EXCIMER FLUORESCENCE OF DINUCLEOTIDES, POLYNUCLEOTIDES, AND DNA

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The ultraviolet-absorption spectrum of DNA closely resembles that of a mixture of its constituent nucleotides,[1] apart from the well-known hypochromism.[2, 3] On the other hand, the emission spectra of DNA and its constituent nucleotides are qualitatively different.

The phosphorescence from the excited triplet state of DNA has been shown[4, 5] to originate from thymine residues which after excitation have transferred a proton across the hydrogen bond to adenine.

In this paper we present the results of a study of the fluorescence at 85°K from the excited singlet states of dinucleotides, synthetic polynucleotides, and DNA. We show that the fluorescence of stacked dinucleotides originates in excimer states and differs considerably from the fluorescence of the component nucleotides. Furthermore, the emission from polynucleotides resembles that of the dinucleotide excimers and not that of the isolated mononucleotides.

Förster and Kasper[6] showed that neighboring aromatic molecules in their excited singlet state may form an *excited dimer* or excimer. At the excimer equilibrium distance, the molecules attract each other in the excited state but repel each other in the ground state. Therefore,[7] excimer fluorescence is red-shifted from the monomers and shows no vibrational fine structure, while the optical absorption remains the same as in monomers. Furthermore, because the energy minimum of the excimer is created by a covalent[6-10] interaction between the π-electron systems, the stacking of the aromatic molecules increases the probability of excimer formation. Excimers have been observed in many different solutes and solutions,[7] in crystals of stacked aromatic molecules,[8, 11] and in solutes in glasses.[12]

The dinucleotides 3′-5′ ApG, GpC, UpA, CpC, and 2′-5′ ApC were obtained from Gallard-Schlesinger Chemical Corp. and used without further purification. Synthetic TpT (deoxy) was obtained[13] from Dr. T. M. Jacob, and TpTp from Dr. F. J. Bollum. A chromatographic analysis of these samples in the isopropanol-ammonia-water system showed less than 2 per cent impurities in ApG, UpA, CpC, ApC, and TpT. Random poly A_2U with $s = 4.2$, synthesized with *M. lysodeikticus* polymerase, was a gift from Professor J. Fresco. Poly d(AT) was extracted from the crab *Cancer borealis* by the method of Davidson *et al.*[14] The calf thymus DNA was from Worthington Biochemical Corp. (Freehold, N.J.) batch #606 and was dialyzed before use. Poly dA:dT, poly dG:dC, and poly rG:rC were prepared by Professor M. Chamberlin using *E. coli* polymerase enzymes as previously described.[15] The DNA from φX174 was generously supplied by Professor R. Sinsheimer.

The optical emission was measured at 85°K with a spectrophosphorograph using Bausch and Lomb 0.5-m grating monochromators for excitation and emission. The experimental curves shown are not corrected for the wavelength dependence of the combined efficiency of the EMI 9558QC photomultiplier and the emission monochromator, but this varied by less than 20 per cent over the wavelength

ranges shown.   Electron spin resonance measurements were made with a Varian Associates X-band spectrometer with 100 kc/sec modulation of ~20 gauss peak-to-peak amplitude.   Osram 200-watt high-pressure Hg lamps in Wild microscope mounts were used for both ESR and luminescence experiments.   Optical rotatory dispersion (ORD) measurements were made on a Durrum-Jasco model ORD/UV-5 spectrophotometer, and UV-absorption spectra were measured on a Bausch and Lomb Spectronic 505.

A study was made by UV absorption, optical rotatory dispersion, and fluorescence of six dinucleotide samples: UpA, ApC, CpC, and TpT, all at pH 7, CpC at pH 2, and TpT at pH 12 in ethylene glycol (EG):H_2O (1:1) glass, and of mixed mononucleotide controls.   Total base concentrations were 3×10^{-3} M.   The optical absorption was measured at 77°K with the samples in a 0.3-mm thick quartz cell. Hypochromism of a few per cent was detected, but not investigated further as errors in the comparative absorption measurements were of the same order.   In no case did we observe major differences between the spectra of dinucleotides and controls; hence there were no electronic ground-state changes.   The ORD measurement was used to evaluate stacking of the bases (Fig. 1).   As a criterion for stacking, we took

$$\text{Max}\left\{ [\varphi(\lambda)]_D - [\varphi(\lambda)]_M \right\} > 10^4,$$

