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Photochemical Reactions of Cytosine Nucleosides in Frozen Aqueous Solution and in Deoxyribonucleic Acid*

A. J. Varghese

ABSTRACT: Irradiation of cytidine in frozen aqueous solution with 254-nm ultraviolet radiation produces two types of dimeric product, Cd_1 and Cd_2. Cd_1 has been identified as a cyclobutane-type dimer. On heating in acidic, neutral, or alkaline aqueous solutions, Cd_1 is converted mainly to cytidine, cyclobutane-type uridine dimer, and uridine, respectively. Mild acid hydrolysis of the uridine dimer obtained from Cd_1 gave cyclobutane-type uracil dimer, the infrared and ultraviolet absorption spectra of which are identical with those of the cis-syn isomer. An aqueous solution of Cd_2 showed an ultraviolet absorption maximum at 314 nm. Acid hydrolysis of Cd_2 yielded 6-(4′-pyrimidin-2′-one)uracil (PO-U) which has absorbance maxima at 305 nm (ϵ 10,800) in acidic and neutral solutions and at 325 nm (ϵ 12,000) in alkaline solutions. Irradiation of deoxycytidine in frozen aqueous solution produced two products having properties similar to those of Cd_1 and Cd_2. PO-U was also found to be present in acid hydrolysates of irradiated DNA. The proposed mechanism of formation of PO-U involves a cytosine adduct probably derived from an azetidine intermediate.

Irradiation of a mixture of thymidine and cytidine, or thymidine and deoxycytidine produced a number of products from which, after acid hydrolysis, thymine dimer, thymine–uracil dimer, uracil dimer, 6-(4′-pyrimidin-2′-one)-thymine, and 6-(4′-pyrimidin-2′-one)uracil were identified. All these products are also present in acid hydrolysates of irradiated DNA.

A major fraction of the lethal and mutagenic effects of ultraviolet light on biological systems has been attributed to photochemical transformations of pyrimidine bases in the nucleic acids (McLaren and Shugar, 1964). While considerable progress has been made in recent years in understanding the photochemical reactions of thymine and uracil derivatives, much less is known about cytosine and its derivatives (Burr, 1968; Fahr, 1968). It has been shown that irradiation of cytidine and cytidylic acid in aqueous solution with ultraviolet light (λ 254 nm) adds water across the C_5-C_6 double bond to form the 5,6-dihydro-6-hydroxy derivatives (photohydrates) (Johns *et al.*, 1965; Miller and Cerutti, 1968). However, attempts to demonstrate the formation of photohydrates in native DNA have not been successful (Setlow and Carrier, 1963). On the other hand, there is substantial evidence to indicate that cyclobutane-type dimerization of cytosine residues occurs in irradiated bacterial DNA and the number of cytosine-containing dimers is comparable to the number of thymine-containing dimers at low doses of ultraviolet

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light (Setlow and Carrier, 1966). In a recent paper we have shown that irradiation of cytosine nucleosides in frozen aqueous solution produces cyclobutane-type dimeric products (Varghese and Rupert, 1971).

From acid hydrolysates of irradiated DNA, cyclobutane-type *cis-syn*-thymine dimer (I) (see Chart I), thymine–uracil dimer (II), 6-(4′-pyrimidin-2′-one)thymine (III), and 5-thyminyl-5,6-dihydrothymine (IV) have been isolated. Since all these products have also been isolated from frozen-solution irradiation of thymine or thymidine (Varghese, 1970), we studied the photochemical reactions of cytosine nucleosides in frozen aqueous solution. Such an investigation should contribute to an understanding of the photochemical alterations of cytosine residues in DNA.

Experimental Section

Materials

Cytidine, deoxycytidine, uridine, uracil, and calf thymus DNA (Sigma, Grade 1) were purchased from Sigma Chemical Corp. Dowex 50W X-12 (H^+, 100–200 mesh), Dowex 1X-8 (Cl^-, 200–400 mesh), and Bio-Gel P-2 (100–200 mesh) were obtained from Bio-Rad Laboratories. All solvents were of reagent grade. [2-^{14}C]Cytidine was obtained from Schwarz BioResearch, Inc.

