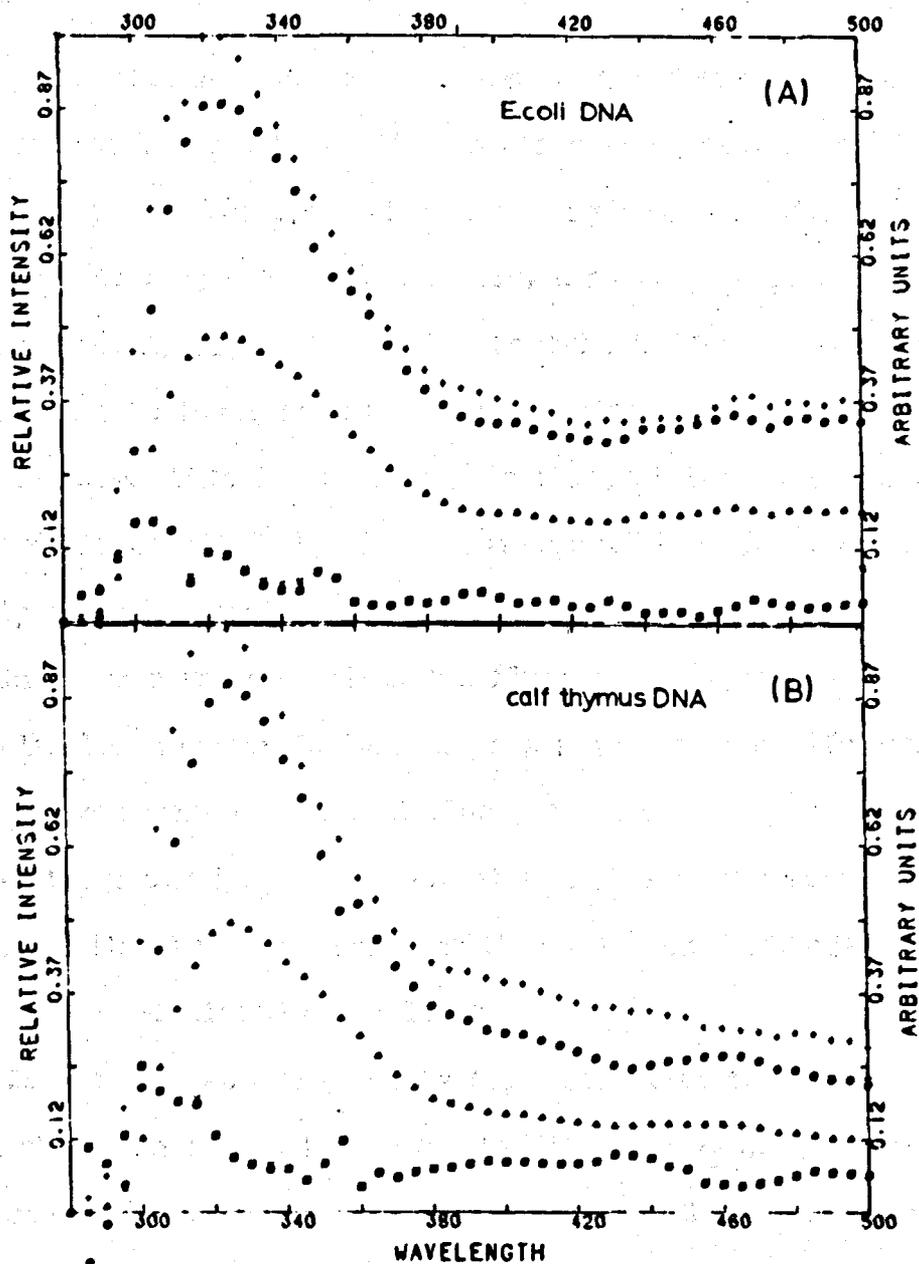


Figure 8. $\lambda_{ex} = 260$ nm, spectra are corrected for photomultiplier sensitivity (+) observed fluorescence without optical filter, (Δ) observed fluorescence with optical filter in excitation light path, (\circ) calculated stray light in spectrum observed without filter, (\square) calculated fluorescence without filter (stray light subtracted out).

the experimental spectra is quite apparent, and the intensity of scattered stray light is small. In other instances, where the apparent intensity of stray light was larger because the samples had not been centrifuged to remove debris, a sharp peak appeared in the calculated stray light at from 340 to 360 nm. This appeared to be an artifact due to lower signal to noise ratios in the spectra obtained using filters. This led to errors in transcribing data, which were increased in magnitude by the denominator of Equation 3 which changes steeply through a minimum in this region.

In these cases the calculated fluorescence spectrum was certainly not reliable. Similar failure of this method to quantify the stray light present were observed at fluorescence wavelengths more than 450 nm. At these wavelengths the signal to noise ratios were low, and errors similar to those producing the peak around 350 often resulted in negative calculated stray light.

It was pointed out to me by Dr. Callis that these difficulties arise where the transmittance of the filter at λ_f and λ_x become the same and it is not possible to solve Equation 3 for the stray light $L(\lambda_f)$. The problems at 350 nm (λ_f) are undoubtedly due to this. The analysis is useful only in fluorescence wavelengths when the filter absorbance is high.

Because of these failures, the spectra reported as the most

reliable are those obtained using chemical filters in the excitation light peak. These spectra are used only when visual inspection shows they are little different from the spectra obtained without use of filter, indicating that the contribution of stray light is small. In some instances there may be a small amount stray light in the region around 350 nm due to the weakness of the optical filter here. Spectra obtained with the optical filter give a more accurate representation of the intensity of the fluorescence at longer wavelengths relative to the intensity at 325 nm.

Heat Denaturation

An abrupt increase in the fluorescence intensity was observed when DNA solutions were heated to about 80°C. This change coincided with the well known increase in absorption that accompanies disruption of the hydrogen bonded double helix (62). The temperature range in which this increase occurred depended on the concentration of both salt and DNA in the DNA concentration range used ($\sim 10^{-3}$ M in nucleotides). The change was observed to a varying extent in all samples examined. Experimental difficulties especially in purification and temperature recording prevented compilation of a large amount of quantitative data. However, the data obtained from deproteinized samples, one calf thymus and one E. coli sample along

with less complete measurements on other samples provide a strong confirmation that the fluorescence observed from the undernated samples is indeed from polymeric DNA.

Seen in Figure 9 is the increase with temperature of the fluorescence at 330 nm ($\lambda_{ex} 260$) from a deproteinized sample of *E. coli* DNA. Also shown for comparison are the proportional increase in absorption at 260 nm of an aliquot of the same preparation observed in a separate experiment. The proportional changes in fluorescence (330 nm λ_f , 260 nm λ_{ex}) of an equimolar mixture of mononucleoside phosphates is shown for comparison. The fluorescence intensities have been normalized to equal absorption and excitation intensities and are shown as the ratio of the quantity at a given temperature to its initial value at 25°C.