where $[\varphi(\lambda)]_D$ and $[\varphi(\lambda)]_M$ are the molar rotations of the dinucleotide and control at wavelength λ, in degree cm^{-1} $(\text{mole/l})^{-1} \times 100$.   Our results are consistent with those of Warshaw and Tinoco,[16] taking into account the unstacking effect of the EG:H_2O solvent, and the increased stacking which occurs upon lowering the temperature.   The ORD measurements indicate that at pH 7 UpA, ApC, CpC, and TpT are stacked.   In the case of UpA max $\left\{ [\varphi(\lambda)]_D - [\varphi[\lambda]_M \right\}$ is smaller than 10^4 at the lower temperature, but the increase in ORD from $+10$ to $-25°C$ gives us confidence that some stacking is taking place.   As expected from the charged states of the bases, very little stacking is indicated in CpC at pH 2 and none in TpT at pH 12.   Warshaw and Tinoco[16] have previously shown that dinucleotides can be unstacked by varying the pH so as to charge the bases.   Changes in stacking upon lowering the temperature below $-30°C$ are expected to be small, as the EG:H_2O (1:1) solvent forms a rigid glass slightly below that temperature.   Therefore, the $-30°C$ measurements of the stacking are considered to be valid at 85°K, the temperature of the fluorescence measurements.   In the stacked dinucleotides the bases are closer together than in the unstacked, and it is more likely they will form excimers upon excitation.

The fluorescence spectra (Fig. 1) of all controls and of the unstacked CpC at pH 2 and TpT at pH 12 have the same wavelength dependence as the summed fluorescences of their constituent mononucleotides.   As expected, no excimers occur.

The fluorescence spectra of the stacked dinucleotides, on the other hand, are considerably broadened and red-shifted from those of their controls.   From this, and the unchanged absorption spectra, we conclude that the emission is from excimer states.   Furthermore, the excitation and absorption spectra of CpC were identical.

A mononucleotide component seems to be present in the fluorescence of TpT at pH 7.   This may reflect the sensitivity of excimer formation to the heterogeneity of the stacking.
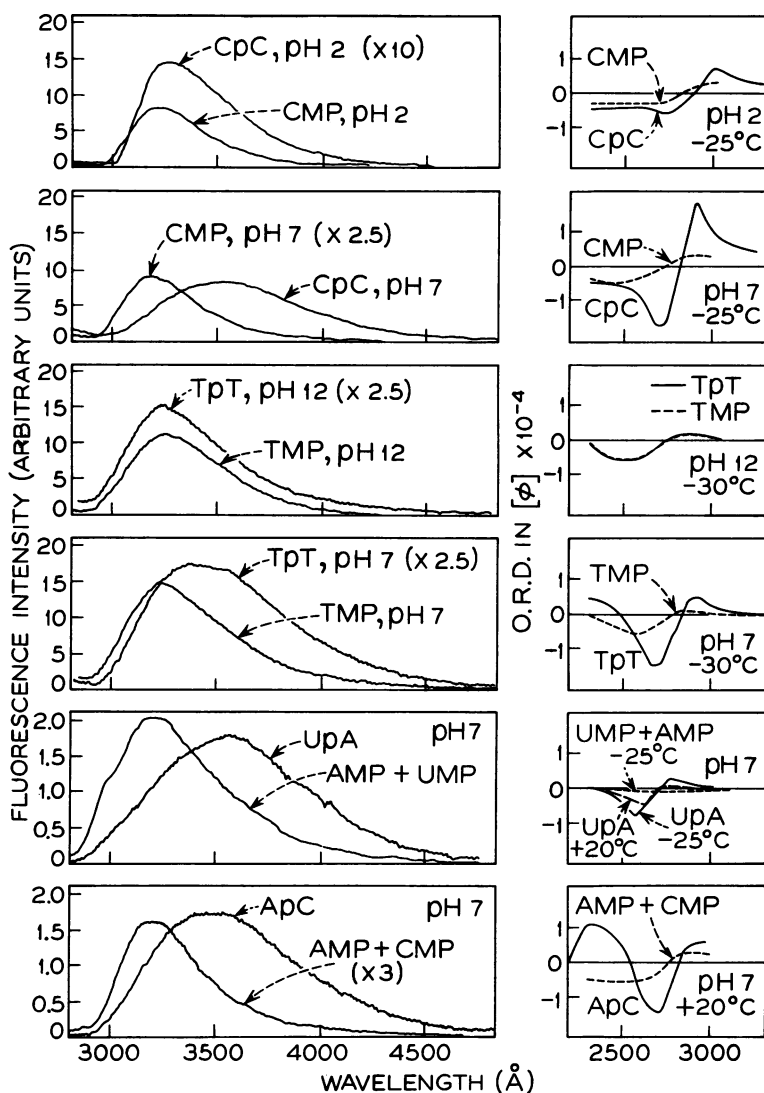
FIG. 1.—Optical rotatory dispersion and fluorescence of dinucleotides and mixtures of mononucleotides. Large differences in ORD between dinucleotides and controls show that ApC, UpA, CpC, and TpT, all at pH 7, are stacked, while CpC at pH 2 and TpT at pH 12 are unstacked. The broad and red-shifted fluorescence of the stacked dinucleotides is evidence for an excimer excited state. Total base concentrations are 3×10^{-3} M in EG:H_2O. The temperature of the fluorescence measurements is 85°K.

