Excited Electronic States of DNA

J. Eisinger and R. G. Shulman

The ultraviolet irradiation of many microorganisms leads to mutations and death (*1*). Since the wavelength dependence of these biological effects has been shown to be identical with the absorption spectrum of DNA, it is concluded that photo-damage centers produced in DNA may have lethal or mutagenic consequences. In the short time between the absorption of an ultraviolet photon by one of the bases of DNA and the final formation of a damage center, many electronic rearrangements can take place, various excited states of different multiplicities may be populated, energy may be transferred between different regions of the DNA, and some covalent bonds may be formed while others may be broken. Here we attempt to trace the paths of excitation energy through this maze, emphasizing, where it is possible, the connections between electronic excitations and photochemical reactions.

It is useful to think of this problem in terms of a series of electronic states of DNA which are formed once the original ultraviolet excitation has taken place. Figure 1 illustrates this view, with the arrows indicating various alternative pathways. The superscripts 1, 2, and 3 refer to the spin multiplicities of the states: ^{1}DNA* and ^{3}DNA* are excited singlet and triplet levels of DNA, which need not correspond to levels found in isolated nucleotides. ^{2}DNA represents a free radical with one unpaired electron. Examples of these states, determined by recent experiments, are discussed below.

DNA consists of a sugar-phosphate chain which does not absorb at wavelengths greater than 200 nanometers and of four heterocyclic bases which do absorb at these wavelengths (*2*). The bases are adenine (A), guanine (G), cytosine (C), and thymine (T). A nucleotide is the subunit of DNA which consists of a base, a sugar, and a phosphate. The absorption spectrum of double-stranded DNA closely resembles the sum of the absorption spectra of the constituent purine and pyrimidine bases in shape but is about 30 percent less intense.

We may conclude from this that the electronic interaction between the bases is weak enough so that it is proper to speak of the absorption of an ultraviolet photon by a single base in DNA. It is therefore natural to start a study on the excited states of DNA by studying the excited states of the monomers. While this approach is fruitful, one should note that bases in their *excited* states have much stronger interactions with neighboring bases than those in their ground states have, and that these interactions can profoundly affect the excited states.

Figure 2 shows the lowest-lying energy levels of a typical organic molecule such as a purine or a pyrimidine and indicates the various radiative and nonradiative transitions which may occur between them. The two excited states of greatest importance are the lowest excited singlet and triplet states. Excitation by direct absorption is usually to the excited-singlet vibrational manifold, because the transition from ground singlet to excited singlet is allowed, in contrast to the singlet-to-triplet absorption, which is spin-forbidden. In both the singlet and triplet manifolds, vibrational relaxation to the lowest vibrational state occurs quickly, and then the excited molecule has the possible paths shown by the arrows in Fig. 2. From singlet or triplet it may decay radiatively (r) or nonradiatively (nr) to the ground state, with specific rate constants of ^1k_r, $^1k_{nr}$, ^3k_r, and $^3k_{nr}$. In addition there is a specific rate constant for intersystem crossing, designated k_{ISC}.

Intersystem-crossing transitions between excited singlet and triplet states described by k_{ISC} are spin-forbidden but commonly take place, since small energy differences, good overlap between vibrational levels, and mixing with higher-lying electronic states all may militate in their favor. Radiative transitions from the excited triplet states to the singlet ground state, ^3k_r (phosphorescence), are also forbidden and have large energy differences to contend with. As a result they occur so slowly that, for nucleotides at room temperature, no phosphorescence is observed, since $^3k_r \ll {}^3k_{nr}$, while at 77°K, where $^3k_{nr}$ is reduced, the lifetimes of the excited triplet states are between 10^{-3} second and 10 seconds. Usually the singlet states of the nucleotides are quenched in solutions at temperatures above approximately 100° to 200°K, except for the protonated purines, which fluoresce at room temperature (*3*). To observe emission of nucleotides, it is therefore usually necessary to dissolve them in aqueous glasses at low temperature. The environment of the nucleotides in such polar glasses [for example, ethylene glycol, 50 percent; water, 50 percent (EG:H_2O)] is thought to resemble the environment in water, but the restrictions a rigid matrix places on the motion of a molecule and its solvent shell can have important effects upon its excited states. This is dramatically illustrated by the large red shift in fluorescence observed in protonated G and protonated A at room temperature.

The emission spectra may be used for determining the energy of the excited states. Since relaxation to the lowest vibrational level of a particular excited electronic state is usually rapid as compared to all other processes, emission shapes are determined by transitions from this level to a series of excited vibrational levels of the ground state. As a result, when the emission is from the electronic state which is being excited, its spectrum shows a peak toward the red relative to the O-O transition line (between the lowest vibrational levels of the excited and ground states) and is, at least approximately, a mirror image of the absorption spectrum, which shows a peak toward the blue relative to the O-O line (*4*).

An important insight into the electronic processes of excited molecules comes from a knowledge of quantum yields for the various radiative and nonradiative processes. The fluorescence and phosphorescence quantum yields φ_F and φ_P are readily obtained from the integrated intensities of the fluorescence and phosphorescence spectra, with the aid of suitable calibrating samples whose quantum yields are known absolutely. The picture remains incomplete, however, until φ_{ISC}, the quantum yield for the singlet-to-triplet intersystem crossing, is found. It can be obtained from the equilibrium number of mole-

The authors are affiliated with the Bell Telephone Laboratories, Murray Hill, New Jersey.

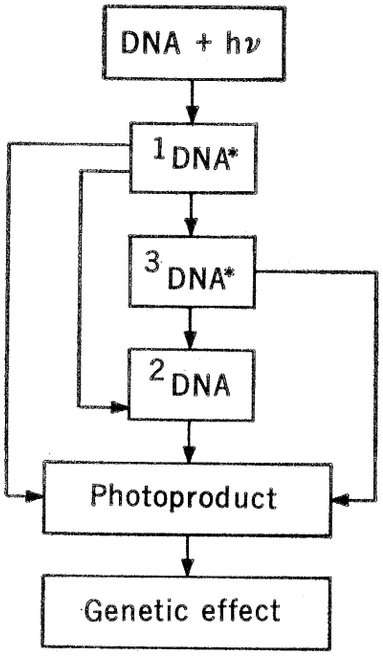


Fig. 1. Schematic representation of various possible paths for dissipation of the energy absorbed by DNA. In the symbols ^{1}DNA*, ^{3}DNA*, and ^{2}DNA, the superscripts, referring to spin multiplicity, describe an excited singlet, an excited triplet, and a free radical, respectively.

cules in the excited triplet state N_T when the molecule absorbs quanta at a known rate. The quantum yield φ_{ISC} and the more commonly cited φ_P will be the same only if nonradiative deexcitations of the triplet level are negligible.