The main feature to note is the coincidence of the abrupt increases in fluorescence and absorption. There is no change in the position of the fluorescence maximum or in the spectral shape as the temperature is raised and denaturation proceeds (not shown in figure). The near coincidence of the absorption and fluorescence increases along with the lack of any change in the spectral shape and position leave little doubt as to the authenticity of the fluorescence observed from native samples. It is difficult to imagine that anything but the nucleic acid could increase the absorbance, cause the observed

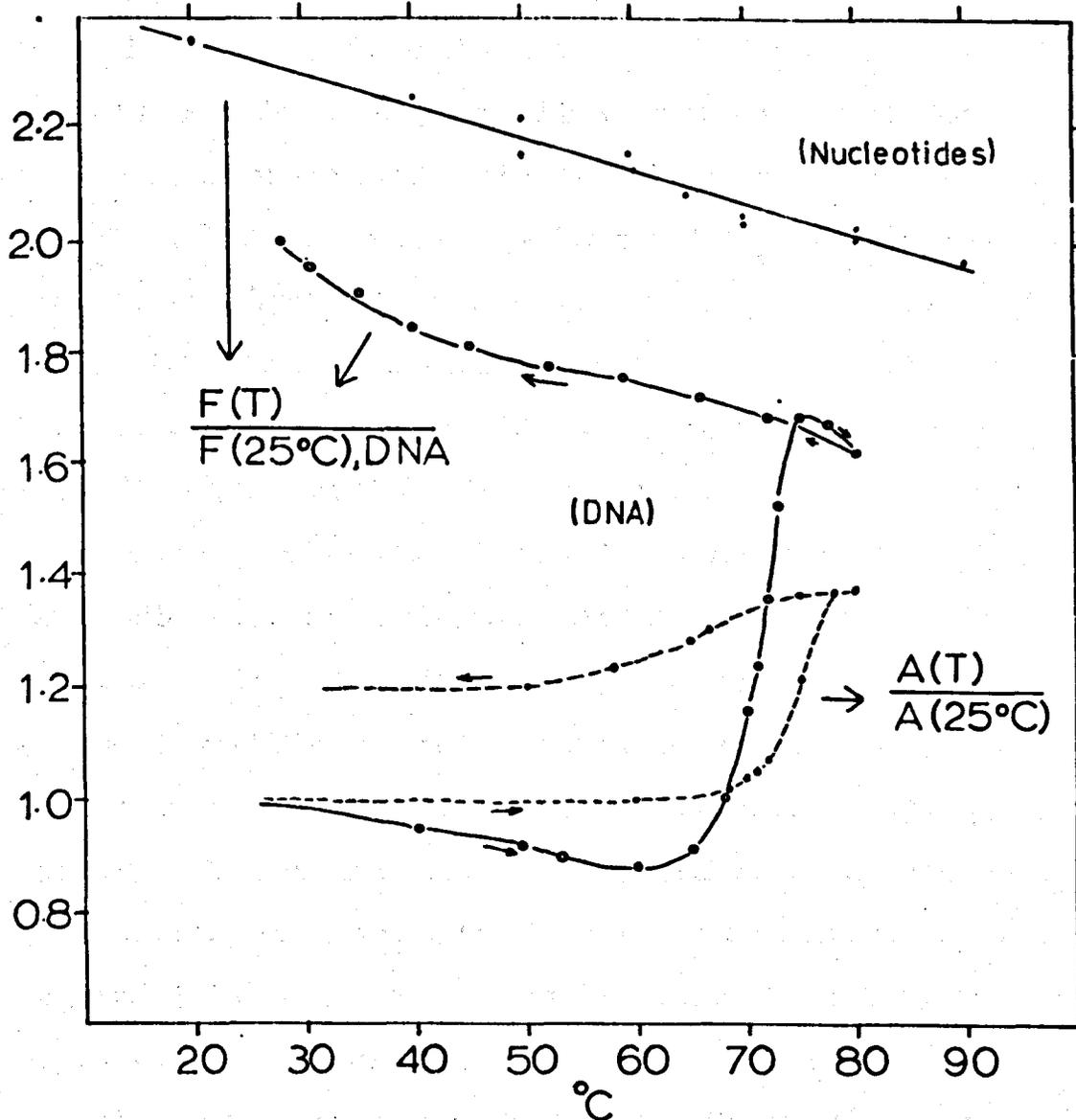


Figure 9. Plot of fluorescence intensity at 325 nm for *E. coli* DNA and nucleotide mixture as a function of temperature. Fluorescence of DNA is normalized to account for absorption changes and is shown as a ratio to that observed at initially at 25°C in native conformation. Absorption shown as ratio to initial state 25°C from a separate experiment. Small arrows show direction of temperature change.

increase in the fluorescence intensity, and cause no significant change in the shape of the fluorescence at 80°C.

An interesting sidelight to this centrally important feature is the dependence of the denaturation temperature on the concentration of the DNA ($\sim 10^{-3}M$). In earlier experiments, absorption measurements were done on aliquots of sample which were diluted approximately 5-fold in .006 M buffer. The changes in absorption occurred about 10° lower than the temperature at which the increases in fluorescence were seen. The absorption changes occurred at about the temperature region expected from previous studies (54). It was necessary to use 1 mm pathlength cells so that absorption at 260 could be observed in undiluted samples. One this was done as described above (Experimental section) it could be verified that the absorbance and fluorescence changes occurred at close to the same temperature. The absorption changes of these concentrated solutions still occur at slightly different temperatures than the fluorescence changes. The difference is probably due to the less uniform temperature under the different experimental conditions of the absorption measurements.

The result of an experiment similar to the one in the preceding figure except that the DNA was from calf thymus is shown in Figure 10. It is readily apparent that the increase in fluorescence from this sample is much less (~25%) than the increase for the E. coli sample

(ca. 70%). Though it is not obvious in the figure the initial fluorescence in the calf thymus sample at 25°C corresponds to a quantum yield of about 5.5×10^{-5} whereas that for the *E. coli* sample gave Φ_f of 3.3×10^{-5} . It is notable too that the increase in fluorescence has a midpoint at ca. 85°C as opposed to ca. 70°C for the *E. coli*. This might be an indication of residual protein contamination of the calf thymus. The final fluorescence intensities are actually nearly the same (at 80°C) for both samples. As the fluorescence of tryptophan is known to decrease about 4-fold between 20 and 80° (32). This is a further indication that the higher initial Φ_f for the calf thymus sample is due to a protein contamination not removed by purification procedures.

It is interesting to note the decrease in fluorescence with temperature in the monomer mixture (Figure 9) as well as in native and denatured polymers. This was seen to be reversible in all cases including the native polymer provided the native samples were not heated past the initial denaturation point. This is as is expected from the decrease in fluorescence of DNA and its components in going from 80° to 300°K.