Methods

Irradiation. The method of irradiation with ultraviolet light (254 nm) in frozen aqueous solution has been previously described (Varghese, 1970). For labeled samples, the solution contained 10 μCi/200 ml of [2-^{14}C]cytidine (33.8 mCi/mmole). In the case of mixtures, the solutions were 1 mM with respect to the individual bases of nucleosides. For DNA irradiation conditions were the same as those used for the isolation of cyclobutane-type *cis-syn*-thymine dimer from DNA (Varghese and Wang, 1967a).

Paper Chromatography. The samples were concentrated and streaked on Whatman No. 3MM paper (46 × 57 cm; about 50 mg/sheet) and chromatographed by the descending technique. The following solvent systems were used: (A) *sec*-butyl alcohol saturated with water and (B) *n*-butyl alcohol–acetic acid–water (80:12:30, v/v).

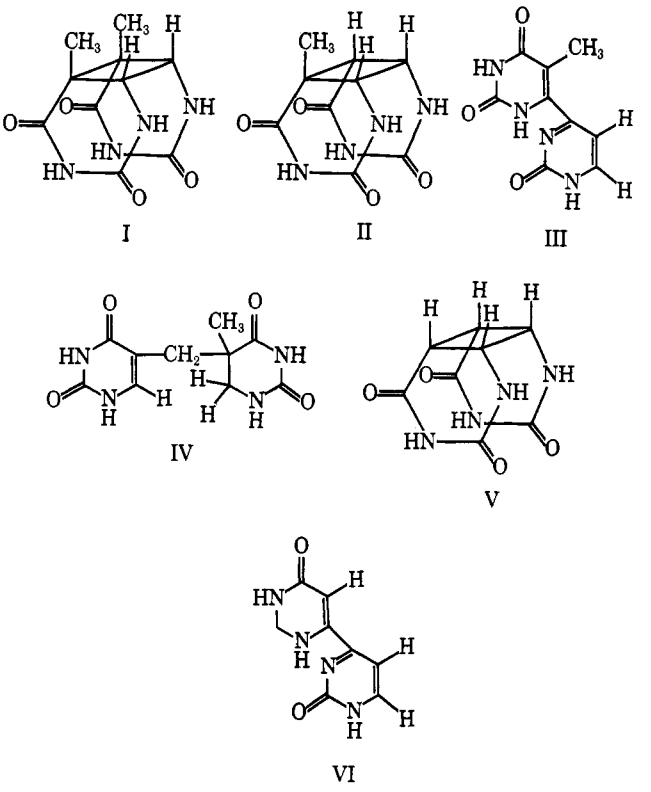
Detection and Isolation of Products. From a chromatogram, strips (1 cm wide) were cut out and eluted with water. The ultraviolet absorption spectrum of each fraction was taken. A spectrum different from that of the starting material indicated the presence of a product. Aliquots of 3 ml from each of these fractions were irradiated for approximately 1 min at a distance of 4 cm from two germicidal lamps and the ultraviolet absorption spectrum was again taken. (If cyclobutane-type dimers were present, the absorbance after irradiation will increase at 270 nm for cytosine and at 260 nm for uracil.) For isolation, strips containing the desired product were cut out and extracted thoroughly with water. The extracts were concentrated and the products purified by rechromatography in the same solvent or by column chromatography.

Acid Hydrolysis. Trifluoroacetic acid hydrolysis was carried out in sealed tubes at 165° for 90 min. The hydrolysate was chromatographed on papers as described above. For mild acid hydrolysis, the samples were heated in a boiling water bath for 30 min in 4 N hydrochloric acid. The hydrolysate was evaporated to dryness, redissolved in water, and chromatographed on paper.

