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*PYRIMIDINE DIMERS IN UV-IRRADIATED POLY dI:dC**

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Ultraviolet irradiation of polynucleotides containing thymine results in the formation of dimers between adjacent thymine residues.[1, 2] The demonstration that these dimers are responsible to a large, but not exclusive, extent for the inactivation of primer DNA[3] and of transforming DNA[4] made use of a specific photochemical property of the dimers, namely, that they may be monomerized by short-wavelength irradiation. The fact that many UV effects are photoreactivable and that treat-

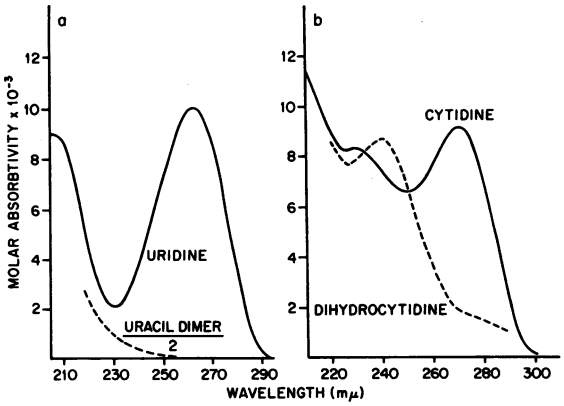

FIG. 1.—Absorption spectra at pH 7 of uridine, cytidine, and typical saturated derivatives. (Data from ref. 18, pp. 61 and 189.)

ment of irradiated DNA with photoreactivating enzyme plus visible light results in the disappearance of the dimers also indicates that many effects of UV arise from dimer formation.[5-7]

Thymine dimers were first identified as a photoproduct formed by the irradiation of frozen solutions of thymine.[8] They are stable to acid hydrolysis. Other acid-stable photoproducts have been detected in DNA, but most have not been identified.[1, 2] Cytosine dimers (detected as the deamination product, uracil dimers) are formed in low yield by irradiating cytosine in frozen solution,[1, 9] and the formation of cytosine dimers in DNA has been inferred from the appearance of uracil dimers in chromatograms of acid hydrolysates of heavily irradiated cytosine-C^{14}-DNA.[10] At very high UV doses some cytosine in DNA is converted to uracil.[10] If a similar conversion holds at low doses, it may explain some of the mutagenic effects of UV.

We have investigated the photochemistry of the organized homopolymer complex polydeoxyinosinic acid:polydeoxycytidylic acid (dI:dC), and for comparison, dA:dT. This report describes methods of detection and identification of cytosine dimers in polynucleotides and describes some of their photochemical properties. We show that cytosine dimers are formed and are deaminated to uracil dimers at measurable rates and that the production of uracil from irradiated cytosine appears to be a two-step process—with uracil resulting from the photochemical splitting of uracil dimers. Both cytosine and uracil dimers are destroyed by visible light in the presence of photoreactivating enzyme. Direct evidence for the enzymic monomerization of the dimer is presented.

Methods.—The polymers we have investigated, dI:dC and dA:dT, are ordered homopolymer complexes that are synthesized by the sequential action of a terminal deoxynucleotidyl transferase and replicative deoxynucleotidyl transferase (DNA polymerase) isolated from calf thymus glands.[11] Some properties of these polymers have been published,[12, 13] and detailed characteristics will be reported separately. Chromatographic analyses were performed on polymers labeled either with H^3C or H^3T. Their activities, as measured in a scintillation counter, were approximately 10,000 counts/min/μg of polymer.

Polymers (10 μg/ml) in 0.05 *M* phosphate buffer, pH 7, were irradiated with

known intensities ($\sim 10^4$ ergs/mm^2/min) of monochromatic UV obtained by use of a large quartz monochromator and a 500-watt high-pressure mercury lamp. Absorbance changes during irradiation were measured in a Beckman model DU spectrophotometer. For chromatographic analysis following irradiation, 0.2-ml samples of radioactive polymers were heated for 5 min at 100°C[14], dried, hydrolyzed with 98% formic acid at 175°C, and chromatographed in butanol-acetic acid-water (80:12:30). The chromatograms were cut into strips, eluted with water, and counted in a dioxane-naphthalene scintillator.

