INHIBITION OF PYRIMIDINE DIMER FORMATION IN DNA BY CATIONIC MOLECULES: ROLE OF ENERGY TRANSFER

B. M. SUTHERLAND *and* J. C. SUTHERLAND

From the Department of Molecular Biology, Walter Reed Army Institute of Research, Washington, D.C. 20012

ABSTRACT In addition to the acridine dyes, acridine orange and proflavine, we find that three other cationic molecules which bind to DNA—ethidium bromide, chloroquine, and methyl green—inhibit the production of cyclobutyl pyrimidine dimers by ultraviolet radiation. Intercalation is not necessary for dimer inhibition. The long range nature of the inhibition implies that energy transfer is responsible. The transfer is between the lowest excited singlet state of DNA and the acceptor singlet, and seems to involve the Förster mechanism.

INTRODUCTION

Cyclobutyl pyrimidine dimers, produced in DNA by ultraviolet (UV) radiation (220–300 nm), are of chemical interest because of their lethal and mutagenic effects. (See, for example, review by Setlow, 1966.) Beukers (1965) and Setlow and Carrier (1967) have shown that the presence of the acridine dye proflavine during UV irradiation of DNA reduces dimer yield. Since the acridines intercalate between the DNA bases (Lerman, 1964 and references cited therein), dimer reduction was attributed to physical blockage (Beukers, 1965) or helix distortion (Setlow and Carrier, 1967). We showed that each proflavine inhibited dimer yield over 12 base pairs and each acridine orange over 14 base pairs. These long distances indicated that energy transfer from DNA to the dye contributed to dimer inhibition (Sutherland and Sutherland, 1969).

If energy transfer is responsible for dimer inhibition, other molecules with appropriate energy levels and which bind to DNA should also reduce dimer yield. We find that the intercalating molecules, ethidium bromide and chloroquine, as well as the nonintercalant methyl green, reduce dimer yield with high efficiency. Analysis of the relative efficiency of dimer quenching, energy levels, and of the spectral properties of the acceptors shows that energy transfer can account for the reduced dimer yield. This transfer occurs between the singlet levels of the donor and acceptors and involves the Förster mechanism.

METHODS AND MATERIALS

Methods of labeling DNA and production of, assay for, and identification of pyrimidine dimers have been described previously (Sutherland and Sutherland, 1969; Sutherland et al., 1968). Briefly, purified thymine-^{3}H labeled DNA was irradiated (4×10^4 erg/mm^2 of 254 nm; fluences corrected for self-absorption of the DNA and for absorption by the dye) in 0.001 M phosphate buffer in the presence of cationic acceptor molecules. The samples were hydrolyzed in formic acid, chromatographed on cellulose thin layers in butanol:acetic acid:water (40:6:15) (Smith, 1963), counted in a scintillation counter and analyzed for thymine and thymine-containing dimers.

Additional procedures used in the current experiments are described in detail in the two sections below.

Preparation of Solutions

Ethidium bromide (2,7-diamino-10-ethyl-9-phenylphenanthridium bromide) was purchased from the California Biochemical Corporation (Los Angeles, California). Concentrations were determined spectro-photometrically using a molar extinction coefficient at 480 nm of 5.6×10^3 (Waring, 1965). Methyl green (methylated hexamethyl pararosaline) was purchased from the National Aniline Division of Allied Chemical Corp. (New York, N. Y.) and purified extensively by Kurnick's (1950) method. Concentrations of stock solutions were determined spectrophotometrically in 0.2 M acetate buffer, pH 4.1, using a molar extinction coefficient of 7.7×10^4 at 632 nm (Kurnick, 1950). Highly purified chloroquine (7-chloro-4(4 diethylamino 1-methylbutylamino)quinoline) was the gift of Dr. L. Kazyak of our Institute. A molar extinction coefficient of 1.89×10^4 at 343 nm (Cohen and Yielding, 1965) was used to calculate stock concentrations.

