MECHANISMS OF INHIBITION OF PYRIMIDINE DIMER FORMATION IN DEOXYRIBONUCLEIC ACID BY ACRIDINE DYES

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

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

ABSTRACT The ultraviolet (UV)-induced formation of cyclobutyl pyrimidine dimers in *Escherichia coli* deoxyribonucleic acid (DNA) in vitro has been investigated in terms of the mechanism of inhibition by acridine dyes, the effect on dimer yield of specific singlet and triplet quenchers, and the mechanism of dimer formation. Our results indicate that (*a*) energy transfer is important in dimer reduction by acridines, (*b*) this transfer occurs from the singlet (S_1) of DNA, and (*c*) at room temperature triplet quenchers do not reduce dimer yield in DNA.

INTRODUCTION

Ultraviolet (UV) radiation (220–300 nm) produces cyclobutyl pyrimidine dimers in DNA both in vivo and in vitro (see reviews by Setlow, 1966; Setlow, 1964; Smith, 1964; Wacker, 1963). The presence of the acridine dye proflavine during UV irradiation decreases dimer formation (Beukers, 1965; Setlow and Carrier, 1967; Setlow and Setlow, 1967). Pyrimidine dimers are of special interest because of their implication in the lethal (see reviews by Wacker, 1963; Smith, 1964; Setlow, 1964; Setlow, 1966) and mutagenic effects of UV (Witkin et al., 1963; Hill, 1965; Witkin, 1966; Kondo and Kato, 1966; Sutherland et al., 1968). Although the mechanisms of dimer production in solutions of mononucleotides, dinucleotides, and synthetic polynucleotides have been investigated (see for example: Greenstock and Johns, 1968; Greenstock et al., 1967; Eisinger and Shulman, 1967; Lamola and Eisinger, 1967; and Eisinger and Lamola,1967), the energetic precursors of the dimer in DNA are uncertain.

Acridine dyes bind to DNA by intercalating between adjacent base pairs and by exterior ionic bonding (Lerman, 1961, 1963, 1964 *a* and 1964 *b*). The mutagenic, carcinogenic, or antimalarial activities of several molecules have been associated with their ability to intercalate (Lerman, 1964 *c*; Isenberg and Baird, 1967; O'Brien et al., 1966). This property has also been used in investigations of supercoiled DNA

(Crawford and Waring, 1967; Clayton and Vinograd, 1967), and as a test for double-strandedness of DNA (Le Pecq and Paoletti, 1967; Allison et al., 1965).

Study of the effect of acridines on dimer yield can give information not only on the mechanism by which intercalants prevent dimer formation, but also on the mechanism of dimer formation. For these reasons we have investigated the effect of acridine dyes and specific quenchers of singlet and triplet states of DNA on the dimer yield. Our results indicate that at room temperature (*a*) energy transfer from DNA to acridines is important in the reduction of dimer yield, (*b*) singlet states of DNA are responsible for this transfer, and (*c*) triplet quenchers do not reduce dimer yield in DNA.

MATERIALS AND METHODS

Thymidine-^3H Labeled DNA

The DNA of *E. coli* 15 T^- was labeled by growing the cells in medium containing 1 mc of thymidine-^3H (New England Nuclear Corp., Boston, Mass., specific activity >15 C/mM) and nonradioactive thymidine to a total concentration of 4 μg/ml (Setlow and Carrier, 1964). The DNA was purified by a modification of Marmur's (1961) method and contained about 10^3 cpm/μg.

Preparation of Solutions

Proflavine sulfate was obtained from the National Aniline Division of Allied Chemical Corp., New York; acridine orange, obtained as a histological stain from Fisher Scientific Co., Pittsburgh, Pa., was purified extensively by the method of Weill and Calvin (1963); Ni^{++} and Mg^{++} were used as their chlorides; the latter was purified by recrystallization from water. Both were of reagent grade. Spectro-quality acetone was obtained from Fisher Scientific Co.