Since molecules in the triplet state have two electrons with paired spins ($S = 1$), they can be detected by electron spin resonance (ESR), usually of the so-called "$\Delta m = 2$" transition (*5*). The magnitude of the ESR signal corresponding to the "$\Delta m = 2$" transition may be used to determine N_T, and hence φ_{ISC}. For this, one monitors the irradiation intensity by means of the ESR signal of a molecule in the triplet state with known φ_{ISC}. This intensity calibration requires considerable care since it depends on the line widths and line shapes of the resonances; a method for using this technique to determine φ_{ISC} has been given by Guéron *et al.* (*6*). The field for resonance H_{min} is a function of the dipole-dipole interaction between the two paired electrons and, as such, is characteristic of the electronic wave function of the triplet state in question. This is a particularly useful method for identifying molecules in the triplet state when a number of different chromophores are being irradiated, as is the case in DNA. It generally offers better resolution, albeit less sensitivity, than an identification based on the phosphorescence spectra and phosphorescence decay times.

The experimental methods used to determine absorption, fluorescence, and phosphorescence spectra, singlet- and triplet-state lifetimes, and ESR spectra are well described in the literature. Here we merely note that, since the nonradiative processes are extremely rapid in nucleic acids and radiative and intersystem-crossing quantum yields are thus often very low, very sensitive emission and ESR spectrometers are required. Unless otherwise indicated, all optical and ESR spectra discussed here were obtained at 80°K in (EG : H_2O) glasses. The use of frozen water solutions in emission studies is to be avoided, since the solute molecules tend to form crystallites. This may profoundly affect the excited states and makes quantum yield determinations virtually imopssible.

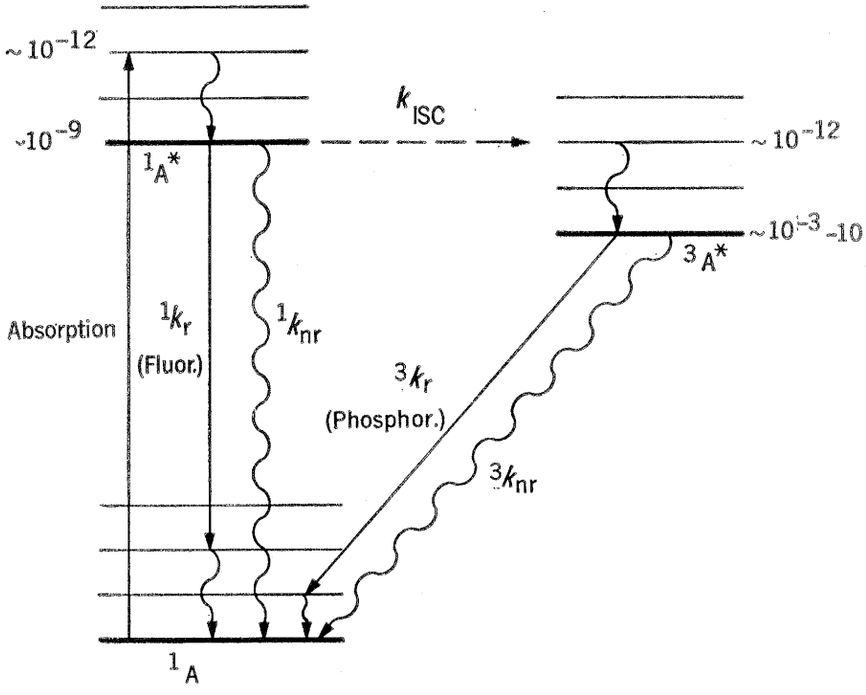


Fig. 2. The canonical singlet and triplet manifolds describing the excited states of an organic molecule A. The specific rate constants are described by the k's which are identified in the text; typical values of the lifetimes of the excited states are given in seconds.

Excited States of Nucleotides

The low-temperature absorption, fluorescence, and phosphorescence spectra of the nucleotides adenosine monophosphate (AMP), guanosine monophosphate (GMP), thymidine monophosphate (TMP), cytidine monophosphate (CMP), and uridine monophosphate (UMP) are shown at various *p*H values in Fig. 3. Various parameters characterizing the excited-state properties are summarized in Tables 1 and 2. These spectra are discussed in detail elsewhere (*7*, *8*). Here we summarize the experimental results, beginning with the singlet states.

The energy of the lowest vibrational level of the excited singlet states, $^1\varepsilon$, may be determined with reasonable accuracy if the fluorescence and absorption spectra have mirror symmetry or if they at least have well-defined thresholds in the red and blue, respectively. In this way we may arrange these O-O transitions in order of decreasing energy: A, U, T, G, C (see Table 1).

If absorption and emission take place between the same two electronic states and the vibrational modes of the molecule are the same in the ground and excited states, there exists a general relationship between the molar absorption coefficient and the radiative lifetime of the excited state. Since the radiative lifetime (τ_R) and the lifetime of the excited state (τ) are related by the equation $\varphi_F = \tau/\tau_R$, τ can be calculated from the absorption properties of a molecule and the experimentally determined value for φ_F. In this way it is found that the calculated values for singlet lifetimes τ are much shorter than the values observed experimentally (see Table 1). The origins of these discrep-

ancies are not understood, but one possibility is that the fluorescence comes from a low-lying state, not observed in absorption because of its weak absorptivity, which has a correspondingly long lifetime.

It may also be seen from Table 1 that in all nucleotides the quantum yields for nonradiative singlet decays ($1 - \varphi_F - \varphi_{ISC}$) are large. This is not often found in aromatic compounds.

The lowest-lying singlet and triplet states which have been identified are π-π^* states, arising from promotion of an electron from a filled π orbital to an empty one. Evidence for the π-π^* nature of the lowest excited singlet state of CMP and TMP may be seen in Fig. 3, which shows that the absorption and fluorescence spectra are mirror images on a wave-number scale. Since the strong absorptions must be π-π^* transitions and the emission appears to come from the same state, one may conclude that the lowest excited state is a π-π^* state. The π-π^* nature of the singlet-state emission in analogs of AMP and GMP have been established by fluorescence depolarization studies (*9*). However these experiments are inconclusive because it is possible that the lowest singlet state is really an n-π^* state and that the intensity and polarization of transitions which involve it come from a π-π^* admixture.

It may be seen from Table 2 that all triplet lifetimes are of the order of 1 second and that none are shorter than 0.3 second. From this it may be concluded that the lowest-lying triplet states of nucleotides all are π-π^* levels, since n-π^* levels would have much shorter decay times. The values of D and E, the dipolar interaction constants for the paired electrons in the triplet states of the nucleotides, appear in Table 2. They agree quite well with the values calculated from π-π^* triplet states (*10, 11*) but not with those expected for n-π^* states (*12*).