As was mentioned before, there was very little change in the shape of the fluorescence spectrum as the sample was heated through the denaturation temperature. However, when denatured samples are

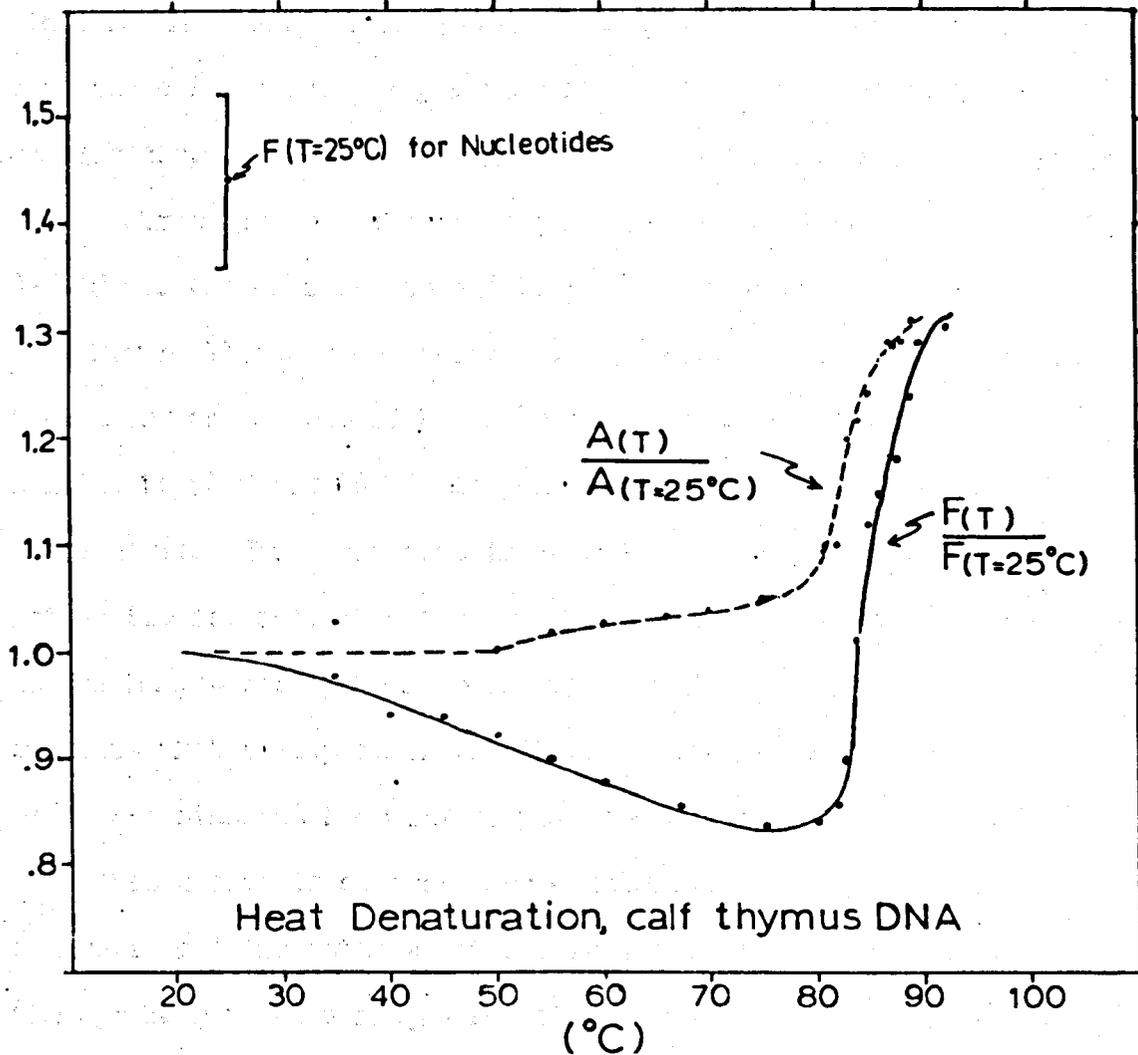


Figure 10. Plot of fluorescence 325 nm and absorption at 260 nm from deproteinized calf thymus DNA as a function of temperature. Same format as Figure 9. Absorption from separate experiment. Fluorescence of nucleotides at 25°C is shown as the ratio to fluorescence of DNA (calf thymus) at 25°C.

cooled back to room temperature, there is a dramatic reversible increase in the fluorescence at wavelengths greater than 400 nm. Since the intensity at the principal maximum increases with cooling also there is an even larger increase in the intensity at longer wavelengths. The normalized intensity at 400 nm relative to that in the spectrum from the native polymer is seen to increase about 2- to 3-fold for the calf thymus and *E. coli*, respectively.

Figure 11 shows the average fluorescence of one sample of calf thymus and one sample of *E. coli* DNA which has been denatured by heating to 95°C for 15 minutes and quickly cooled back to room temperature. The excitation is at 260 nm using optical filters. The native fluorescence is shown for comparison. This reversible increase in the long wavelength component upon cooling of the denatured polymer suggests that this part of the fluorescence may be from exciplexes which are hindered from forming in the native state.

This notion is supported by consideration of spectra from synthetic polynucleotides notably poly A at room temperature (4,14). The spectrum of this single stranded synthetic polymer has a maximum at ~400 nm. The intensity of the peak is sharply dependent on temperature decreasing by a factor of 2 in going from 4 to 24°C.

A similar spectrum ($\lambda_{\text{fmax}} \sim 400 \text{ nm}$) and dramatic temperature dependence is seen in poly C when half the cytidines are protonated