Solutions were prepared by dissolving the thymine-^3H labeled DNA in 0.001 M PO_4 buffer, pH 7.0, to give a final concentration of about 3×10^{-5}M in phosphate, and adding either stock solutions of the dye or ion to a final concentration of 1.5×10^{-7} to 3×10^{-5}M, or acetone to a final concentration of 0 to 1% v/v (to 0.15 M). The dye solutions were allowed to equilibrate for at least ½ hour (Freifelder et al., 1961). The vessels containing acetone were tightly covered to prevent evaporation of the acetone.

Irradiation

The exposure for each sample was corrected for self-absorption according to Morowitz's (1950) calculations and for the fraction of the radiation absorbed by the DNA. The absorbances of the DNA solutions at 254 nm were measured in a Beckman DU Spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.) before and after addition of the quencher. Samples of 2.0 ml were irradiated in 3.5 cm diameter plastic petri dishes. When the irradiated solutions contained acetone, the petri dish was covered with a quartz plate to prevent evaporation. The samples were exposed to 254 nm radiation from a low pressure mercury lamp (General Electric Company, Schenectady, N. Y., G15T8). The exposure rate at the top surface of the solution was 10^4 erg mm^{-2} min^{-1} as calculated from Jagger (1961) meter readings. The dose delivered to the DNA of each sample was 4×10^4 erg mm^{-2}. All irradiations were carried out at room temperature.

Assay for Dimers

After irradiation the samples (containing about 20 μg of *E. coli* DNA) were chilled; 20 μg of calf thymus DNA were added for aid in chromatographic identification. An equal volume of 10% trichloroacetic acid was added, and the samples were centrifuged at 5000 rpm for 10 minutes, then drained briefly. The precipitates were hydrolyzed in formic acid at 175° C for 30 min. The hydrolysates were dried, spotted in a small amount of water on Brinkmann (Brinkmann Instruments Inc., Westbury, N. Y.) Cellulose MN 300 F_{254} thin layer plates and chromatographed in a Brinkmann Sandwich tank in butanol :acetic acid: water (40:6: 15) (Smith, 1963). After chromatography, the markers were located by a germicidal lamp, the chromatograms were sliced into ½ cm strips, eluted with water, and counted in a dioxane-based scintillation fluid (Setlow et al., 1963). Each sample contained about 2×10^4 cpm in the thymine region. None of the additives interfered with hydrolysis, chromatography, or counting efficiency.

Identification of Dimers

The identity of the dimers was confirmed as previously described (Sutherland et al., 1968): (*a*) chromatographic mobility in several solvents, (*b*) the kinetics of their irradiation in solution to give thymine as the only radioactive monomer, and (*c*) co-chromatography with dimers formed by irradiation of ^{14}C-thymine in ice. Both thymine-thymine and uracil-thymine dimers (formed from the deamination of cytosine-thymine dimers during acid hydrolysis (Setlow and Carrier, 1966) were measured in the total dimer yield.

Spectrophotometry

A Cary Model 14 recording spectrophotometer (Cary Instruments, Monrovia, Calif.) was used to measure absorbance spectra; a Beckman DU with Gilford (Gilford Instruments, Oberlin, Ohio) recorder and automatic temperature control was used in all thermal denaturation studies.

Fluorometry

Fluorescence measurements were made on an American Instrument Co., Inc., Silver Spring, Md., double monochromator spectrophotofluorometer. The fluorescence monochromator passed a 6 nm band centered at 410 nm. The exciting monochromator was adjusted to pass wavelengths between 250 and 280 nm with a resolution of 12 nm. All measurements were made in 3×3 mm quartz sample cells and were corrected for absorption inside the cell.

Gel Filtration

DNA and acetone in 0.001 M phosphate buffer were separated by gel filtration on Sephadex G-100 (Pharmacia Fine Chemicals, Piscataway, N.J.) in a 2.5×29 cm column previously equilibrated with the same buffer. Thirty-two 0.5 ml fractions were collected, covered immediately, and counted in a liquid scintillation counter.