The field at which the "$\Delta m = 2$" line is observed in ESR studies can be used to determine $D'[\equiv (D^2 + 3E^2)^{1/2}]$, which is also shown in Table 2. From the D' values of the mononucleotides it may be seen that the resolution between purines and pyrimidines is excellent, but that it is not easy to differentiate between T and T^- on the basis of the ESR measurements alone. The similarity between D and E for these two molecular species is particularly surprising because infrared studies in the ground state (*13*) have shown different tautomeric forms for these different states of ionization. Molecular orbital calculations which employ Gaussian basis functions are being made (*14*), in an effort to find why the excited states are similar even though the ground states are different. Pullman and Kochanski (*11*) have reported that the calculated values of D for T and T^- are the same.

Table 1. Properties of the excited singlet states of nucleotides at 80°K in ethylene glycol and water, showing f, the oscillator strengths; $^1\varepsilon$, the energy of the O-O transition; φ_F, the fluorescence quantum yields; φ_{ISC}, the intersystem-crossing yields measured by electron spin resonance; $(1 - \varphi_F - \varphi_{ISC})$, the yield of internal conversion from the first excited singlet level; $^1\tau_R$, the radiative lifetime calculated from f on the basis of the Einstein relation; $^1\tau$ (calc), the singlet lifetime calculated from $^1\tau$ (calc) $= \varphi_F{}^1\tau_R$; and(finally, $^1\tau$ the observed value for the singlet liftime. [From Blumberg *et al.* and Gueron (*42*)]

Sample*	f	$^1\varepsilon$ (10^3 cm^{-1})	φ_F	φ_{ISC}	$1 - \varphi_F - \varphi_{ISC}$	$^1\tau_R$ (10^{-9} sec)	$^1\tau$ (calc) (10^{-9} sec)	$^1\tau$ (obs) (10^{-9} sec)
AMP (*p*H 7)	0.3	35.2	0.01	0.02	0.97	3.	0.02	2.8
GMP (*p*H 7)	.08	34.0	.13	.15	.72	12.	1.6	~ 5.
CMP (*p*H 7)	.18	33.7	.05		.95	5.5	0.3	
TMP (*p*H 7)	.22	34.1	.16	~ 0.	.84	4.5	.6	3.2
TMP (*p*H 12)	.18	34.35	.24	0.15	.61	5.5	1.2	2.9
UMP (*p*H 7)	.2	34.9	~ .01		~ .99	4.5	0.03	
UMP (*p*H 12)	.2	35.0				4.5		

* *p*H values before freezing.

It was pointed out above that, by measuring the intensities of the "$\Delta m = 2$" transitions, one can calculate φ_{ISC}. Experimentally determined values for φ_{ISC} are shown in Table 2, and they reveal that the probability of internal conversion for triplets,

$$^3k_{nr}/(^3k_r + {}^3k_{nr}) = (\varphi_{ISC} - \varphi_P)/\varphi_{ISC},$$

is appreciable for the nucleotides even at 80°K. The energies of the triplet states of the nucleotides were determined from the blue edges of the phosphorescence spectra for the molecules with observable phosphorescence; these energies are listed in Table 2. When phosphorescence or electron spin resonance is not observed for an irradiated molecule (as was the case for TMP and protonated AMP), this does not mean that a triplet level with an energy below the energy of the first excited singlet state does not exist; in fact Hund's rule states that such a triplet state must exist. Nor does it necessarily mean that the nonradiative lifetime of the triplet is too short to allow detection. It may mean that the intersystem-crossing rate is too low to permit appreciable population of the triplet state. Such cases lend themselves admirably to study by means of sensitizers. A triplet sensitizer is a molecule with a large value of φ_{ISC} whose triplet energy is high enough and whose lifetime is long enough to facilitate triplet transfer to the acceptor molecule under study (*15*). It was through the use of acetone and acetophenone as sensitizers that the T triplet state was populated and then characterized by its ESR and phosphorescence properties (*8*). Since phosphorescence is not observed from isolated T molecules in the absence of sensitizers, it may be concluded that the intersystem-crossing rate is low as compared to the singlet-state decay rate.

Sensitizers are useful not only in establishing the existence and properties of triplet states which cannot be populated by intramolecular processes but also in determining the relative energies of triplet states of the nucleotides. While these energies can, in principle, be determined from the thresholds of the phosphorescence spectra of the sensitized or directly excited triplets, this method is not infallible, since the O-O transition may be missing as a result of an unfavorable Franck-Condon factor. When this is the case, the threshold will give too low an energy for the triplet state. This was, in fact, suggested to be the case for adenine derivatives

Table 2. Properties of the excited triplet states of nucleotides at 80°K in ethylene glycol and water, showing $^3\varepsilon$, the energy of the O-O transition; φ_P, the phosphorescence quantum yield; $[1 - (\varphi_P/\varphi_{ISC})]$, the fraction of triplets formed which decay without emitting; $^3\tau$, the observed triplet lifetime; and the parameters of the spin-Hamiltonian function where $D' = (D^2 + 3E^2)^{1/2}$.

Sample*	$^3\varepsilon$ (10^3 cm^{-1})	φ_P	$1 - (\varphi_P/\varphi_{ISC})$	$^3\tau$ (sec)	D' (cm^{-1})	D (cm^{-1})	E (cm^{-1})
CMP (*p*H 7)	27.9	0.01		0.34	0.194		
GMP (*p*H 7)	27.2	.07	0.5	1.3	.145	0.141	0.017
TMP (*p*H 12)	27.0	.03	.8	0.5	.198	.196	.010
AMP (*p*H 7)	26.7	.015	.35	2.4	.126	.121	.027
TMP (*p*H 7)	26.3			0.33	.200	.203	.010

* *p*H values before freezing.

(*16*). The triplet-state energies given in Table 2 were obtained from the blue edge of the phosphorescence spectra and have been confirmed by observation of energy transfer from acetone to all triplets of lower energy listed in Table 2, and from acetophenone to T only.

Knowledge of the order of triplet levels is of course important for estimating the possibility of triplet energy transfer in DNA and other polynucleotides; generally only transfer to levels with the same or lower energy is possible. All cases of triplet energy transfer between nucleotides which have been reported (*17, 18*) are consistent with this kind of transfer except for the triplet energy transfer from A to C in the dinucleotide ApC, reported by Hélène, Douzou, and Michelson (*19*). However, their result has been contradicted by more recent observations (*17, 18*) in which it has been shown that the transfer of energy goes from C to A, not from A to C as they claimed.