Column Chromatography. For the purification of cyclobutane-type dimeric products of cytosine nucleosides, an aqueous solution containing about 10–15 mg of the sample was applied to a 4 × 90 cm Bio-Gel P-2 column. The column was eluted with water and 20-ml fractions were collected. PO-U was purified by using a Dowex 50W X-12 (H^+, 100–200 mesh) column. The sample was applied in aqueous solution to a 2 × 30 cm column. The column was eluted with water and 10-ml fractions were collected. Cyclobutane-type dimeric uridine was purified by a Dowex 1X-8 (formate, 200–400 mesh) column. About 50 mg of the sample was dissolved in 5 ml of 0.1 N ammonium hydroxide and applied to a 2 × 30 cm column. Elution was performed with a linear ammonium formate gradient (Weinblum and Johns, 1965). The mixing chamber contained 1.25 ml of formic acid, 5 ml of ammonium hydroxide, and 1 l. of water, while the reservoir contained 2 ml of formic acid, 7.5 ml of ammonium hydroxide, and 1 l. of water. Fractions of 10 ml each were collected and the ultraviolet absorbance spectrum of each fraction was taken. Cyclobutane-type dimeric products were detected by irradiating aliquots of each fraction at 254 nm and observing the increase in absorbance, as mentioned above. Fractions containing the desired product were combined and evaporated to dryness.

Cyclobutane-Type cis-syn-Uracil Dimer (V) and 6-(4′-Pyrimidin-2′-one)Uracil (PO-U;[1] VI) from Uracil. Uracil solution irradiated in the frozen state was evaporated to dryness on a rotary evaporator at 40–45°. The residue (from 1 g of uracil) was extracted with water until the extract showed no ultraviolet absorption maximum above 250 nm. The residue so obtained upon cyrstallization from hot water amounted to 420 mg. A portion (25 mg) of the residue was dissolved in 20 ml of 0.1 N NH_4OH and subjected to Dowex 1X-8 (formate) column chromatography.Two uracil dimers were detected. The fractions constituting the individual peaks were pooled and con-

CHART I



[1] Abbreviation used is: PO-U, 6-(4′-pyrimidin-2′-one)uracil.

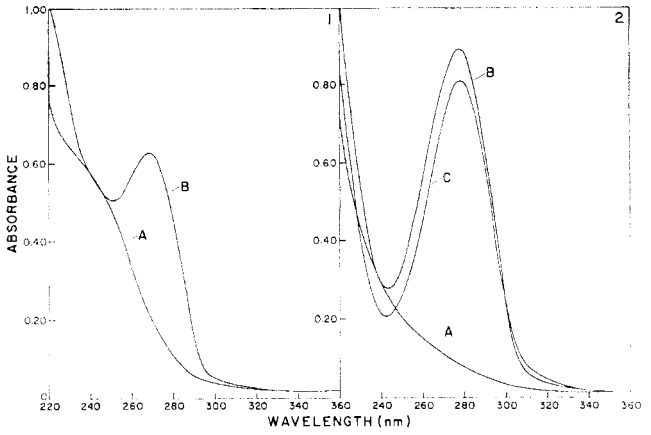
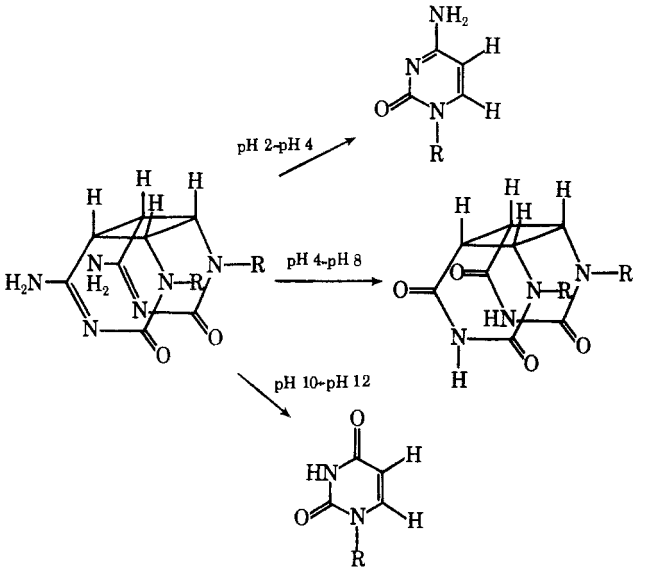