RESULTS AND DISCUSSION

Mechanisms of Dimer Inhibition

The presence of proflavine at high dye:DNA phosphate ratios during UV irradiation of DNA has been shown to reduce dimer yield greatly (Beukers, 1965; Setlow

and Carrier, 1967; Setlow and Setlow, 1967). Setlow and Carrier (1967) showed that the inhibition of dimer formation resulted from a decreased rate of dimerization and not from an increased rate of monomerization. There are five mechanisms by which this reduction might occur: (*a*) Absorption of UV by the dye instead of the DNA is a trivial possibility and has been eliminated by correcting for the increased absorption due to the dye (see Materials and Methods). (*b*) Dimers could have been broken by energy absorbed by the dye and transferred to the dimer, since the dye absorbs much more strongly at 254 nm than does the dimer. However, Setlow and Carrier (1967) have found that in the presence of proflavine the action spectrum for dimer splitting does not resemble the absorption spectrum of the dye. (*c*) Physical blockage of dimer formation due to the interposition of the proflavine molecule between two potentially dimerizable pyrimidines as suggested by Beukers (1965) would be an extremely short range effect. That is, each intercalant would prevent dimer formation over two base pairs, at the most. (*d*) Intercalation of an acridine dye is thought to distort the DNA helix for a short distance on each side of the dye molecule (Lerman, 1964 *a*). Since the quantum yield for dimer formation in polynucleotides is very small (<0.01), helix distortion might further reduce the quantum yield for dimer production over the distorted segment (Setlow and Carrier, 1967). The distance over which each dye could reduce dimer formation would depend on the length of the distorted segment. Lerman (1961) found that an intercalated molecule distorted the DNA helix to the extent that intercalation was prevented at the two adjacent binding sites. However, the distortion was not sufficient to prevent intercalation at the two second nearest sites. That is, only every other site can be filled. This model agrees with the experimental result (Peacocke and Skerrett, 1956) that the upper limit for intercalatory binding for proflavine is 0.22 dyes per base (i.e., one dye per two base pairs). Thus, one intercalated dye distorts a segment of the helix about four base pairs long. (*e*) The intercalated molecule might also act as a trap for energy which otherwise would produce dimers. If each dye molecule inhibited dimer formation over a segment much greater than four base pairs, we would expect energy transfer to contribute to dimer reduction.

Proflavine: Effect on Dimer Yield

We have investigated dimer yield in *E. coli* DNA as a function of proflavine concentration. Fig. 1 shows the large reduction in dimer yield with increasing dye:DNA base pair ($D:P$) ratios. Up to $D:P = 0.1$, virtually all the proflavine is bound to the DNA and all the bound molecules are associated with the DNA by the strong, or intercalatory, mode (Peacocke and Skerrett, 1956).

For small dye to base pair ratios, there is no overlap of the segments of the DNA in which each dye affects dimer formation. Therefore, β, the effective distance over which each intercalant acts, is given by:

$$N = N_0(1 - \beta D),$$

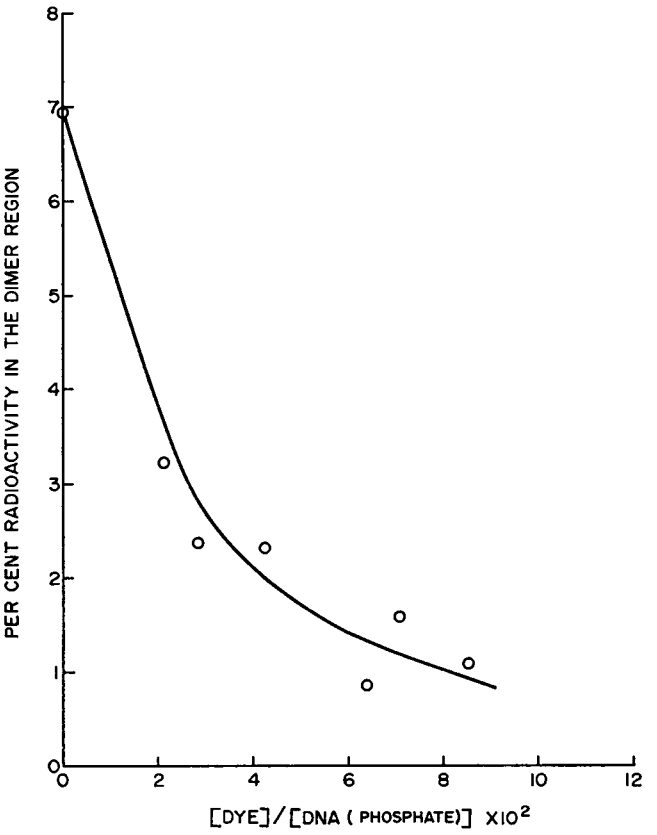

FIGURE 1 A typical experiment that shows the reduction in dimer yield produced by proflavine. The value for DNA alone is in agreement with that obtained by Wulff (1963) for *E. coli* DNA exposed to 4×10^4 erg mm^{-2} of 254 nm radiation. All experiments with the acridine dyes were carried out in subdued or yellow light to prevent possible photodynamic action.

where N_0 is the per cent of dimers in the absence of proflavine, and N is the per cent of dimers at a dye:base pair ratio of D. This relation is similar to that used by Isenberg et al. (1967) for the distance over which paramagnetic ions quenched DNA phosphorescence. β is calculated from the initial slope of the plot of dimer yield vs. dye to base pair ratios. Fig. 1 shows that β is approximately 12 for proflavine. Preliminary results with acridine orange give values of β between 10 and 14. These large values of β suggest that neither physical blockage nor helix distortion was entirely responsible for dimer reduction and argued for transfer of energy from DNA to the dye.

Confirmation of Energy Transfer

If energy transfer is involved, (*a*) β for inhibition of dimerization should agree with that for energy transfer from DNA to the quencher, and (*b*) molecules other than

the acridines should also inhibit dimer formation. Weill and Calvin (1963) found that at room temperature DNA sensitizes the fluorescence of proflavine and acridine orange, and that each acridine orange accepts energy from 10 to 20 base pairs. Thus, their value for β agrees with our value of 10 to 14 base pairs for the inhibition of dimers by acridine orange. In addition to proflavine and acridine orange, ethidium bromide which differs in ring structure from the acridines but is thought to intercalate (LePecq and Paoletti, 1967), also inhibits dimer formation.[1] The value of about 10 for β found by LePecq and Paoletti is in reasonable agreement with the value of 16 obtained from dimer inhibition. Further, methyl green which is nonplanar and does not intercalate, but does bind to DNA (Neville and Davies, 1966), also inhibits dimer formation.[1] Molecules which can accept energy from DNA, but do not bind to it (and thus neither distort the helix nor physically block dimer formation) should also reduce dimer yield. Acetone meets these criteria as follows: (*a*) The lowest singlet energy level (S_1) of acetone lies below the lowest singlet level of DNA (Lamola et al., 1967; Borkman and Kearns, 1966); thus, transfer from DNA to acetone is energetically possible. Further, Lamola et al. (1967) found that thymidine monophosphate (TMP) in solution transfers energy to acetone. We found that the fluorescent emission of 0.1 M acetone excited by 250 to 280 nm radiation was enhanced by about 5% by the presence of 3×10^{-5} M (in phosphate) *E. coli* DNA. Under these conditions DNA accounts for about 10% of the total absorbance; thus, a 5% increase in fluorescence corresponds to transfer of roughly half of the energy absorbed by DNA to acetone. (*b*) Several lines of evidence indicate that acetone could not change the quantum yield for dimer production by altering the structural conformation of DNA. Acetone does not have a planar conjugated ring system as do intercalating molecules. Thus, acetone should not physically block dimer formation nor distort the helix. Concentrations of acetone used in the dimer experiments neither changed the melting temperature nor broadened the melting profile of *E. coli* DNA. The observed per cent hyperchromicity was that expected for native *E. coli* DNA (Marmur and Doty, 1959). Further, difference spectra taken on the Cary Model 14 of DNA in buffer, plus increasing amounts of acetone vs. buffer, plus the same amounts of acetone showed no hyperchromic effect at the concentrations used in the dimer experiments. The UV absorption spectrum of a mixture of acetone and DNA was the sum of the spectra of solutions of the same concentrations of the two components separately. In addition, Yamafugi et al. (1956) have found that acetone derivatives do not cause depolymerization of DNA even after extended exposures. Further, the exposures of UV radiation used in the quenching experiments do not complex acetone and DNA. Fig. 2 shows that after gel filtration of an irradiated DNA-acetone mixture, no ^{14}C (acetone) counts appeared in the DNA (^{3}H) fractions. If 1% of the acetone were bound, 400 cpm would