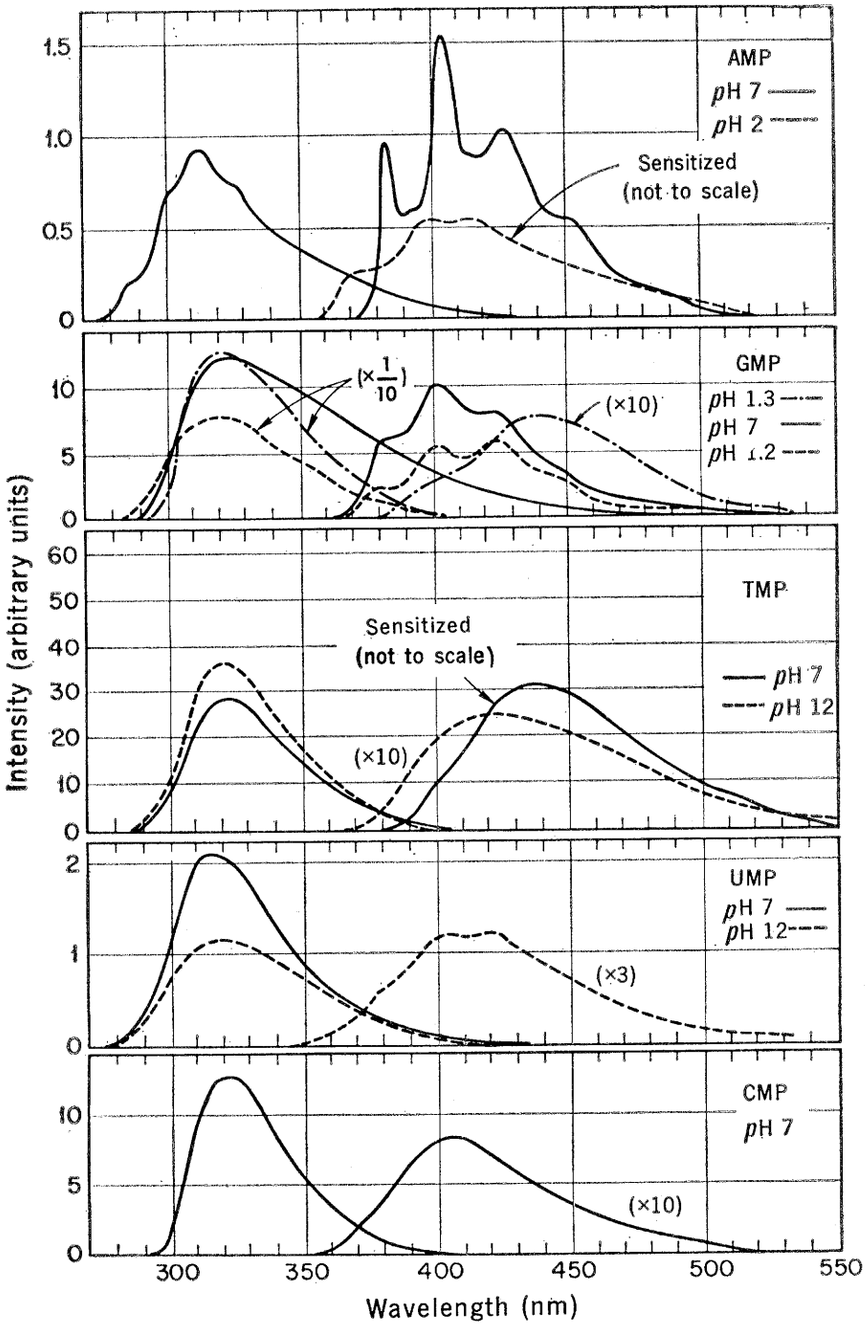


Fig. 3. Fluorescence and phosphorescence spectra of common nucleotides in ethylene glycol and water at 80°K. Except for the phosphorescence spectra of protonated adenine and neutral thymine, which were obtained by sensitization, the scale is the same for all emission spectra. See Tables 1 and 2 for more quantitative information.

Dinucleotides

The next step in attempting to understand the excited states of polynucleotides is a study of the excited states of dinucleotides. We denote these by the expression XpY, which normally means that between the bases X and Y there is a 3′–5′ sugar-phosphate-sugar linkage (*19a*). Such a dinucleotide has virtually the same absorption spectrum as an equimolar mixture of the mononucleotides of X and Y. On the other hand, it often has a very different optical-rotary-dispersion (ORD) spectrum, and the difference indicates appreciable stacking (base-base interactions) between the bases X and Y (*20*). Such ORD spectra give only a qualitative measure of stacking, since the theory does not allow one to interpret these measurements in terms of a unique geometry. Indeed, dinucleotides in solution can be expected to have a considerable distribution of geometries even when strong stacking is indicated by ORD measurements, because of thermal motion whose energy is comparable to stacking energies. Presumably some of this randomness persists in the low-temperature glasses which have been used for measuring the emission spectra of dinucleotides.

We first discuss the excited singlet states of dinucleotides, then their triplet states.

Singlet States of Dinucleotides

Stacked dinucleotides often have a broad structureless emission band which is greatly shifted toward the red from the fluorescence of the mononucleotides. This band has been ascribed to "excimer" emission (*21*).

Excimers, or excited-state dimers, were discovered by Förster and Kasper (*22*), who observed the appearance of a blue fluorescence in pyrene solutions as the concentration was increased (the absorption remained unchanged). At low concentration, pyrene fluoresces in the violet and the fluorescence spectrum is structured and has mirror symmetry with the absorption spectrum. Förster and Kasper explained the new band which appeared at longer wavelengths at high concentration as coming from excimers, or dimers formed between an excited chromophore and a nearby unexcited one. The energy of this complex is lower than that of the excited monomer, as a result of a stabilizing electron-electron interaction. In the ground