Solutions were prepared by dissolving the thymine-^{3}H labeled DNA in 0.001 M PO_4 buffer, pH 7.0, to give a final concentration of about 5×10^{-5} M in DNA phosphate, and adding dye stock solutions to final concentrations of 5×10^{-6} to 10^{-4} M. All procedures were carried out in the dark or in subdued light to prevent possible photodynamic effects or fading of the dyes. The solutions were allowed to complex for at least 12 hr.

Since free methyl green, but not that bound to DNA, fades on exposure to light (Kurnick, 1950), special procedures were followed to prevent changes in absorption of methyl green-DNA solutions during UV irradiation. Methyl green and DNA were allowed to complex at 5°C for at least 12 hr, then exposed to a fluorescent light for 24 hr to allow complete fading of the unbound dye.

Long-Wavelength UV Irradiation

Samples were exposed to wide-spectrum long-wavelength UV from a lamp fitted with two FT8-B1B bulbs (General Electric Co., Schenectady, N. Y.). A 4 mm thick pyrex plate was placed between the samples and the light to exclude wavelengths below 300 nm. A Jagger (1961) meter calibrated for black light was used to determine exposure rates. Exposure times were adjusted for absorption by the dyes according to the absorbance of the solution at 360 nm.

RESULTS AND DISCUSSION

Acridine dyes bind to DNA in solution by intercalating between adjacent base pairs and by exterior ionic bonding (Lerman, 1964 and references cited therein).

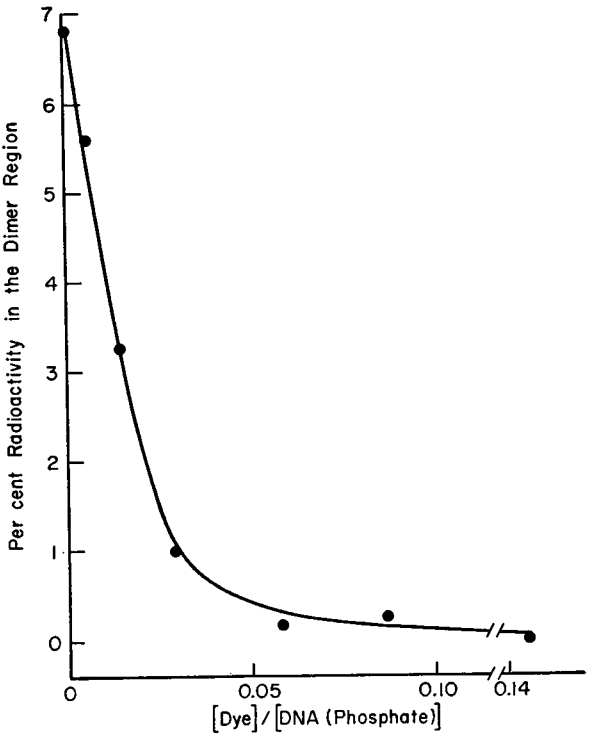

FIGURE 1 A typical experiment showing the effect of ethidium bromide on dimer yield. For D less than 0.1 all the dye is bound to the DNA by intercalation (Waring, 1965).

The presence of acridines during UV irradiation of DNA inhibits pyrimidine dimer formation (Beukers, 1965; Setlow and Carrier, 1967; Setlow and Setlow, 1967). Energy absorbed by DNA may be transferred to a bound acridine (Weill and Calvin, 1963; Lerman, 1963). The effective number of base pairs over which each dye molecule inhibits dimer formation, β, is too large to be due to physical blockage or helix distortion and implicates energy transfer (Sutherland and Sutherland, 1969).