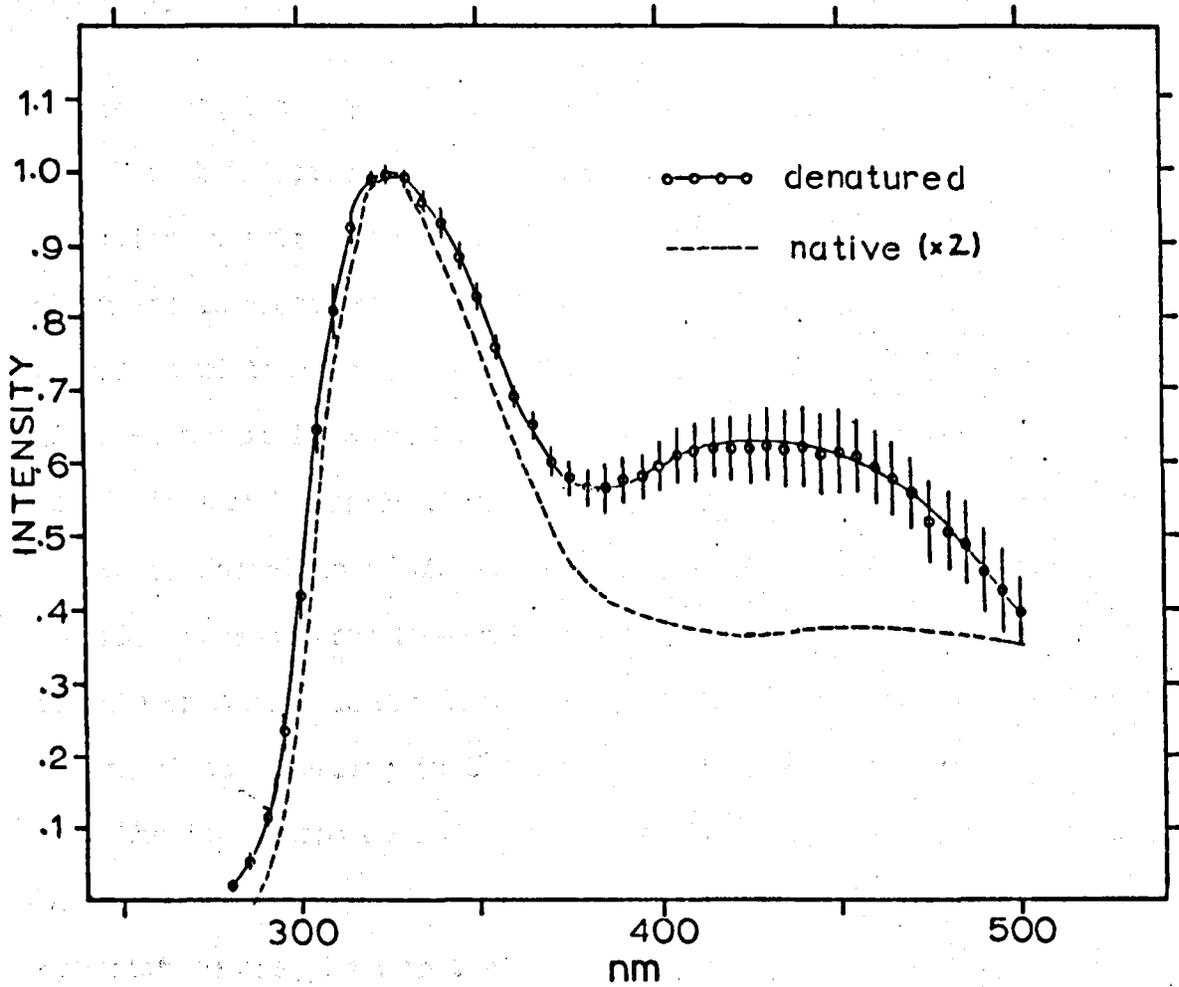


Figure 11. Fluorescence spectrum of *E. coli* and calf thymus DNA heated for ~15 min at 95°C then cooled in ice water to 25°C. $\lambda_{ex} = 260$ nm .006 M phosphate buffer pH 7. Average of 2 spectra using optical filter in excitation beam.

around pH 4.2, and for the dinucleotide ApA and CppC in neutral solution.

Fluorescence Changes as a Function of pH

Abrupt increases in the fluorescence of DNA were observed with titration to extremes of pH. These changes occurred at pH values which are in excess of the values expected from the ionization constants of the nucleotides. The pattern of changes in the DNA fluorescence is in accord with both the fluorescence behavior of the nucleotides and earlier studies which have demonstrated that the native conformation of DNA suppresses the ionization of the bases. The fluorescence from DNA at high and low pH offers additional support to data from neutral samples, and indications of limited excited state interactions occurring in the ionized and disordered forms.

The ionization constants of the nucleosides and nucleotides have been measured and compiled by Jordon (63). Relevant values from his compilation are given in Table 2.

Though not shown, there is a considerable decrease in the acidic pK_as in going from nucleotides to nucleosides. A similar but smaller effect is seen for the 2' and 3' phosphates. Both can be explained in terms of the negative charge that would be on the phosphate group in the pH range 3-4 (64). The difference is not likely to be important in highly polymerized samples. There is also a 0.4 to 0.6 pH unit

Table 2. Dissociation Constants of Deoxynucleotides (63).

	Acidic	Basic
Deoxyadenosine 5' phosphate	4.4	-
Deoxyguanosine 5' phosphate	2.9	9.7
Deoxycytidine 5' phosphate	4.5-4.6	-
Thymidylic acid	-	10.0
Phosphoric acid	-1, 5.9-6.4	

decrease for purine nucleotides when the sugar is ribose instead of deoxyribose. Barring the event that all the material studied is RNA and not DNA this should not be relevant. To my knowledge, there is no reason to believe this is so.

It was first noted by Peacocke and coworkers (65-67) that the uptake of hydrogen or hydroxide ions by native DNA doesn't occur at the pH values expected from the ionization constants of the bases. They found that in titration from neutral solution to basic pH's the observed pKa's were well above 11.0. The observed acidic pK's were from 2.8-3.5, the lower pH values corresponding to higher ionic strengths .15 to .02, respectively. Back titration from the extremes of pH corresponded closely with the values that would be predicted from ionization constants of the four nucleotides. The displacement

of the curves for titration from neutrality is apparently caused by hydrogen bonding between Watson-Crick base pairs. Later work has assigned the sites for protonation and removal of protons from the nucleotides and seem to be in accord with this reasonable notion (64). In base, the protons are removed from the N_3 nitrogens of guanosine and thymidine. There is some question however concerning the site of proton addition in acidic solutions. Absorption measurements have clearly indicated that there is considerable binding of protons on cytosine before the denaturation of the native form occurs (68-70). The site is apparently the N_3 nitrogen, and since this site is involved in base pair hydrogen bonding, the question arises as to whether such a large proportion (~80%, pH 3.1, 20°C, $\mu=0.1$) (68) of the cytidines can be protonated without disrupting the native conformation.

The fluorescence data to be reported here does not foster any new conclusions regarding this question mostly because the fluorescence of CMP does not change appreciably in this pH range. Comparison of fluorescence from the free nucleotides and polymeric DNA at various pH's indicated the same behavior as was observed in the absorption and potentiometric work. However, proceeding from this similarity, some new aspects of the effect of polymerization on the excited states of the nucleotides can be approached.

With protonation only the purine nucleotides are known to change their fluorescence characteristics. For both adenine and guanine nucleotides, a sharp increase in quantum yield is observed and the wavelength of the fluorescence maximum shifts to longer wavelengths. Only the fluorescence of GMP is seen to increase with loss of a proton. The changes observed in the fluorescence of the nucleotides are summarized in Table 3. The values presented there are in good agreement with previous study by Vigny and Duquesne (4). Comparison of quantum yields for the individual nucleotides and a mixture of them

Table 3. Quantum Yields of DNA and Nucleotides

	pH 2.4		pH 7		pH 12	
	λ_{fmax}	$\phi_f \times 10^5$	λ_{fmax}	$\phi_f \times 10^5$	λ_{fmax}	$\phi_f \times 10^5$
AMP (or Adenosine)	390	80±10	312	3-4	315	3-4
GMP (or Guanosine)	370	1700±200	330	7±1	330	50±1
CMP	325	8	325	9±2	330	8±1
TMP	No Change Expected		327	10±1	330	12±1
Nucleotide Mixture	375	500±50	325	6±.5	325	17±1
DNA	362	58±4	325	3-4	325	16±1

ϕ_f of nucleotides independent of excitation wavelength. Excitation of nucleotide mixture and DNA both at 260 nm.

with DNA Φ_f provides a good starting point for discussion of the fluorescence of the polymer.

Figure 12 compares the changes in fluorescence as a function of pH for a mixture of nucleotides with that from two samples of deproteinized DNA. The nucleotide mixture's fluorescence increases much as would be expected from the ionization constants of AMP and GMP. Titration from neutrality with acid or base for both DNA samples is displaced to more extreme pH values. This is much in accord with potentiometric results. It is strong confirmation of the polymeric origin of the fluorescence to note that the increase in fluorescence coincides with the loss of hypochromicity, and that the low levels of fluorescence observed initially can not be regained with neutralization. The precision of experiments was such that the denatured DNA quantum yields should be regarded as approximately the same as that from the monomer mixtures in neutral solutions. The high fluorescence yield from the back titration of acid calf thymus DNA above pH 4 may be due to instrumental error or impurities in NaOH.