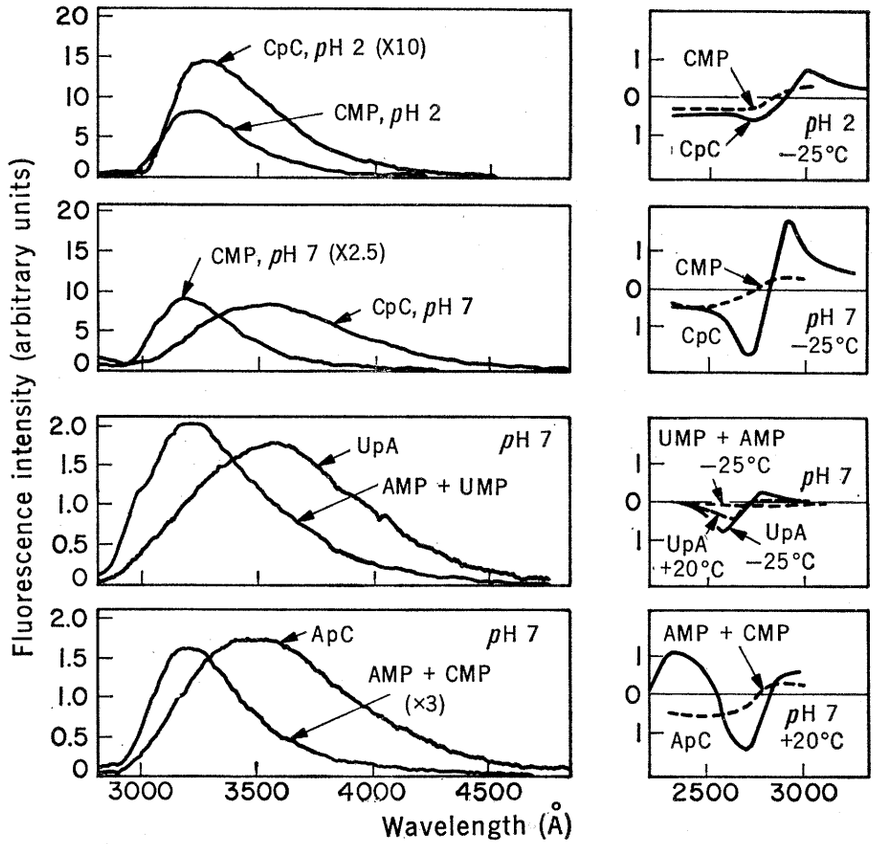
Fig. 4 (right). Fluorescence of the indicated dinucleotides compared with that from an equimolar mixture of their constituents. The extent of departure of the ORD curves, at right, from the y axis is a measure of base-base interactions, or stacking. Note particularly in the top two frames that when CpC at pH 7 is stacked, it emits at longer wavelengths than the mixture of its constituent monomers does, whereas, when it is unstacked by electrostatic repulsion at pH 2, it emits just as its constituent monomers do.

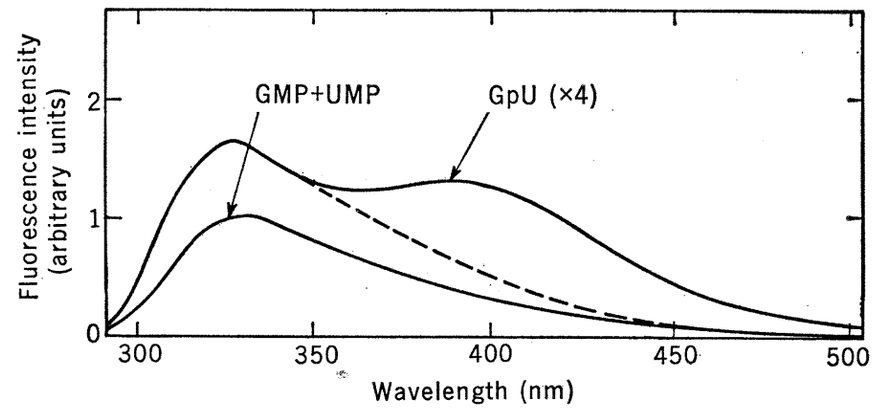
state the two molecules normally repel each other at distances shorter than the sum of the van der Waals radii, a sum which, for molecules of aromatic compounds, is about 3.4 angstroms. For molecules in the excited state, however, there may exist a potential minimum at a shorter intermolecular distance. Emission from this excited-state dimer or excimer (*22a*) will invariably be at longer wavelengths than the emission from the monomer, for two reasons. (i) The excited-state energy is lowered by the dimer interactions; (ii) at the smaller internuclear separation, the ground-state energy is raised by the intermolecular repulsion. Since no dimer is formed in the ground state, the absorption spectrum will be unchanged from that of the monomer. Moreover, since the ground state of the dimer is unbound, it has no discrete vibrational levels, thus excimer emission is always broad and featureless.

The appearance of excimers in dinucleotides (XpY) could be confirmed by a comparison of the dinucleotide's fluorescence and absorption spectra with those of an equimolar mixture of XMP and YMP. In all cases the absorption spectra of the dinucleotides resembled those of these equimolar mixtures. Figure 4 shows the emission of a number of dinucleotides, along with their ORD spectra. Note that in CpC, at pH 2, where both bases are protonated, the ORD spectrum indicates no stacking (presumably because of charge repulsion), and the excimer emission observed at pH 7 has been replaced by monomer fluorescence. Table 3 summarizes these results and gives measured values for excimer quantum yields, φ_F.

When excimer emission dominates, excimer formation rates are faster than the monomer emission rates, which have been measured to be in the range 10^8 to 10^9 per second.

The excimer state formed between two bases is seen to be dependent on their relative geometry. Thus ApC(3′–





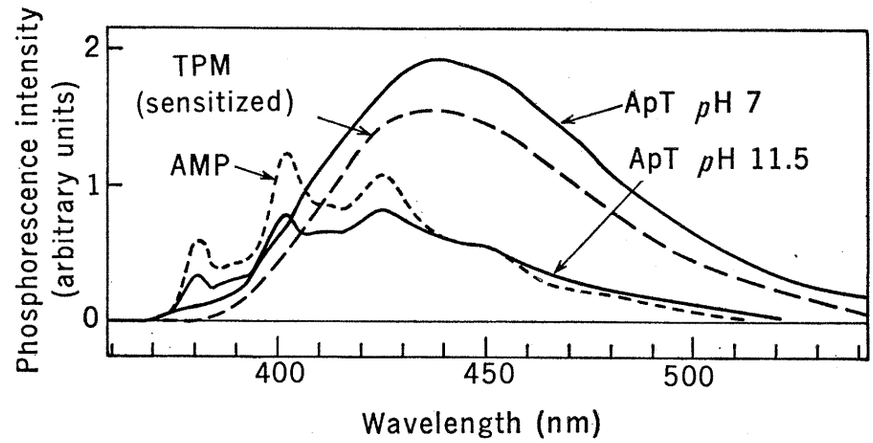


Fig. 5 (above, middle). Fluorescence of GpU, showing monomer and excimer contributions, as compared to fluorescence of the monomer mixture. Fig. 6 (bottom). Comparison of the phosphorescence spectra from ApT at pH 7 and pH 11.5 with those from AMP and sensitized TMP.