Enzymic photoreactivation was achieved by illuminating samples containing photoreactivating enzyme from yeast[15] (purified 500-fold) with light from two "black light" lamps at 37°C. The incident intensity was 10^4 ergs/mm^2/min in the wavelength interval from 330 to 410 mμ.

Results and Discussion.—Absorbance changes in dI:dC: The dimerization of adjacent thymine residues in a polynucleotide has been shown to be a photochemically reversible reaction: T,T $\rightleftharpoons$ $\widehat{\text{TT}}$, driven to the right by long- and to the left by short-wavelength irradiation.[16, 17] This reaction is readily followed by absorbance changes resulting from saturation of the 5,6 double bond upon formation of dimers of thymine or uracil (Fig. 1*a*). However, saturation of this bond in cytidine results in a decrease only at long wavelengths and an increase at 240 mμ (Fig. 1*b*).[18] Such absorbance changes are found as a result of photohydration of cytidine and have been predicted to occur as a result of dimerization.[18] If cytosine dimers are formed, we expect not only to find an absorbance increase at 240 mμ in irradiated dI:dC, but, because the dimer has a high absorption coefficient, that (1) the absorbance decrease at 270 mμ observed after 280 mμ irradiation will be appreciably smaller than for thymine polynucleotides, and (2) the rate of increase of absorbance produced by short-wavelength irradiation (following long-wavelength irradiation) will be much larger than that observed for thymine or uracil dimers. Figure 2 shows that these phenomena are observed for irradiated dI:dC. These absorbance changes arise only from alterations in cytosine because irradiation of dI separately produces no change (<0.2%).

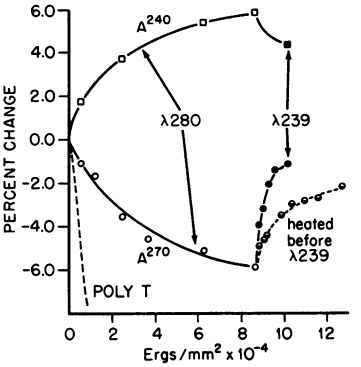

FIG. 2.—Absorbance changes in dI:dC produced by irradiation at 280 mμ followed by 239 mμ. Heating after 280 mμ, but before 239 mμ, was at 60°C for 60 min. The absorbance change in poly T (ref. 17) is shown for comparison.

One may rule out photohydration of cytidine as contributing to the absorbance changes shown in Figure 2 by the following argument. The hydrate is unstable and reverts to cytidine at an appreciable rate at room temperature.[18] Such a temperature-dependent absorbance change is *not* observed in the organized polymer dI:dC at the doses we have used. Moreover, the wavelength-reversible changes in Figure 2 are not found for the hydrate.[19] Poly rI:rC irradiated with 254 mμ shows absorbance changes similar to those expected for dimer formation,[20] and irradiation of poly rC produces absorbance changes that consist of both heat- and wavelength-reversible parts,[19] parts characteristic of hydrate and dimer formation. These results indicate that the principal action of UV radiation on ordered homopolymer

TABLE 1

MEAN (1/e) DOSES FOR MONOMERIZATION OF DIMERS BY 239 mμ RADIATION

Dimer		1/e Dose (ergs/mm² × 10^{-4})	Type of measurement
Thymine:	In poly T	2.0	Absorbance[17]
Uracil:	Isolated from ice	3.1	Absorbance[24]
	Isolated from dI:dC	3.6	Chromatographic
	In poly U	2.5	Absorbance[24]
	In dI:dC	1.9	Absorbance
	In dI:dC	2.2	Chromatographic
Cytosine:	In dI:dC	0.31	Absorbance
	In dI:dC	0.30	Chromatographic

complexes containing cytosine is the formation of dimers, and that hydration does not take place in an ordered polymer but does take place in disordered or denatured polymers.[21]

Further evidence for the formation of cytosine dimers in dI:dC comes from the effects of heating on the rate of absorbance change produced by 239 mμ. Saturated cytosines may be easily deaminated to yield saturated uracils.[22, 23] Therefore, if irradiated dI:dC is heated before 239 mμ irradiation, the cytosine dimers should be converted to uracil dimers. As a result, the rate of absorbance increase should be characteristic of uracil dimers in polynucleotides,[24] i.e., much lower than that of the unheated polymer, because of the lower absorbance of uracil dimers (Fig. 2 and Table 1).