It should be noted that in the acidic extreme of pH 2.4 the fluorescence of the polymer is a factor 8 or 9 less than from the monomer mixture. A similar though less extreme relationship can be noted in the back titration curves from both acid and base. Since the potentiometric studies indicated that back titration of the denatured

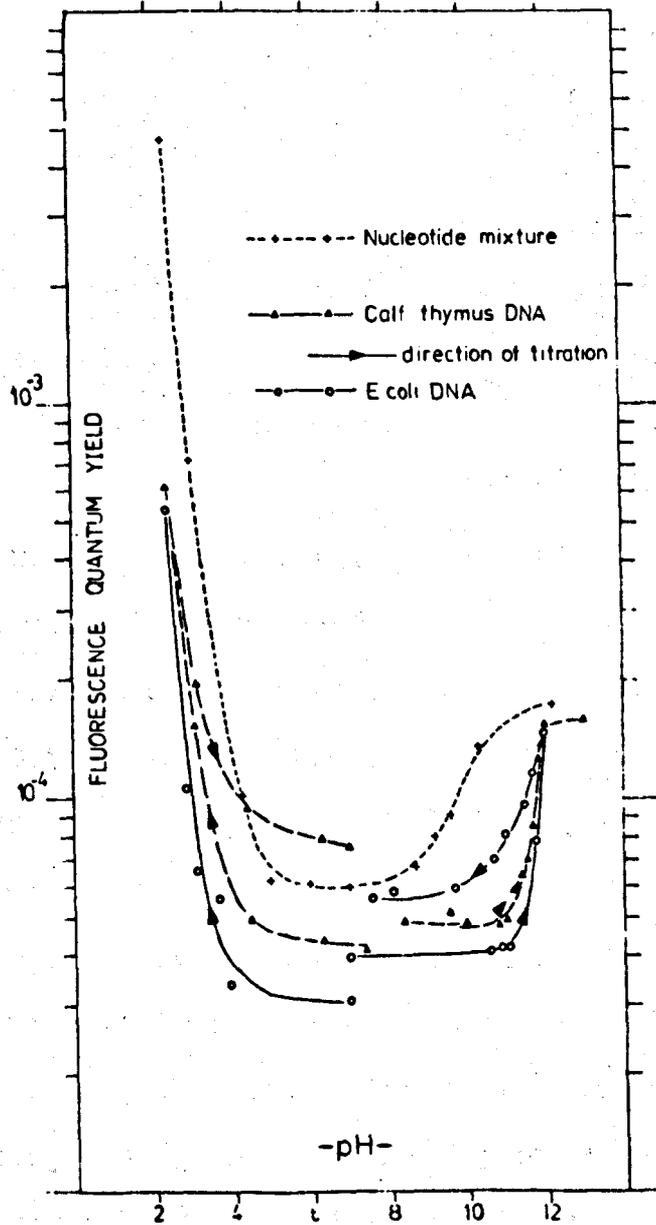


Figure 12. Plot of Φ_f for *E. coli* and calf thymus DNA as a function of pH. Back titration from acidic extreme done only for calf thymus. Back titration in base done only for *E. coli*.

polymer was quite similar to the behavior expected of free nucleotides, it might be reasonable to explain this difference in terms of quenching in the polymer. However, the reason for the diminished intensity of the polymer fluorescence in these instances can not be stated unequivocally. Since the intense fluorescence of the GMP cation is almost certainly the reason for the increased fluorescence seen in both the polymer and nucleotide mixture at pH 2.4, decreasing the degree of protonation of GMP in its ground state would have the same effect. Suppression of the initial uptake of protons by the native double helix has been noted. It is not known if the same conformational factors which caused the initial suppression are still operative at this pH. Because accurate measurement of the consumption of titrant were not taken, it is not possible to estimate independently the degree of protonation in the polymer or the nucleotide mixture.

Lacking the measurement of proton uptake, it can only be speculated that the weaker fluorescence seen in the polymer is due to quenching of the GMP cation excited state. This is reasonable in that the high quantum yield of the GMP cation implies a long lived excited state. A long excited state lifetime may allow for quenching to be observed in the polymer. However, it is just as reasonable to speculate that the relatively weaker fluorescence of the polymer is

due simply to the presence of a smaller proportion of the GMP moieties in their protonated forms in the polymer at pH 2.4. The decrease in the quantum yield for the polymer at pH 2.4 relative to the nucleotide mixture is somewhat at odds with results in synthetic homopolymers (12). The single stranded synthetic polymer quantum yields (except poly U) are seen to exceed those from the monomers in neutral, room temperature aqueous solution (14). The polymer environment apparently is a less favorable environment than room temperature solvent for the radiationless relaxation of excited nucleotides. At pH 2.4, the DNA is presumably single stranded over almost all its chain length, so that the decrease in Φ_f due to hydrogen bonding seen at low temperature in DNA, and hydrogen bonded double stranded polymers at room temperature should not occur.

There is no way to differentiate between the two explanations with the existing data. The fluorescence and fluorescence excitation spectra give some evidence of order in the polymer at pH 2.4, but they do not enable one to distinguish between events occurring before and after the absorption of a photon in regards to the weaker fluorescence from the polymer.

The increase in fluorescence intensity that occurs at low pH is simply explained as the protonation of guanosines and adenosines. This is demonstrated in Figure 13 which compares the fluorescence