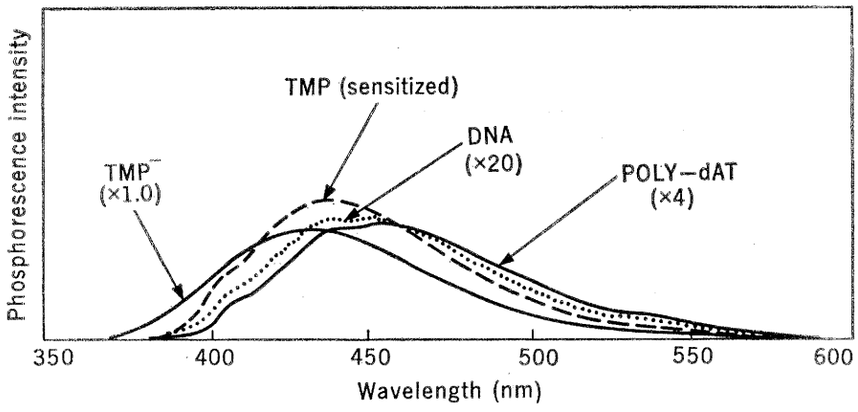


Fig. 7. Comparison of the phosphorescence spectra of DNA, poly-dAT, TMP (sensitized), and TMP⁻, showing how the first three spectra resemble each other and are reproducibly different from the spectrum of TMP⁻.

5′), ApC(2′–5′), and ApC(5′–3′) have different emission spectra, as indicated by the different values of λ_{max} and φ_F listed in Table 3. This presumably is caused by different stacking conformations. While CpC and polyC both form excimers, they emit at slightly different wavelengths. It seems reasonable not to endow these excimers with the well-defined characteristics of a single molecular state but, rather, to think of them as having a potential minimum whose depth and positional coordinates are determined by the exchange and charge-transfer interactions which the geometrical constraints permit. An extreme case of this variability is illustrated by the emission spectra of GpU (see Fig. 5), which is a combination of monomer and excimer fluorescence.

The absorption threshold of A is at a distinctly shorter wavelength than that of C. By comparing the excitation spectra of ApC fluorescence and monomer fluorescence, it was therefore possible to conclude (*17*) that, for this and other dinucleotides, photon absorption by either of the constituent bases is equally likely to lead to excimer formation. Furthermore, in the same experiments it was found that, for an excimer, the ratio of fluorescence intensity to phosphorescence intensity was a constant, independent of exciting wavelength. The simplest explanation of this is that the excimer is a precursor of the triplet state and that all the energy absorbed by the dinucleotide is channeled to the excimer, whence a certain fraction goes to the triplet by one particular path.

Table 3. Fluorescence and phosphorescence observed from dinucleotides and their corresponding equimolar nucleotide mixtures for excitation at wavelength of 265 nanometers. All dinucleotides are (3′–5′) unless it is stated otherwise. The red shift in the wavelength of the fluorescence maxima λ_{max} shows that many dinucleotides form excimers. In CpA and GpU, monomer and excimer fluorescence peaks have been resolved, while in other cases, marked with an asterisk, the broad peaks observed may indicate two kinds of emission. The fluorescence of ApA is shifted slightly toward the red but retains the vibrational structure of AMP. This red shift is probably due to an exciton interaction which is also observable in absorption and does not result from excimer emission. The fluorescence quantum yields φ_F can be higher or lower than those of the monomers but are of the same order of magnitude. The phosphorescence spectra and decay times of dinucleotides are, in all cases, like those of that constitutent base which has the lowest-lying triplet state (see Table 2). The values for φ_F and φ_P contain experimental uncertainties of about 15 percent.

Sample	Fluorescence		Phosphorescence	
	λ_{max} (nm)	φ_F	Type of emission	φ_P
AMP + CMP	319	0.024	A	0.008
ApC	358	.062	A	.07
ApC(2′–5′)	333	.042	A	.06
CpA	320 and 380	.001 and 0.01	A	.008
AMP + GMP	327	.044	A + G	.030
ApG	335	.085	A	.11
GpA	360*	.032	A	.11
GpA(2′–5′)	346	.047	A	.10
AMP + UMP	312	.008	A	.0056
ApU	362	.028	A	.042
UpA	360*	.010	A	.031
GMP + UMP	325	.054	G	.026
GpU	323 and 405	.01 and 0.008	~ G	.009
CMP + UMP	317	.041		.002
CpU	341	.019		.003
CMP	320	.06	C	.011
CpC	356	.09	C	.010
AMP + TMP	322	.064	A	.006
ApT	355	.058	T	.021
TpA	330	.052	T	.009
AMP	313	.011	A	.015
ApA	317	.037	A	.055

Triplet States of Dinucleotides

Dinucleotides generally show phosphorescence emission, ESR signals, and triplet-state lifetimes characteristic of only one of their constituent monomers. In a few cases a small contribution from the triplet state of the other base remains. The base which has the preponderant triplet-state population is, in all cases, the one with the lower triplet energy level (see Tables 2 and 3). It can be seen in Table 3, for the dinucleotides containing A and C or A and G, that the triplet excitation is found on A. This is in accord with the relative energies of the triplet states shown in Table 2. From the results on dinucleotides containing A and U, we would infer that the A triplet is lower in energy, but we have no independent confirmation of this. The results on ApT and TpA shown in Table 3 locate the triplet on T, once again in agreement with their relative energies, while an equimolar mixture of A and T shows only A-like phosphorescence (*23*). The T-like nature of the *p*H 7 ApT phosphorescence is illustrated in Fig. 6 by a comparison with sensitized TMP and with AMP. In contrast, a careful analysis of the spectrum and decay times of *p*H 11.5 ApT, where the T is deprotonated, shows that the phosphorescence comes from A and T in the same proportion as it would in an equimolar mixture of A and T at *p*H 11.5. The fluorescence of ApT at *p*H 11.5 shows monomer emission from T⁻ (φ_F is smaller by an order of magnitude for A than for T⁻). No excimer emission is observed. In the charged molecule, the bases might be more than 10 angstroms apart. The absence of interactions in both the excited singlet and the excited triplet states is consistent with a nonstacked conformation, in contrast to the results at *p*H 7, where stacking is indicated.

It is difficult to know by what mecha-

nism the lower-lying triplet state is populated in most dinucleotides. As mentioned above, in cases where excimers are formed, intersystem crossing from the excimer singlet state may be responsible. An alternative hypothesis would be that, no matter where the intersystem crossing occurs, the lowest-lying triplet will be populated as a result of triplet energy transfer. The range over which such triplet transfer can be expected to be effective is not well known; it is reasonable to suppose, however, that it would occur between nearest-neighbor bases for which the exchange interaction is appreciable.