If this interpretation is correct then other molecules which bind to DNA and have suitable energy states should also reduce dimer yield with β's similar to the acridines. Ethidium bromide, which differs in structure from the acridines, intercalates between adjacent base pairs (Waring, 1965). LePecq and Paoletti (1967) have shown that in solution at room temperature UV energy absorbed by DNA sensitizes the fluorescence of ethidium bromide, thus implying energy transfer. Fig. 1 shows dimer yield as a function of D, the number of bound dyes per DNA base pair. Low values of D reduce dimer yield sharply. At these low D values, virtually all the ethidium bromide is bound to the DNA by intercalation and there is no overlap in the segments of DNA affected by each dye. Under these conditions, N, the per cent dimers formed at a given D, is related to N_0, the per cent dimers formed in the

absence of dye, by the equation

$$N = N_0(1 - \beta D). \quad (1)$$

Thus, β can be calculated from the initial slope of the N vs. $2D$ curve.[1] The data in Fig. 1 give a β of 17 for ethidium bromide.

Methyl green is a triphenyl methane dye. These dyes are nonplanar and have been shown not to intercalate by X-ray diffraction (Neville and Davies 1966) and by

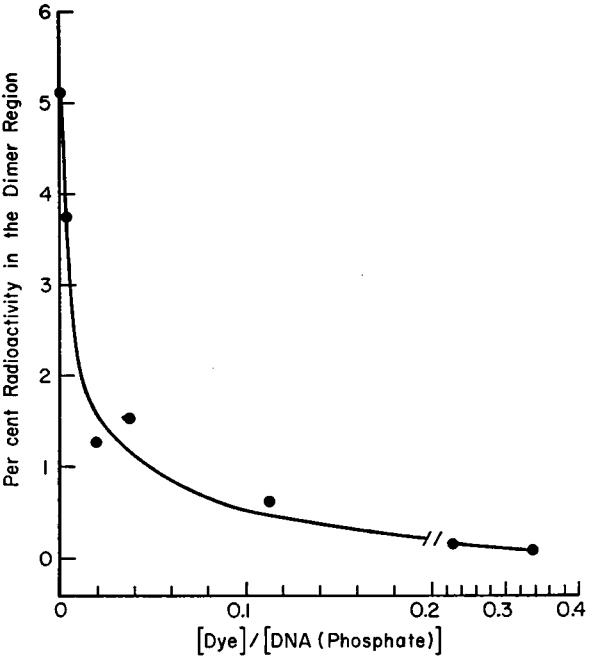

FIGURE 2 Effect of methyl green on dimer yield. For D less than 0.1 all the dye is bound to DNA. Methyl green does not intercalate.

flow dichroism.[2] Their failure to disturb the layer line spacing of DNA (Neville and Davies, 1966) indicates that they do not distort the helical structure. The lowest excited singlet, S_1, of methyl green lies below both the S_1 and T_1 (lowest excited triplet) of DNA and thus transfer from DNA to methyl green is energetically possible. As Fig. 2 shows, small concentrations of methyl green greatly reduce dimer yield. These data give β of about 20 base pairs. The high β's for dimer reduction for these two nonacridines and the ability of the nonintercalating molecule, methyl

[1] The factor 2 converts the data in Figs. 1, 2, 4, and 5 (given in DNA phosphate) to base pairs as required by equation 1.

[2] Hahn, F. E., and A. Krey. In preparation.

green, to inhibit dimer formation strongly support our hypothesis (Sutherland and Sutherland, 1969) that energy transfer reduces dimer yield.

Setlow and Carrier (1967) have shown that proflavine reduces dimer yield by changing the rate of formation and not the rate of monomerization. Similarly, we have ruled out photosensitized monomerization by ethidium bromide, chloroquine or methyl green as follows: labeled-^{3}H DNA was irradiated with 4×10^4 erg mm^{-2} of 254 nm radiation. Then ethidium bromide, chloroquine, or methyl green was added to a final dye/DNA (phosphate) of about 0.5, the samples were exposed to about 10^5 erg/mm^2 of black light (which only the dyes absorb) and analyzed for dimers. Controls (+254 nm, no dye) were also exposed to black light and analyzed for dimers. The black light irradiation did not change dimer content in samples with or without the dye. Thus, dye-sensitized monomerization is not responsible for dimer inhibition.