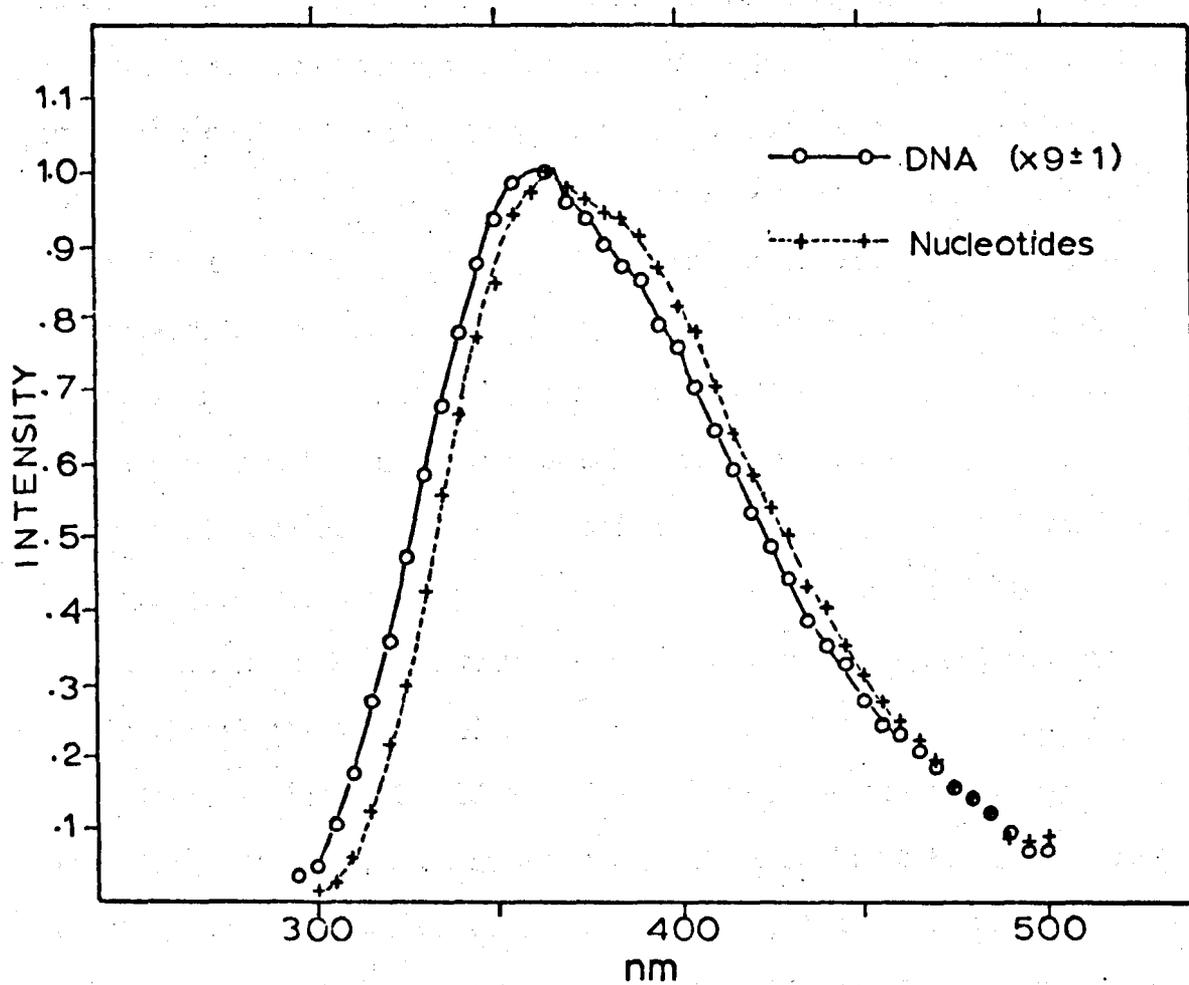


Figure 13. Fluorescence spectra for DNA and nucleotide mixture pH 2.4, $\lambda_{\text{ex}} = 260$ nm. Nucleotide mixture is in 50:50, glycerol:water. Fluorescence corrected for photomultiplier response.

spectra of DNA, a nucleotide mixture and of GMP. The nucleotide mixture is identical to GMP's in shape though its intensity is about .3 of the GMP spectrum with excitation at 260 nm, in agreement with the value expected from the fraction of light absorbed by GMP at 260 nm. The nucleotide spectra were obtained from samples in 50:50 glycerol water solution. This was done to observe the effect of viscosity on the fluorescence. The increase in viscosity produced only about a 5 nm blue shift in the nucleotide spectra. The DNA spectrum is clearly shifted about 10 nm to the blue of the nucleotides which are in this quite viscous solvent. This suggests that the polymer hinders solvent reorientation around the protonated purines. Polarization measurements indicate this also in that the fluorescence of DNA is more polarized throughout the fluorescence band and with different excitation wavelengths.

There is limited evidence of excited state interactions given from the excitation spectra at low pH. Figure 14 is the excitation spectra for fluorescence at 365 nm of the nucleotide mixture and DNA all at pH 2.4 and from the same samples as Figure 16. The nucleotide spectrum is virtually identical to that from GMP⁺. Both follow the absorption spectrum of GMP⁺ quite closely. The absorption of DNA is shown as the dashed line. The small increase in the DNA excitation around 270 nm (relative to that of the nucleotides) may indicate a

limited amount of energy transfer from pyrimidines to the GMP cation.

A different situation is seen at the high pH extreme where the Φ_f of nucleotide mixture and denatured polymer are essentially equal. Ionic forms of guanosine are probably responsible for the bulk of the fluorescence in both cases. The similarity of the Φ_f of the polymer and monomer, and their fluorescence spectra seen in Figure 15 indicates that there is little excited state interaction in DNA at high pH.

The excitation spectrum of two samples of calf thymus DNA at pH 12 are shown in Figure 16. In Figure 17 is a similar spectrum from an equimolar mixture of nucleotides for comparison. The polymer spectrum was obtained without the use of the optical filters discussed previously, and the same stray light scattering problems discussed in connection with excitation spectra of the neutral native DNA may be occurring here. That is, the coincidence of the absorbance and excitation may be partially due to a light scattering artifact, but stray light scattering is expected to be less serious as the quantum yields are 2 to 3 times greater in this case. With this caution in mind the close coincidence of the absorbance and excitation spectra of the polymer at pH 12 may indicate a limited amount of energy transfer, but it cannot be very significant.