The triplet quantum yields, like the singlet quantum yields, are often larger in dinucleotides than in the constituents. However, this increase generally follows an increase in fluorescence upon excimer formation, indicating that φ_F and φ_{ISC} have both increased because the internal conversion rates at the singlet level have decreased.

Polynucleotides

Among the polynucleotides, the alternating copolymer poly-d(A-T):d(A-T)—or poly-dAT for short—is particularly easy to understand because its emission properties resemble those of the dinucleotide ApT. Its singlet state is an excimer which is excited equally well by photons absorbed by A and photons absorbed by T. The triplet state is localized on T (*8*) and is the triplet of neutral T (rather than of the anion, as we first reported it to be). In the following paragraphs we summarize the properties of these two triplet states, showing how these properties are used to identify the form of the T triplet observed in poly-dAT and DNA.

The phosphorescences of T, T^-, poly-dAT, and DNA are compared in Fig. 7. The phosphorescence of T was sensitized with acetone, as described below. Note that, with respect to the blue edge of the spectrum and the characteristic "bump" at 400 nanometers (which is reproducible), both polynucleotides resemble T more closely than they resemble T^-. The decay times are given in Table 4. Here, too, although the differences are slight, the triplet states of the polymers have decay times like that of T.

When the nucleotides are dissolved in deuterated solvent, the decay times change as indicated, and, once again, the isotope effect of DNA and poly-dAT is like that of T. Finally, in Table 2 are listed the values of D and E, the two parameters of the spin-spin interaction in the triplet state determined by observing the $\Delta m = 1$ and "$\Delta m = 2$" transitions in the ESR spectrum. Here, too, the same comparison can be made, and once again we find that, while the triplet-state properties of T and T^- do not differ very much, the triplets of DNA and poly-dAT resemble the triplet of T more closely than they resemble that of T^-. For contrast we have also listed in Table 2 the ESR parameters of the AMP and GMP triplets, just to

Table 4. The effects of deuteration upon triplet lifetimes ${}^3\tau$; H and D refer to samples dissolved in EG : H_2O and EG-d_2 : D_2O, respectively.

Sample	${}^3\tau$		${}^3\tau(D)/{}^3\tau(H)$
	H	D	
DNA (calf thymus)	0.30 ± 0.03	0.45 ± 0.02	1.50 ± 0.17
TMP (sensitized)	$.35 \pm .02$	$.52 \pm .04$	$1.49 \pm .20$
TMP^- (pH 12)	$.45 \pm .02$	$.55 \pm .02$	$1.22 \pm .10$
DMT (sensitized)	.73	.79	1.08

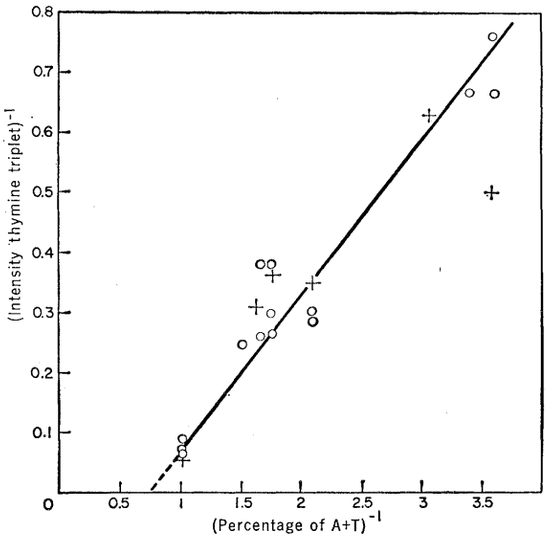
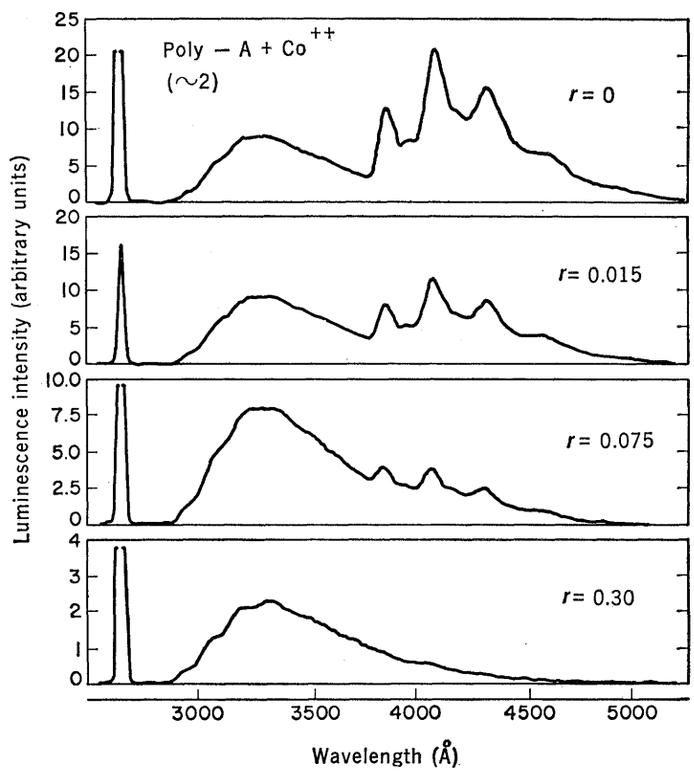


Fig. 8 (above). Graph of the inverse of the observed T triplet intensity (from optical and ESR experiments) plotted against the inverse of the A-T base-pair content of DNA's from various sources. (Circles) Data from phosphorescence measurements; (crosses) data from ESR experiments. Fig. 9 (right). Quenching of the phosphorescence in poly-rA by Co^{++}, which hardly affects the fluorescence. The range of phosphorescence quenching is limited in this case by the low molecular weight of the poly-rA ($s_{20} \sim 2$) and is more extensive in longer poly-rA molecules (*29*). The amount of metal found, M_b, and the total phosphate concentration, P_t, are related by the parameter $r = M_b/P_t$.



show how different these triplets are from the two forms of T.

To return now to poly-dAT, one possible difference between it and ApT is that it offers the opportunity for long-range energy transfer. However, quenching experiments (*24*) with Mn^{++} at 77°K show that each metal ion quenches the phosphorescence of only five or six bases; this limited range is probably explainable in terms of direct Förster-type transfer to the metal ion.

The singlet state of DNA is an excimer. Because of their broad unstructured shapes, it is not possible to use the excimer spectra to identify the pairs of bases responsible for the emission. Since the G-C base pairs are quenched (*17, 25*) at the singlet level in poly-dG:dC and poly-rG:rC, it is likely that the DNA excimer involves the A-T bases rather directly.