The principal changes observed in dI:dC may be summarized in the scheme

$$C,C \overset{h\nu}{\rightleftharpoons} \widehat{CC} \overset{\text{heat}}{\longrightarrow} \widehat{UU} \overset{h\nu}{\rightleftharpoons} U,U.$$

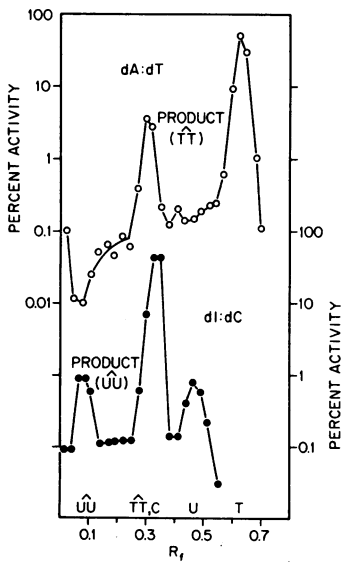

FIG. 3.—Chromatograms of acid hydrolysates of dA:dT(H³) and dI:dC(H³) irradiated with 5 × 10^3 ergs/mm² at 280 mμ. The mobilities of known compounds are indicated on the abscissa.

A quantitative analysis of the absorbance changes in terms of numbers of cytosine dimers in dI:dC is difficult because of changes in hypochromicity that result from UV irradiation of this low-melting-point polymer (T_m = 47°C in 0.01 *M* phosphate buffer, pH 7.0). Nevertheless, the following approximate calculation of the number of cytosines dimerized is useful. The absorbance decrease at 270 mμ following 4 × 10^4 ergs/mm² of 280 mμ is about 4.5 per cent. Since the absorbance of the dimer is probably not zero (Fig. 1*b*), and cytosine represents only 60–70 per cent of the UV absorbance of the polymer at 270 mμ, the 4.5 per cent decrease should represent dimerization of about 8 per cent of the cytosines. However, because the formation of a dimer reduces the hypochromic effect, the amount of cytosine in dimers is probably closer to 10 % a number similar to that found by the chromatographic analysis (below).

Chromatographic analysis: Chromatograms of acid hydrolysates of irradiated dA:dT(H³) and

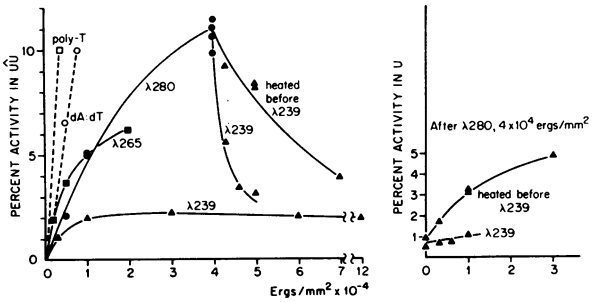

FIG. 4.—The percentage of total radioactivity in uracil dimer and uracil, as functions of wavelength and dose, that appear in acid hydrolysates of irradiated dI:dC(H^3). The amounts of thymine dimers in dA:dT(H^3) and in poly T (ref. 17) are shown for 280 mμ (○) and 265 mμ (□). Heated samples were at 60°C for 60 min.

dI:dC(H^3) are shown in Figure 3. Note that, as for the absorbance changes, the amount of photoproducts in dI:dC is less than in dA:dT. The product from irradiated dI:dC that we call uracil dimer arises from deamination of cytosine dimers. It has been identified by the following two criteria. (1) In three different solvents,[25] it has chromatographic mobilities similar to the uracil dimer prepared by irradiating uracil in ice. (2) The isolated photoproduct is monomerized, when irradiated in solution, to yield only uracil with a 1/e dose that is the same as that for the compound made in ice[26] (Table 1). Most of the uracil (usually ~0.7%) that appears in the chromatogram in Figure 3 arises from the hydrolysis of dI:dC and not from the action of radiation, because it also is found in unirradiated samples. An estimate from a number of experiments is that a dose of 10^4 ergs/mm^2 at 280 mμ results in the conversion of 0.1 per cent of the cytosine to uracil.