Excimer fluorescence is also observed in polynucleotides. The assignment of the excimer origin rests upon the similarity between the emission from polynucleotides and dinucleotides. In Figure 2a this similarity can be observed for UpA and the random single-stranded poly A_2U. In Figure 2b the comparison between CMP, CpC, and poly C at pH 5 where it has been reported[17] to be an ordered double helix, leaves no doubt about this assignment. Note further that excimer fluorescence is observed, at these low temperatures, for both the single-strand and double-strand form of polynucleotides. This indicates that at low temperatures there is
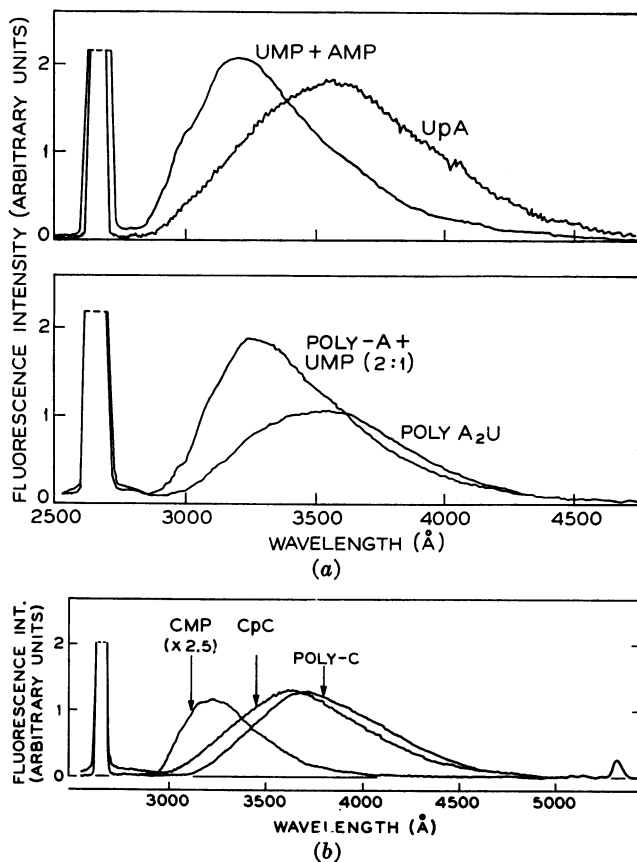
FIG. 2.—(a) A comparison of the fluorescence from UpA and poly A₂U and their controls all measured in EG:H₂O glass at 85°K, showing excimers in a single-stranded polynucleotide. (b) A comparison of poly C in its ordered state at pH 5 with CpC and CMP both at pH 7. The very small changes in the position of CMP fluorescence with protonation are negligible compared to the excimer shifts observed.
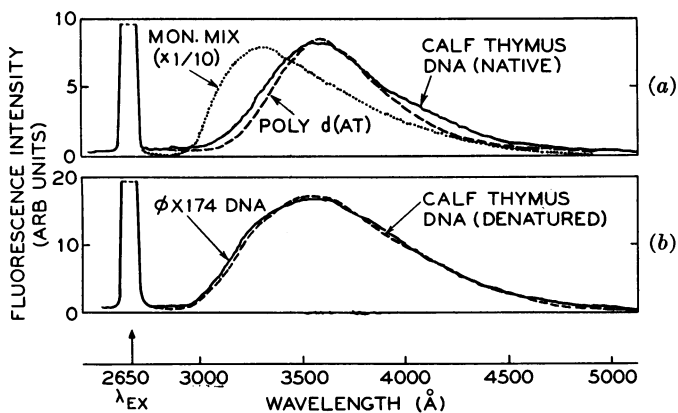