The dependence of the T-triplet intensity upon the percentage of A-T base pairs is shown in Fig. 8. The curve for the inverse of triplet intensity plotted against the inverse of the fractional A + T content is a straight line, in accordance with a very recent analysis (*26*). This analysis distinguishes between the relative probabilities that energy is *absorbed* by A-T or G-C base pairs and the relative probabilities that the energy is *trapped* by A-T or G-C base pairs. Once trapped by the G-C base pair, the energy is assumed to be quenched; when the energy is trapped by the A-T base pair, there is a certain probability that a T triplet will form. From the intercepts of the straight line it is possible to calculate that the G-C base pairs are four times as effective as the A-T base pairs in trapping the energy; by contrast, the probabilities of absorption are known to be approximately equal. Hence it is apparent that, some time after the absorption occurs but before the triplet is formed, the DNA excitation is delocalized. However, this experiment does not determine either the extent or the mechanism of the delocalization.

It is worth noting that experiments have shown sensitized fluorescence of acridine dyes bound to DNA at 300°K (*26a*) and also at 77°K (*26b*); this fluorescence can be explained (*26*) as indicating a Förster transfer (*27*) and not a long-range base-base energy transfer in the DNA. These and other experiments concerning energy transfer have been critically reviewed recently (*26*).

In contrast to the uncertainty about triplet energy transfer in DNA, the metal-ion quenching experiments on poly-rA (in which all the bases are A's) first mentioned by Bersohn and Isenberg (*28*) do show (*29*) that triplet excitation can move from base to base. Figure 9 shows extensive quenching of the triplets in poly-rA by Co^{++}, which hardly reduces the fluorescence. The limitation upon the range of triplet quenching in this experiment has been shown to be the length of the poly-rA chains (*29*). With sufficiently long strands of poly-rA, one transition metal ion bound to poly-rA quenched the phosphorescence of approximately 130 bases. Furthermore, this is a lower limit for the range of triplet migration during the triplet's 2.4-second lifetime, the range in these experiments being limited by a random arrangement of lengths along which transfer can occur. These lengths may be straight segments separated by kinks in the polymer.

To summarize, we have presented evidence for singlet energy transfer, to excimers, between neighboring bases. Calculations of the singlet energy-transfer distance by the Förster mechanism have been made for purines and pyrimidines in the Watson-Crick structure of DNA and they show that transfer over distances of a few bases is possible at low temperatures (*10*) but unlikely at room temperature since the lifetimes are much shorter. Long-range transfer at the triplet level, on the other hand, has been demonstrated in poly-rA. The existence and range of energy transfer must, therefore, be investigated separately for each polynucleotide under consideration.

Photoproducts and Photomechanism

One important reason for studying the excited electronic states of DNA is the insight this gives about the subsequent photochemistry. In fact, it was an identification (*30*), by electron spin resonance, of the T free radical in irradiated DNA which aroused our interest in the excited states of DNA.

The present knowledge of the photoproducts in DNA is limited. Some five or six acid-stable products have been separated chromatographically, but only the pyrimidine dimers (*31*), dihydrothymine (*32*), and a CT compound (*33*) have been identified chemically. Of these, the T dimer (TT) has been studied most extensively. Even for this product there are still formidable gaps in our knowledge. Here we discuss the excited-state precursor of the T dimer. The discussion is largely based on results for T and dimethylthymine (DMT) dimerization, but other pyrimidine dimers, such as CC and CT, may dimerize by a similar mechanism.

The T dimer was discovered by Beukers and Berends (*34*) in frozen aqueous solution of T, and, since then, all four forms of dimers have been prepared and identified (*35*). Their structure can be described as follows: two T molecules whose 5-6 bonds are joined by two covalent bonds to form a cyclobutane ring in the *cis* or *trans* configuration, each of which configurations may have the T molecules parallel (head-to-head) or antiparallel (head-to-tail). In irradiated frozen aqueous T solutions the *cis* head-to-head, or ice, dimer is formed, and the same dimer is isolated from irradiated DNA (*35, 36*). In irradiated TpT solutions, all four dimers are found, albeit with different abundances (*37*).

Is the excited-state precursor of TT a singlet or a triplet level of T? First it is well to state that the answer may differ for different systems (for example, TpT solution, T solution, frozen T solutions, or DNA). In fact it can be shown that, if dimer formation occurs with reasonable yield between monomeric solute molecules in solution, the dimer must have a triplet precursor, because singlet lifetimes simply are not long enough to permit excited-state bimolecular reactions to occur. However, we emphasize that the same reaction which proceeds via a triplet state in solution may have a singlet-state precursor when the chromophores are held closer together—that is, in frozen solutions or in a polymer.

From a rough estimate for $10^{-3}M$ solutions, we conclude that the time required for two solute molecules to diffuse together is of the order of 10^{-8} second, while even for $1.0M$ solutions the time is about 10^{-10} second. The lifetime of the T singlet state at 77°K, where its quantum yield is 0.16, is about 10^{-9} second, and since the fluorescence quantum yield is less than 10^{-3} in water, the lifetime must be less than 10^{-11} second. Dimerization in solution via the singlet state is therefore extremely unlikely. On the other hand, the T triplet state, with its longer lifetime, is a possible precursor of the T dimer. Recent experiments on triplet quenching and sensitization in solutions of monomers have shown triplet-state precursors

(*38*), in accordance with this analysis.

The situation is quite different when we turn to systems in which the monomers are always in close proximity to each other. There the fluorescence of the monomer is commonly quenched, and dimerization quantum yields are high. Thus (*39*), frozen aqueous T solutions, which consist of microcrystalline aggregates of T, have a fluorescence quantum yield of less than 10^{-3}, while isolated T molecules in ethylene glycol and water have a yield of 0.16. The corresponding quantum yields for dimerization are approximately unity and zero. Similarly, DMT dimers dissolved in ethylene glycol and water can, after being broken by light of 248-nanometer wavelength, be re-formed, with a quantum yield of unity (*40*), so long as the monomeric units are kept in close proximity to each other in the frozen glass.

From these experiments it is concluded that, in condensed T systems such as frozen solutions and solutions of broken dimers in aqueous glass, a singlet state is the most likely precursor of the dimer. This is possibly the case for the dimer formed between neighboring T bases in DNA also, but this has not been demonstrated. Lamola and Yamane (*41*) have shown that the triplet state *can* lead to the formation of T dimers in DNA by producing T dimers by triplet sensitization without exciting the singlet state of DNA. Since this method produced DNA containing T dimers and virtually no other acid-stable lesions, it should prove helpful in experiments undertaken to probe the biological consequences of ultraviolet irradiation.

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