The amount of uracil dimer observed in acid hydrolysates of irradiated dI:dC depends on wavelength and dose as shown in Figure 4. Some data for thymine-containing polynucleotides are shown for comparison. As with the absorbance changes shown in Figure 2, the amount and rate of monomerization produced by 239 mμ depends on whether the polymer was heated after 280 mμ irradiation. The 1/e doses for 239 mμ monomerization are given in Table 1. Irradiation by 239 mμ after the irradiated polymer has been heated results in an increase in the amount of uracil approximately equal to the amount of dimer that disappears. With no heating there is only a small increase in uracil—an increase that may result from a slight amount of deamination during or as a result of short-wavelength irradiation. These data provide direct evidence for the photochemical and heat reactions given above.

We may use the values for the amount of monomerization by 239 mμ to estimate the extent of deamination of cytosine dimers by interpolating between values found for no heating (rapid monomerization) and those found after 100°C (slow monomerization). Data such as those in Table 2 indicate that deamination is complete in 60 min at 60°C but that in 2 hr at 37°C only about half the dimers are deaminated. (We cannot distinguish between deamination of both cytosines in one dimer or two cytosines on two dimers.) Likewise, Green and Cohen[23] found that the half-time for deamination of dihydroderivatives of cytosine was 2–3 hr at 37°C.

Enzymic hydrolysis of irradiated dI:dC: Thymine dimers interfere with the enzymic hydrolysis of polynucleotides, and the limit digest of venom phosphodi-

TABLE 2

MONOMERIZATION OF DIMERS* IN IRRADIATED dI:dC BY 239 mμ AFTER VARIOUS TREATMENTS

Treatment†	Activity in $\widehat{UU}$ (%)	Activity in U (%)	Change in U per change in $\widehat{UU}$
No irradiation at 239 mμ	11.0	0.6	—
10 min, 100°C	9.8	0.9	−0.25
1 × 10^4 ergs/mm², 239 mμ following			
No heat	3.2	1.1	−0.06
60 min, 37°C	3.9	1.6	−0.15
180 min, 37°C	5.5	2.3	−0.3
60 min, 60°C	8.3	3.2	−1.0
10 min, 100°C	8.2	3.1	−0.9

* Formed by 280 mμ, 4 × 10^4 ergs/mm².
† All samples heated 60 min, 60°C before acid hydrolysis.

esterase treatment has been shown to contain trinucleotides containing a thymine dimer.[27] Irradiated dI:dC(H^3) (4 × 10^4 ergs/mm² 280 mμ) was hydrolyzed with venom phosphodiesterase and chromatographed on DEAE paper.[27] Eight and eight-tenths per cent of the radioactivity was found in the region corresponding to trinucleotides. If the trinucleotides had the form pCpC$\widehat{pC}$, one would expect that acid hydrolysis of them would give 0.67 × 8.8% = 5.9% of the radioactivity in dimers. We found 5.5 per cent of the activity in dimers. This value is close to the value of 5 per cent obtained by acid hydrolysis alone and indicates that the procedures we have used for isolating the dimers do not result in large losses.

Enzymic photoreactivation: It is known that enzymic photoreactivation results in the disappearance of thymine dimers from DNA,[5-7] but it has not been proved that the reaction is a monomerization of the dimer. With irradiated dI:dC one can measure photoreactivation of cytosine and uracil dimers. In the latter case, as for 239 mμ irradiation, monomerization of the dimer would result in the appearance of uracil, a compound easy to detect against the background of cytosine labeling in the polymer. Table 3 shows that the photoreactivation of irradiated dI:dC results in the disappearance of dimers in the acid hydrolysate and that if the polymer is heated before photoreactivation, not only do the dimers disappear but there is an increase in uracil after photoreactivation. This is direct evidence that uracil dimers are monomerized by photoreactivating treatment and, by inference, that cytosine dimers are also monomerized. It is not appropriate to compare the absolute rates of photoreactivation of cytosine and uracil dimers from the data in Table 3 because the three-dimensional structure of the polymer dI:dC may be altered by the heat treatment necessary to deaminate cytosine dimers.[28] The finding that cytosine and uracil dimers, as well as thymine dimers, are substrates for the photoreactivating enzyme is an explanation for Rupert's observation[5] that irradiated dG:dC contains a substrate for the enzyme.