FIG. 3.—(a) The excimer emissions of double-stranded DNA and poly dAT are compared with the emission from an equimolar mixture of AMP, GMP, TMP, and CMP. Note particularly the shift and quenching in the polynucleotides. (b) A comparison between heat-denatured DNA and single-strand φX174 DNA.

enough stacking of the bases, even in the single-strand form, to allow considerable interaction of the excited singlet states. Subtle differences of stacking change the degree of excimer emission: in general, we have observed fewer excimers in the more denaturing solvents like ethylene glycol.

As shown in Figure 3a, excimer fluorescence is also observed in poly dAT and native calf thymus in $EG:H_2O$ glass. Upon denaturing the DNA by heating, or going to the single-stranded $\varphi X174$ DNA, the emission becomes still broader although it remains peaked around 3600 Å. In addition, the fluorescence quantum yield is several times larger in the single-stranded DNA samples.

The broadening and increased intensity of the single-stranded DNA samples can be understood in terms of the singlet quenching processes which the following experiment shows to exist in native DNA. We compared the fluorescence intensities of poly dG:dC and poly rG:rC with equimolar mixtures of the monomers. In both ordered polynucleotides the fluorescence was quenched by more than a factor of 10 with respect to the nonhydrogen-bonded GMP and CMP controls. This presumably accounts for the previously[5] observed quenching of the guanine phosphorescence in poly G:C. This quenching of guanine-cytosine pairs will not occur in denatured or single-stranded DNA, hence the larger fluorescence quantum yields shown in Figure 3. The additional width of the single-strand fluorescence may be caused by the more heterogenous nature of its excimers which can include guanine and cytosine residues.

In summary, we have the following picture of the excited singlet states of DNA at low temperatures in $EG:H_2O$ glass. The observed fluorescence is from excimer states. In native DNA, only adenine and thymine fluoresce. The absence of guanine and cytosine fluorescence is explained by the observed quenching of the excited singlets in poly dG:dC and poly rG:rC. From available measurements of quantum yields,[18] the singlet lifetimes of the bases in native DNA can be estimated as $<10^{-12}$ sec for guanine and cytosine and $\simeq 10^{-11}$ sec for adenine and thymine. In denatured DNA, all four bases probably contribute to the fluorescence.

More quantitative studies are in progress including attempts to extend these experiments and conclusions to physical conditions of more biological interest.

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HALIDE IONS AS CHEMICAL PROBES FOR NMR STUDIES OF PROTEINS*

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There has been considerable recent interest in the application of nuclear magnetic resonance techniques to the study of biological macromolecules.[1] NMR is in general not sufficiently sensitive for the direct study of biomolecules in solution at realistic concentrations. Some of the most promising developments have therefore involved the use of nuclear relaxation effects to probe gross molecular structural features.[2] These techniques frequently rely on the effects of small concentrations of paramagnetic atoms on the relaxation times of solvent nuclei,[3-5] or on the binding and exchange of small molecules with proteins.[6] Although paramagnetic relaxation effects have been widely exploited to yield structural information on relatively simple inorganic complexes in solution,[7-9] these techniques usually suffer from the inherent complexity of the relaxation process.[2] The observed effects on nuclear relaxation are usually the result of several competing mechanisms of comparable significance, which makes interpretation of line shapes in terms of molecular events hazardous.

For nuclei with spin greater than $1/2$, the interaction of the nuclear electric quadrupole moment with fluctuating field gradients at the nucleus can provide a simple and dominant relaxation mechanism.[10] It is thus of interest to explore the possible application of this mechanism. In this paper it is shown that the relaxation and exchange of quadrupolar nuclei at suitable sites can provide a general technique for the study of biomolecules in solution. Suitable sites for the binding and exchange of halide ions can be readily inserted at interesting places in proteins, and information analogous to that accessible from spin-labeled biomolecules by ESR[11] can be inferred from the line width of the halide nuclear resonance.

Line-Width Theory.—Fluctuations in the orientation and magnitude of the electric field gradient q at the site of a nucleus with electric quadrupole moment Q provide an efficient nuclear relaxation mechanism. In the extreme narrowing approximation, the contribution to the nuclear resonance line width from quadrupole relaxation is[10]

$$\Delta\nu = K(e^2qQ)^2\tau_c, \tag{1}$$