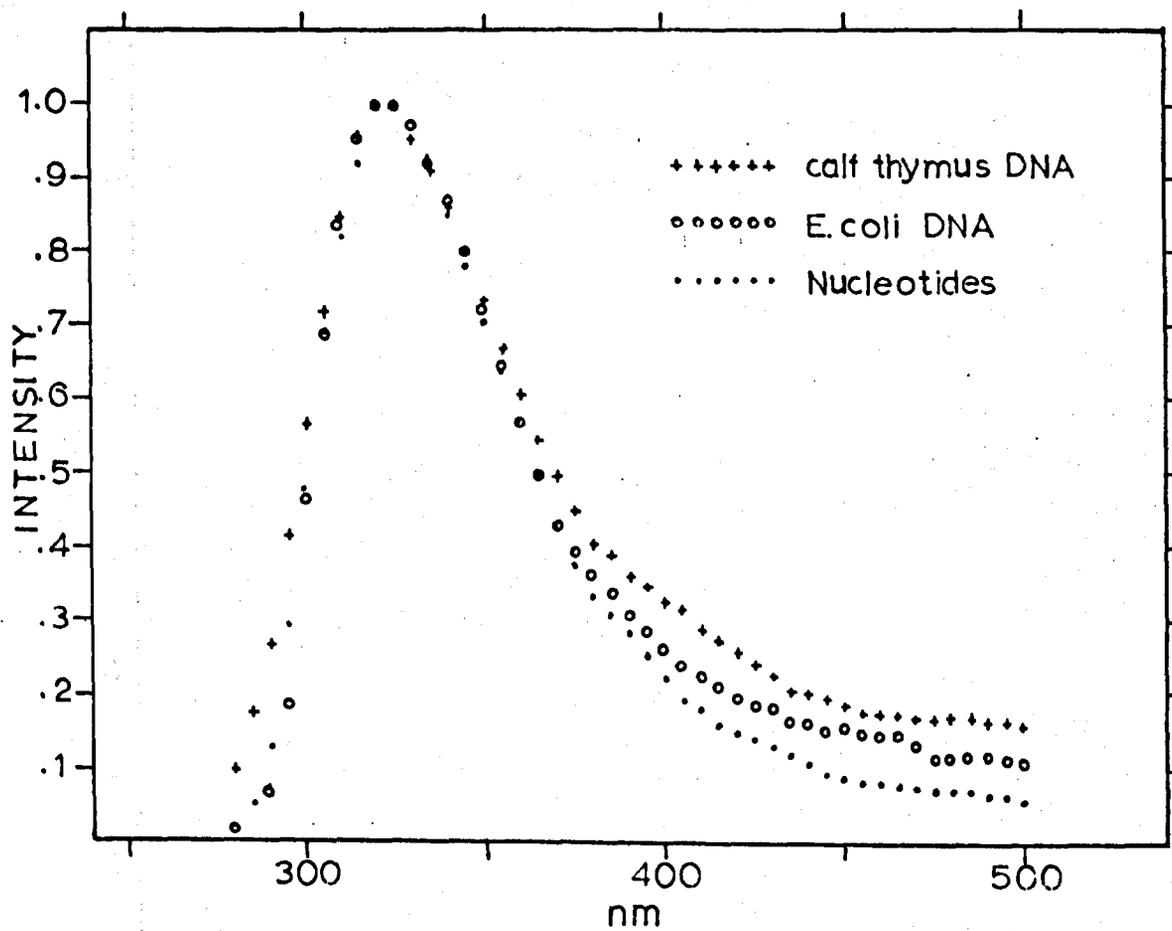


Figure 15. Fluorescence of two samples of DNA and nucleotide mixture pH 12. Intensities are all the same $\pm 10\%$. Spectra corrected for photomultiplier response.

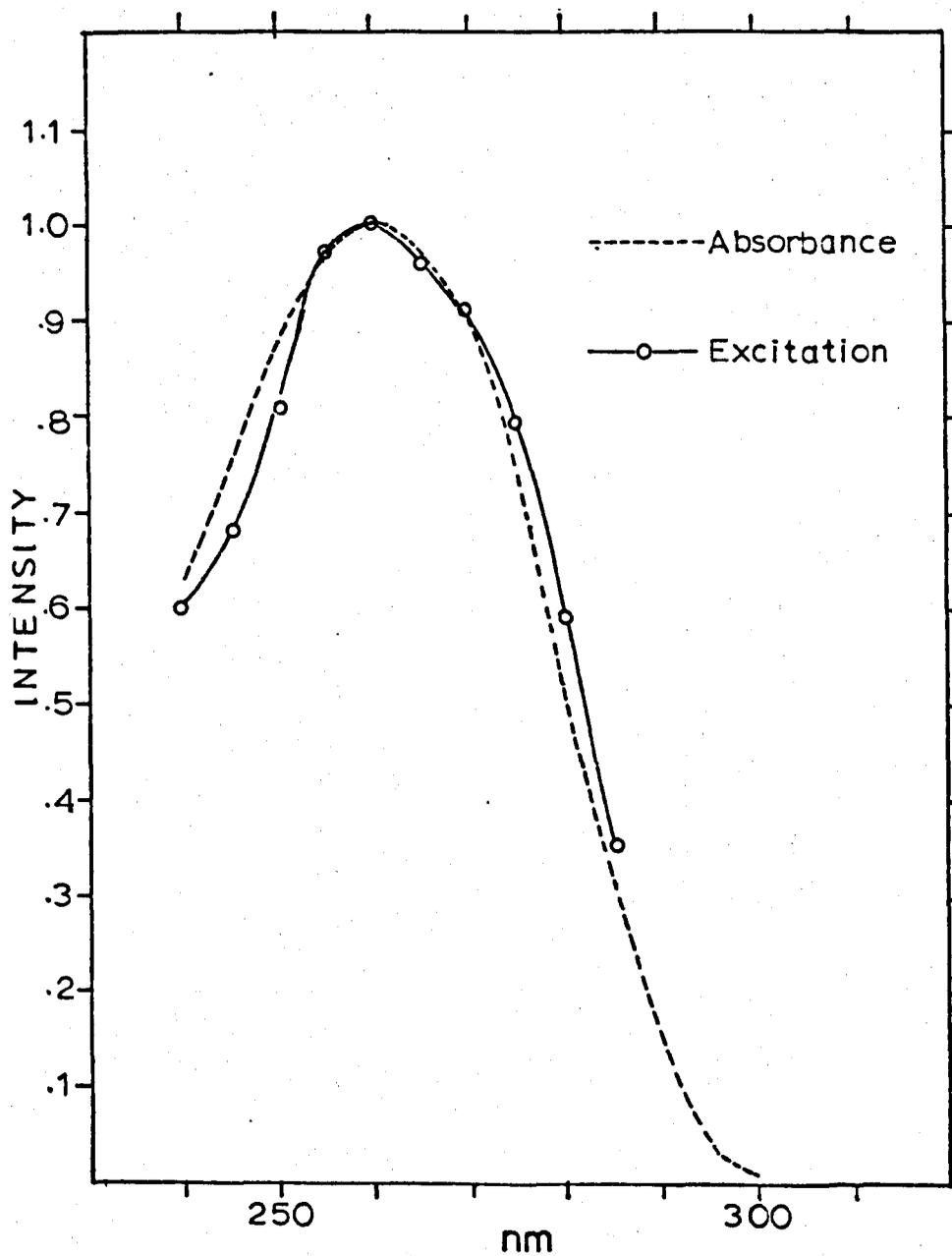


Figure 16. Excitation spectrum of calf thymus DNA, pH 12, $\lambda_e = 330$ nm. No optical filter is used. Absorption spectrum is shown for comparison. Both are average of two samples.