Singlet-Singlet Transfer

All the dyes shown to reduce dimer yield—proflavine, acridine orange, ethidium bromide, and methyl green—have their S_1 and T_1 levels below both the S_1 and T_1 of the DNA bases. Several types of energy transfer from DNA to bound dyes have been demonstrated optically: (*a*) transfer of singlet DNA energy to the singlet of the dye (singlet-singlet transfer), observed in solution at room temperature as sensitized fluorescence (Weill and Calvin, 1963; Lerman, 1963; LePecq and Paoletti, 1967); (*b*) transfer from the DNA triplet to the acridine triplet (triplet-triplet transfer), observed at 77°K as sensitized phosphorescence (Galley and Davidson, 1966; Galley, 1968); and (*c*) transfer from the DNA triplet to the dye singlet (triplet-singlet transfer), observed at 77°K as delayed fluorescence (Isenberg et al., 1964; Galley, 1968).

These transfers have been associated with two mechanisms, the long range or Förster mechanism, caused by interaction of the transition dipoles of the donating and accepting states, and characterized by transfer ranges up to 5–10 nm, and the charge exchange mechanism (for discussion see Dexter, 1953, and Galley, 1968), which requires overlap of the wave functions of the donor and acceptor, and is thus a short range interaction.

Galley (1967, 1968) has attributed long range triplet-triplet transfer at 77°K to repeated charge exchange reactions between adjacent bases and then to an intercalated dye. Methyl green does not intercalate and thus does not have extensive π-electron overlap with the DNA bases. From Galley's reasoning, we would expect that if charge exchange were the only mechanism of dimer inhibition, methyl green should not inhibit dimer formation as well as an intercalated dye. However, Fig. 2 shows that methyl green is one of the most effective inhibitors of dimer formation. Therefore, charge exchange alone cannot be responsible for the efficient dimer inhibition by methyl green. Since triplet-triplet transfer can occur only by the charge

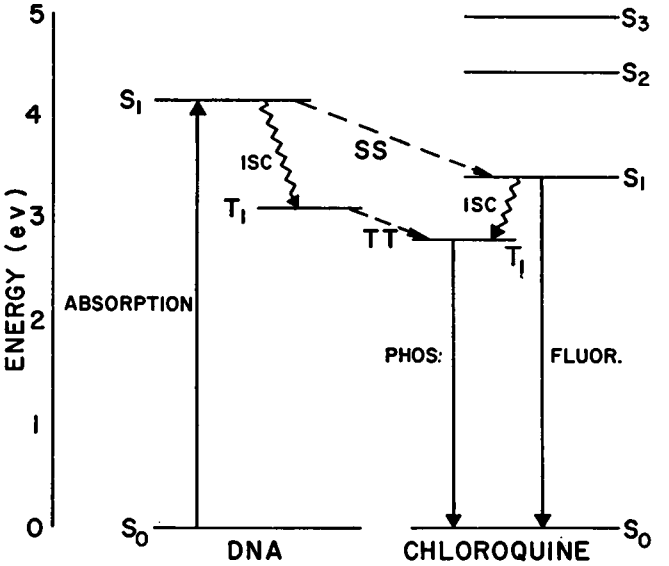

FIGURE 3 Energy level diagram for DNA and chloroquine. The energy of the lowest singlet (S_1) and lowest triplet (T_1) levels of DNA relative to the ground state (S_0) are taken from Lamola et al. (1967). The energies of the three lowest excited singlet states (S_1, S_2, S_3) of chloroquine relative to the ground state (S_0) were determined from the wavelengths of the red edges of the corresponding absorption bands; the energy of the lowest triplet (T_1) was determined from the blue edge of chloroquine's phosphorescent emission (J. C. Sutherland and B. M. Sutherland, 1969). The singlet state of chloroquine may be populated either by direct absorption or by singlet-singlet (SS) transfer, while the triplet state can be populated by inter system crossing (ISC) or triplet-triplet (TT) transfer. Since the S_1 of chloroquine lies above the T_1 of DNA, triplet-singlet (TS) transfer is not possible.

exchange mechanism (Galley, 1967, 1968), our evidence suggests that triplet-triplet transfer does not contribute to dimer inhibition by methyl green.