[1] Sutherland, B. M. and J. C. Sutherland. Paper in preparation.

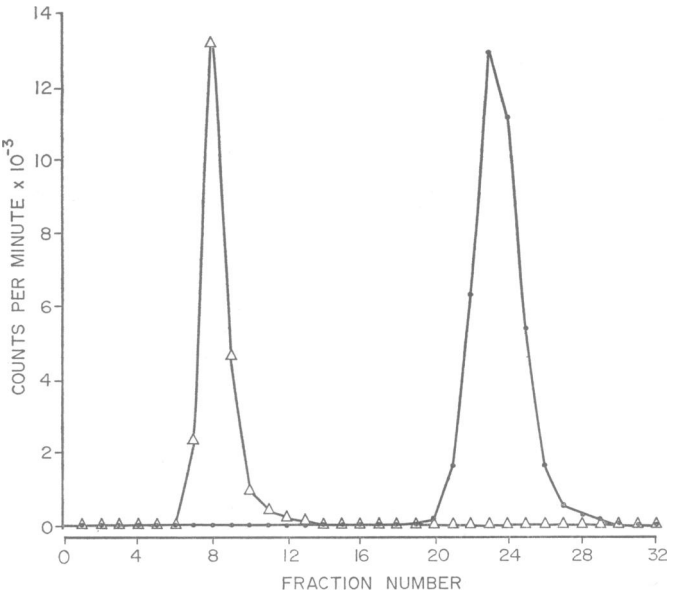

FIGURE 2 Separation of DNA-^{3}H (△) from acetone-^{14}C (●) by gel filtration on Sephadex G-100. The mixture (1.2×10^{-4} M DNA, 0.135 M acetone in 0.002 M phosphate) was exposed to 4×10^4 erg mm^{-2} of 254 nm radiation.

appear in the DNA region. Thus, in our experimental conditions, acetone did not add photochemically to DNA.

The lowest triplet state of acetone (T_1) lies above the triplets of the DNA bases (Lamola et al., 1967). Thus, it is energetically possible for energy absorbed by acetone to be transferred to DNA. Transfer of energy at the triplet level from acetophenone to DNA has been used by Lamola and Yamane (1967) to form thymine dimers. Also, Rosenthal and Elad (1968) have monomerized dimethyluracil dimers by transfer from the triplet of chloranil. However, for transfer from the triplet level of a donor to an acceptor which is not bound to the donor, all oxygen must be excluded, since oxygen strongly quenches the triplet state of the donor and thus prevents the reaction. In our experiments oxygen was present during irradiation and energy absorbed directly by acetone could neither form nor destroy dimers. Hence, any reduction of dimer yield by acetone should be due to energy transfer.

Fig. 3 shows a typical experiment on the effect of acetone on dimer yield. At 0.135 M acetone, dimer yield was reduced to about 40% of the initial value. The decrease in dimer yield supports our hypothesis that energy transfer from DNA to other molecules can reduce dimer formation. Thus, two major lines of evidence support our energy transfer hypothesis: (*a*) the similarity of the β values for energy transfer and for dimer inhibition, and (*b*) inhibition of dimer yield by molecules which interact with DNA in such diverse ways as the acridines and ethidium bromide, methyl green, and acetone.