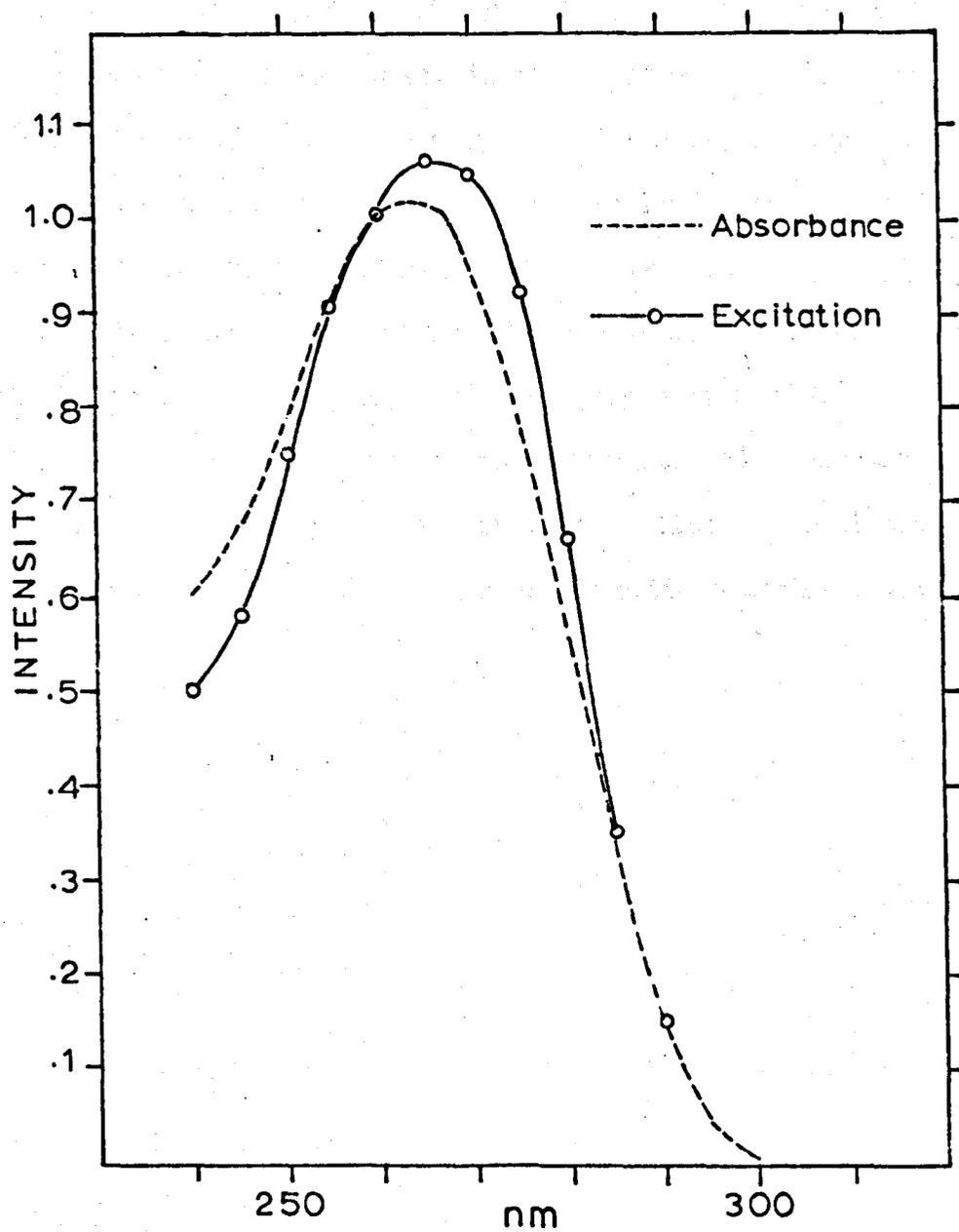


Figure 17 Excitation spectrum of nucleotide mixture pH 12. $\lambda_F = 330$ nm.

In summary, the changes observed in the fluorescence of DNA samples as a function of pH are in agreement with previous studies of the acid base behavior of the polymer and the fluorescence increases seen for the purine nucleotides. The data lend strong support to the authenticity of the weak fluorescence observed at neutral pHs. Fluorescence quantum yields at pH 2.4 may indicate either quenching of excited GMP cations, or suppression of protonation of GMP but the mechanism is unknown. Fluorescence spectra and fluorescence excitation spectra at pH 2.4 and pH 12 give little unambiguous indication of interaction of excited nucleotides with one another.

SUMMARY AND CONCLUSIONS

The fluorescence of DNA from a variety of biological sources has been observed at approximately physiological conditions of pH and temperature. The results are quite different from those observed at 80°K. The quantum yields from the most reliably purified samples are $3-4 \times 10^{-5}$. This is a factor of about 2 less than the quantum yields from equimolar mixtures of mononucleotides. The decrease in the polymer quantum yield relative to the monomer mixture is much less than that observed at 80°K (about 10-fold). Both the diminished degree of quenching in the polymer and low quantum yields are explained in terms of the enhanced radiationless relaxation to the ground state seen in the mononucleotides under these conditions.

Careful purification of commercial DNA preparations produced a 2- to 3-fold decrease in the quantum yield and smaller variations in the quantum yield with wavelength of excitation. This indicates that commercial preparations usually contained some residual protein contaminants. Smaller variations in fluorescence characteristics were seen between batches treated in similar manner. Inference from experiments under heat denaturing conditions and at extremes of pH suggest that some of the variation in fluorescence of purified samples may be due to disruption of the double helical conformation.

The major peak in the fluorescence spectrum resembles very

closely the spectrum of the nucleotide mixture, with the maximum occurring at 325 nm. The fluorescence attributed to exciplexes with a maximum at 350-360 nm seen at low temperature and by Daniels (20) and by Anders (21) at room temperature is not seen. However, a component (ca. 25% of the fluorescence) is seen with a maximum around 430-440 nm. The explanation favored for this is in terms of long lived exciplexes in which the solvent and/or the emitting bases have relaxed to much lower energy conformation in the excited state, the relaxation being facilitated by the more fluid microscopic environment. The long wavelength fluorescence seen in the native samples and more clearly in heat denatured ones is similar in wavelength and temperature dependence to excimer fluorescence seen in synthetic polynucleotides. Little supporting evidence for detailed interpretation is available and the possibility of artifacts introduced during isolation and purification procedures are not excluded. A tempting speculation is that the long wavelength component is due to a naturally occurring but heretofore undetected constituent of the biological polymer.

The excitation spectrum for fluorescence at the principal maximum at room temperature and neutral pH is similar to the spectrum for the nucleotide mixture. It is shifted to the red of the absorption in both cases, and can be explained most simply by the greater proportion of absorption above 260 nm and higher quantum yields of the pyrimidine

bases. The similarity excitation spectra of DNA and the nucleotide mixture indicated that the polymer fluorescence is due mostly to emission from independent bases.

Observations of fluorescence under conditions of extreme pH and heat denaturation offer strong support for the authenticity data and interpretations at neutral pH and ambient temperature. The changes in fluorescence characteristics observed coincided closely with the well known increase in absorption associated with disruption of the double helix. At pH 12 of the fluorescence of the denatured polymer closely resembles that from the nucleotide mixture in spectral shape and quantum yield. At pH 2.4 the fluorescence spectral shape is similar to that of the nucleotide mixture but is blue shifted about 10 nm. The quantum yield is a factor of about nine less for the polymer than for the monomers. The difference between the fluorescence characteristics of the polymer and nucleotide mixture at pH 2.4 are best explained by the long lived excited state of guanine cations being quenched and constrained from reorienting in the polymer. At pH 12 and at 80°C there is less evidence of excited state interaction.

Throughout the research, there was little evidence of extensive excited state interactions. It appears that the radiationless relaxation of the individual nucleotides is so rapid at room temperature that there is little chance for this to occur. The

fluorescence characteristics of the native polymer might be taken to indicate that at room temperature the double helical conformation inhibits excited state interaction such as the formation of exciplexes as seen at 80°K. The overall results here and elsewhere indicate that excited states interactions are limited almost exclusively to nearest neighboring bases. This characteristic may have conferred necessary degree of photostability for evolution to have proceeded in its most primitive stages.

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APPENDIX

Table of Materials

Material	Vendor	Lot #
DNA, from calf thymus	Sigma	39C-9520
	"	48C-9560
	"	89C-9650
	"	99C-9530
	"	99C-9540
	"	126C-9570
	Cal Biochem	901660
DNA from E. Coli bacteria	Sigma	57C-6830
	"	21F-9005
DNA, from hen erythrocytes	Cal Biochem	703064
DNA, from salmon sperm	Sigma	106C-7470
DNA, from extreme thermophile bacteria	Gift from Dr. Julian	
Guanosine 5' Monophosphate (GMP)	Cal Biochem	300712
Adenosine	Sigma	58C-0179
Thymidine 5' Monophosphate (TMP)	Sigma	
Cytidine 5' Monophosphate (CMP)	Sigma	
L-Tryptophan	Sigma	34C-1290
Proteinase K	Beckman	B90-334
Cation X	Cal Biochem	620322