In contrast to the dyes studied, chloroquine has its S_1 above the T_1 of the DNA bases (see Fig. 3). O'Brien et al. (1966) have reported that it binds to DNA by intercalation. We have observed that DNA sensitizes chloroquine fluorescence.[8] Since transfer from the DNA triplet to the chloroquine singlet is energetically impossible, this sensitization must be due to singlet-singlet transfer. If triplet-singlet transfer were the only path leading to dimer inhibition, chloroquine could not affect dimer yield. Fig. 4 shows that chloroquine inhibits dimer formation with a β of about 8. Thus, in the case of chloroquine, triplet-singlet transfer does not contribute to dimer inhibition. Our results are consistent with the hypothesis that dimer inhibition by bound dyes is due to singlet-singlet transfer. Further support for this interpretation comes from the agreement of our β values for dimer inhibition with those of Weill and Calvin (1963) and LePecq and Paoletti (1967) for sensitized

[8] Sutherland, J. C., and B. M. Sutherland. In preparation.

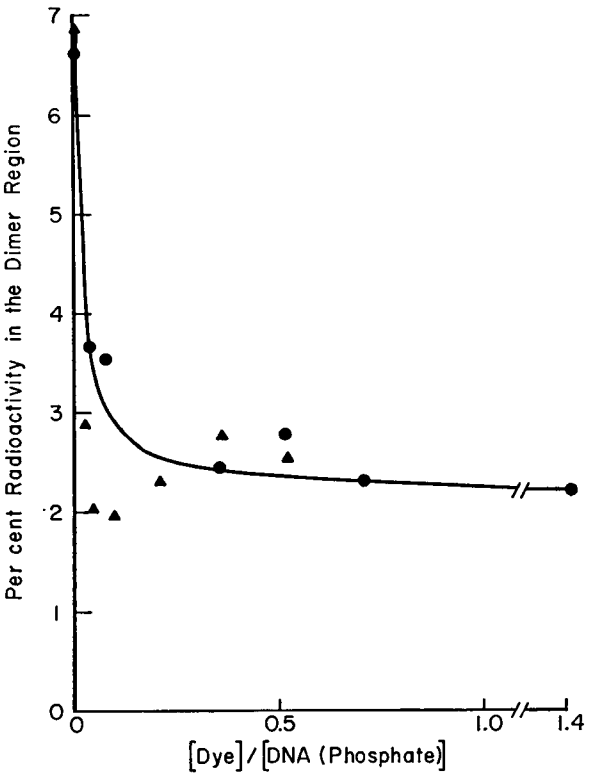

FIGURE 4 Two experiments showing the effect of chloroquine on dimer yield. The values of 6.6 and 6.75% dimers for DNA exposed to 4×10^4 erg mm^{-2} of 2537 A radiation in the absence of dye agree with those obtained by Wulff (1963) and by Sutherland and Sutherland (1969).

fluorescence. Weill (1965) and Burns (1969) have measured the time required for energy transfer from DNA to bound dyes (proflavine and ethidium bromide, respectively). Weill found that the transfer time for proflavine was less than 0.1 nsec; Burns (1969) estimates a maximum transfer time of 1 nsec. These results show that sensitized fluorescence is due to singlet-singlet transfer.

Mechanism of Singlet-Singlet Transfer

Either the charge exchange or the Förster mechanism can be responsible for singlet-singlet transfer. However, following Galley's reasoning, charge exchange cannot be entirely responsible for dimer inhibition by methyl green. Further, Dexter (1953) has pointed out that if the transitions of the donor and acceptor are allowed (i.e. singlet-singlet in the case of the DNA and the acceptors), the transfer mechanism will usually be the Förster mechanism. Thus, it seems likely that the singlet-singlet transfer involves the Förster mechanism.