Dimers with photochemical properties similar to those found in irradiated dI:dC have been observed in irradiated DNA.[29] However, they are not as numerous as thymine dimers. The effects of such dimers on the competitive ability of DNA for the photoreactivating enzyme are consistent with the above reactions and form the content of another paper.[30] The photoreactivable lesions of cytosine in DNA, whose existence was hypothesized on indirect grounds,[7, 31, 32] certainly include cytosine dimers. It is obvious that photoreactivating treatment after deamination

TABLE 3

MONOMERIZATION OF DIMERS* IN IRRADIATED dI:dC BY PHOTOREACTIVATING ENZYME PLUS 330–410 mμ RADIATION

Protein concentration (mg/ml)	Time (min)	Activity in $\widehat{UU}$ (%) (Not heated)	Activity in U (%)	Change in U per change in $\widehat{UU}$
2	0	10.7	0.6	—
2	30	7.8	2.1	−0.5
1	60	7.1	1.0	−0.1
2	60	4.5	1.8	−0.2
	(Heated 60 min at 60°C before photoreactivation)			
2	0	10.7	0.8	—
2	30	6.4	3.4	−0.7
1	60	3.8	6.0	−0.8
2	60	1.7	5.5	−0.6

* Formed by 280 mμ, 4 × 10^4 ergs/mm².

of cytosine dimers could result in the production of mutants in DNA with biological activity.[6]

Summary.—The effects of UV radiation on the ordered polynucleotide dI:dC have been investigated by absorption spectroscopy and by chromatographic analysis of hydrolysates of the irradiated polymers. Dimers between adjacent cytosine residues have been identified as stable photoproducts. They deaminate slowly (50% in 2 hr at 37°C) to uracil dimers. Cytosine dimers are formed at lower rates than thymine dimers between adjacent thymine residues, but they are monomerized at more rapid rates by short-wavelength radiation than thymine or uracil dimers. The photoreactivating enzyme from yeast in the presence of visible light can monomerize cytosine and uracil dimers as well as thymine dimers. The production of uracil from cytosine by UV irradiation at 280 mμ takes place at about one fiftieth the rate of production of cytosine dimers.

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[15] We are grateful to Amir Muhammed for furnishing us the purified enzyme, and to Jane K. Setlow for assistance with this phase of the experiments.

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*ANILIDES AND PHENYLUREAS: CORRELATION BETWEEN CALCULATED PI-ELECTRON STRUCTURE AND INHIBITION OF PHOTOSYNTHESIS**

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In the 14 years since 3-(4-chlorophenyl)-1,1-dimethylurea (CMU) was first synthesized and discovered to be a potent herbicide,[1] substituted chlorophenylureas such as CMU and 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) have come into general use as specific poisons of an early step in photosynthetic energy conversion. The evidence suggests that these poisons, and others in the phenylurea and anilide families, block an electron transfer step near the oxygen-evolving photoreaction (system II).[2, 3] However, the nature of the interaction between these poisons and the photosynthetic system is unclear. It is thus important to determine which properties of these molecules are related to their biological activity.

Good,[4] in 1961, investigated the poisoning potency of a series of about 100 known and newly synthesized anilides and phenylureas, but no clear-cut correlations between the structure of the molecules and their potency as inhibitors could be made. Good suggested, in agreement with Wessels and van der Veen,[3] that the poisoning process involved a physical obstruction by the poison of some site on its biological substrate. However, Good reported that 2-chloroanilides were ineffective as poisons, and we now present evidence that the pi-electron structure of the poison at the 2 position of its phenyl ring is involved in the poisoning process. From our calculations it appears that chemical reactivity at this site is important, and