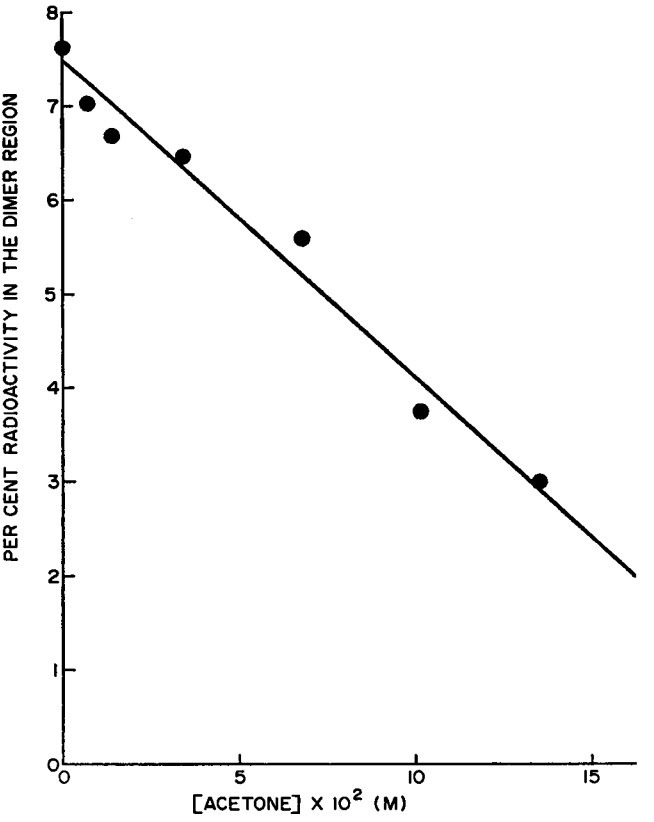

FIGURE 3 A typical experiment that shows the reduction in dimer yield in *E. coli* DNA produced by acetone.

Mechanism of Energy Transfer

The lowest singlet (S_1) and lowest triplet (T_1) of both proflavine and acridine orange lie below both the S_1 and T_1 states of DNA (Lamola et al., 1967; Isenberg et al., 1964). Therefore, either the S_1 or T_1 of DNA could donate energy to the dye.

The following types of energy transfer from DNA to acridines have been observed optically: (*a*) transfer of singlet DNA energy to the singlet of the dye, resulting in sensitized fluorescence (Weill and Calvin, 1963; Weill, 1965); (*b*) transfer from the DNA triplet to the acridine triplet, observed at 77° K as sensitized phosphorescence (Galley and Davidson, 1966; Galley, 1967); and (*c*) transfer from the DNA triplet to the dye singlet, observed at 77° K as delayed fluorescence (Isenberg et al., 1964). Any of these processes, singly or in combination, could be responsible for the reduced dimer yield.

Acetone quenches only singlet states of DNA since its S_1 lies below the S_1 of DNA, while its T_1 lies above the T_1 of DNA (Lamola et al., 1967). Inhibition of

dimer formation by acetone (Fig. 3) thus results from quenching of DNA singlets. Since acridine dyes quench DNA singlets at room temperature (Weill and Calvin, 1963), singlet-singlet transfer contributes to the inhibition of dimer formation by acridines. Weill and Calvin also found that each acridine could accept singlet energy from 10 to 20 base pairs. Fig. 1 shows that each acridine inhibits dimer formation over about 12 base pairs. The similarity of these values shows that singlet-singlet transfer can account for all the dimer inhibition by acridines.

Quenching of DNA triplets by acridines has been observed only at 77° K (Isenberg et al., 1964; Galley and Davidson, 1966); however, it is conceivable that these processes could contribute to the inhibition of dimer formation at room temperature if a triplet were a precursor of the dimer. Since triplets are formed from singlets by intersystem crossing, a reduction in the number of singlets also leads to a decreased number of triplets.

The effect of triplet quenchers such as paramagnetic ions on dimer yield provides evidence that transfer from a DNA triplet is not important in dimer inhibition by acridines. At room temperature, paramagnetic ions (Cu^{++}, Ni^{++}, Cr^{+++}, Co^{+++}, Fe^{++}, Mn^{++}) and O_2, which also is paramagnetic, quench dimer formation in solutions of orotic acid and thymine (Beukers and Berends, 1960; Greenstock et al., 1967), while diamagnetic ions do not (Beukers and Berends, 1960).