The curve of N vs. D shows a rapid decrease in N for low values of D, followed by a plateau (see Figs. 1, 2, and 4). The shape of these curves can be analyzed in terms of transfer by the Förster mechanism. In this mechanism the transfer rate, k_T, is inversely proportional to the sixth power of the distance between the donor and acceptor, R. That is,

$$k_T = k_{T_0} R^{-6} \tag{2}$$

where k_{T_0} is a function of the spectral properties of the donor and acceptor and the angular orientation of their transition moments (see Förster, 1965). The probability that an excitation will be transferred is

$$\Phi_T = (k_T)\,(k_T + \Sigma k)^{-1} \tag{3}$$

where Σk represents the sum of the rate constants for all other de-excitation paths. If R_0 is the range for which Φ_T is $\frac{1}{2}$,

$$R_0^6 = k_{T_0}/\Sigma k. \tag{4}$$

Thus R_0 and also β are large if $k_{T_0}/\Sigma k$ is large.

The maximum transfer rate, $k_{\max}$, occurs when R is the effective distance of closest approach, $R_{\min}$, and the corresponding maximum transfer probability is

$$\Phi_{\max} = \left(1 + \frac{\Sigma k}{k_{T_0}} R_{\min}^{6}\right)^{-1}. \tag{5}$$

At high D there is an acceptor within a distance $R_{\min}$ of each donor and thus the transfer probability is $\Phi_{\max}$. The fraction of dimers in the plateau region is

$$\widehat{\text{PyPy}}_{(\text{plat})}/\widehat{\text{PyPy}}_{(0)} = 1 - \Phi_{\max}. \tag{6}$$

$$= 1 - \left(1 + \frac{\Sigma k}{k_{T_0}} R_{\min}^{6}\right)^{-1}. \tag{7}$$

$R_{\min}$ is about the same for all of the intercalating molecules (see, for example, Lerman, 1964; Peacocke and Skerrett, 1956; and Waring, 1965). Equation 7 shows that if $k_{T_0}/\Sigma k$ is large, the plateau is small. However, as discussed previously, large values of $k_{T_0}/\Sigma k$ correspond to large values of β. Consideration of equations 1 and 7 indicates that steep initial slopes (large β's) should correspond to low plateaus.

Fig. 5 shows the normalized curves of N vs. D for ethidium bromide and chloroquine taken directly from the curves in Figs. 1 and 4, and that previously reported for proflavine (Sutherland and Sutherland, 1969). The data show that steep initial slopes (large β) correspond to small plateaus, and vice versa. We can also analyze the data of Fig. 5 intuitively. If k_{T_0} is large, then the transfer mechanism can compete

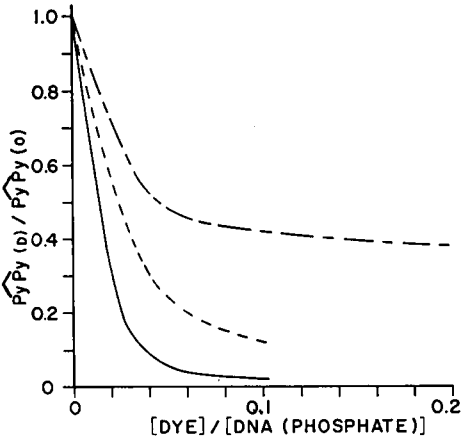

FIGURE 5 Normalized dimer yield as a function of the acceptor to DNA phosphate ratio. Ethidium bromide, ——; proflavine, ——; chloroquine, — — —. A steep initial slope corresponds to a low plateau.

with the other de-excitation mechanisms even when R is large. Thus, β will be large. As the acceptor approaches the donor, the transfer rate increases (equation 2). If the efficiency of the transfer mechanism is comparable to the other processes at long range, it will overwhelm them at short range. For high values of D, when there is an acceptor in the immediate vicinity of each DNA base, most of the excited bases will transfer their energy to a dye and very few dimers will be formed. The reduction of dimer yield by ethidium bromide is such a case: β is about 17 and the presence of one dye for every 4 or 5 bases virtually eliminates dimer formation.