At 77° K paramagnetic ions reduce the intensity of DNA phosphorescence but do not affect fluorescent intensity (Isenberg et al., 1967). That is, paramagnetic ions quench only the triplet state of DNA. Each ion can quench the phosphorescence of about 10 base pairs, thus indicating that at liquid nitrogen temperature the DNA triplets are delocalized over about 10 base pairs and could transfer energy over the required distances.

Fig. 4 shows that neither paramagnetic ions (Ni^{++}) nor diamagnetic ions (Mg^{++}) affect dimer yield. These results, together with those on acetone, imply that energy transfer from a singlet of DNA is responsible for dimer inhibition by acridines.

We have shown that energy transfer contributes to the reduction of dimers by proflavine and acridine orange. The effective distance of 12 base pairs for dimer prevention agrees with the effective energy transfer length of 10–20 base pairs found from sensitized fluorescence of acridine orange (Weill and Calvin, 1963). Acetone, which can quench the singlets, but not the triplets, of DNA, also reduced dimer yield. However, at concentrations equal to or greater than those required to quench DNA phosphorescence at 77° K completely (Isenberg et al., 1967), paramagnetic ions did not reduce dimer yield. Greenstock et al. (1967) found that paramagnetic species reduced dimer formation in solutions of TMP but not in thymidyl-(3′,5′)-thymidine (TpT). This result may mean either that the triplet is not the precursor of the dimer in TpT or, as Greenstock et al. suggested, that the dimer may be formed faster than the triplet can be quenched. Similarly, we cannot entirely exclude the possibility that dimer formation in DNA proceeds via triplet states which at room temperature are so short-lived that they cannot be quenched by paramagnetic ions.

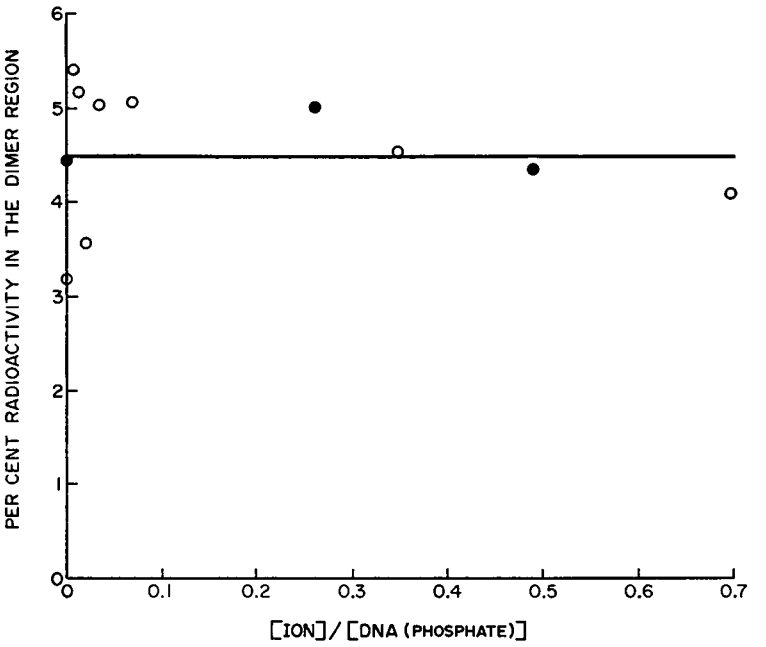

FIGURE 4 Two typical experiments that show the effect of a paramagnetic ion, Ni^{++}, (○), and a diamagnetic ion, Mg^{++}, (●) on dimer formation. Neither ion changed dimer yield.

However, our results suggest that only singlets are energetic precursors of cyclobutyl pyrimidine dimers in DNA as proposed by Eisinger and Lamola (1967) and Eisinger and Shulman (1967). Our results also show that energy transfer from singlets of DNA to acridine singlets inhibits dimer formation.

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