Conversely, when k_{T_0} is smaller, β is smaller, and even if an acceptor is very near each base, there is a finite probability of de-excitation via one of the normal paths, including, of course, dimer formation. For proflavine β is about 12 and Fig. 5 shows that the plateau is about 0.1 at $D = 0.1$. Even at very high values of D (3.74), dimer yield does not drop below a tenth of its initial value (Setlow and Carrier, 1967). For chloroquine β is about 8 and dimer yield is not reduced below three-tenths of its initial value even for $D = 1.4$. Thus, an explanation of dimer inhibition based on the Förster energy transfer mechanism is in qualitative agreement with our data.

The Förster mechanism can be described quantitatively as follows: If the donor undergoes thermal relaxation before transfer, the transfer rate is given by

$$k_T = AR^{-6}(\cos\theta_{DA} - 3\cos\theta_D \cos\theta_A)^2 J \qquad (8)$$

for

$$J = \int_0^\infty f^D(\nu)\sigma^A(\nu)\nu^{-4}\, d\nu. \qquad (9)$$

where A is a constant; R is the separation between donor and acceptor; θ_{DA} is the angle between the dipole moments of the donor and acceptor; θ_D and θ_A are the

TABLE I

VALUES OF THE OVERLAP INTEGRALS AND THE LOWEST EXCITED SINGLETS FOR THE FIVE ACCEPTORS

Acceptor	β Base pairs for dimer inhibition	$J \times 10^{-7}$	S_1
		nm^6	cm^{-1}
Methyl green	20	7.76	14.7
Ethidium bromide	17	2.20	17.4
Acridine orange	14	1.27	19.2
Proflavine	12	2.53	22.2
Chloroquine	8	1.80	27.8

angles between the dipole moments of the donor and acceptor and the line joining the donor and acceptor; $f^D(\nu)$ and $\sigma^A(\nu)$ are the normalized fluorescent intensity of the donor and cross-section for absorption of the acceptor at frequency ν (Förster, 1965). J is the spectral overlap integral between the donor and acceptor. We have calculated J for all five acceptors, using the absorption spectra of completely bound dyes under the same conditions used in the dimer experiments and the values of Eisinger et al. (1966) for fluorescence from native DNA. The values of these integrals and the lowest excited singlets for the five acceptors are shown in Table I. The quenching ability of the dyes increases with increasing energy gap between the S_1 of DNA and the S_1 of the dye. However, the values of J and β do not increase in the same order. Presumably, this results from small differences in the orientation factors of the intercalants and a larger difference in the orientation factors of the nonintercalant methyl green. The J values were calculated assuming vibrational relaxation and transfer from the DNA exiplex; the relative values of J could be affected if either condition were not fulfilled.

We have shown that nonacridines such as ethidium bromide and chloroquine and even the nonintercalant methyl green reduce dimer yield. The data on methyl green and chloroquine, the agreement between the β's for dimer inhibition and those for sensitized fluorescence, as well as the nanosecond transfer times, implicate singlet-singlet transfer. Our data can be explained qualitatively in terms of the Förster mechanism. Thus, we conclude that energy transfer from the DNA singlet to an acceptor singlet, involving the Förster mechanism, inhibits dimer formation.

Dr. B. M. Sutherland's present address is the Department of Molecular Biology and Virus Laboratory, and Dr. J. C. Sutherland's present address is the Laboratory of Chemical Biodynamics, Lawrence Radiation Laboratory. Both of these laboratories are at the University of California, Berkeley, California 94720.

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