FIGURE 1: Ultraviolet absorption spectra of Cd_1 (A) in aqueous solution; (B) after exposure to 254-nm radiation.

FIGURE 2: Ultraviolet absorption spectra of Cd_1 (A) in aqueous solution at pH 2; (B) after exposure to 254-nm radiation; (C) after heating for 5 min at 100°.

SCHEME I



centrated to crystallization. The cyrstalline products were recrystallized from hot water. The major product (16 mg) appeared in fractions 28–40 and the minor one (1.5 mg) appeared in fractions 45–55. On the basis of acid stability, photoreversal to uracil and infrared absorption spectra, the major dimer has been identified as the *cis-syn*-uracil dimer (Blackburn and Davies, 1967; Adman *et al.*, 1968; Khattak and Wang, 1968) and the minor one as the cis-anti isomer (Konnert et al., 1970).

The combined aqueous extract from 1 g of irradiated uracil was evaporated to dryness. The residue was dissolved in trifluoroacetic acid and chromatographed on paper using solvent system B. Uracil dimer, 6-(4′-(pyrimidin-2′-one)uracil, and uracil have R_F values 0.10, 0.20, and 0.50, respectively, in this solvent system (Smith, 1963). From the chromatograms, strips (R_F 0.15–0.25) were cut out and extracted with water. The extract was concentrated and chromatographed on a Dowex 50W X-12 (H^+) column as described above. Those fractions having an absorbance maximum at 305 nm were combined and evaporated to dryness. The residue, on crystallization from hot water, deposited yellow needle-like crystals of PO-U (Khattak and Wang, 1968) and amounted to 27 mg.

Infrared and Ultraviolet Absorption Spectra. Infrared absorption spectra were recorded on a Perkin-Elmer 337 grating spectrophotometer in potassium bromide pellets. Ultraviolet absorption spectra were recorded using a Hitachi Perkin-Elmer Coleman 124 double-beam spectrophotometer equipped with a Sargent SRG recorder.

Results and Discussion

Analysis of paper chromatograms, developed in solvent system A, of cytidine irradiated in frozen aqueous solution, revealed the presence of two products. In the order of chromatographic mobilities, they are referred to as Cd_1 and Cd_2. These products were purified by rechromatography in the same solvent. When viewed under an ultraviolet lamp (λ 254 nm), Cd_1 appeared as a narrow dark band (R_F 0.09), while cytidine (R_F 0.32) appeared as a broad dark band. When eluted from the chromatograms, Cd_1 was found to be contaminated with cytidine and Cd_2 (R_F 0.14). Final purification of Cd_1 was accomplished by chromatography on the Bio-Gel P-2 column, as described in Methods. On elution from the column, Cd_2, Cd_1, and cytidine appeared in that order. In a typical experiment about 40 mg of Cd_1 was obtained from 3 g of cytidine.

The ultraviolet absorption spectrum of Cd_1 in neutral and alkaline solutions shows a poorly resolved peak at 244 nm, which disappears in acidic solution (Figures 1 and 2). Similar spectral characteristics have been previously reported for C_5–C_6-saturated cytosine derivatives (Sinsheimer, 1957; Wierzchowski and Shugar, 1962). From a series of absorption spectra taken at pH values from 2 to 8 the pK of Cd_1 was determined by plotting A_{240} *vs.* pH as 5.4. Though this value is about 1.12 units higher than reported for cytidine (Cavalieri, 1952), it is lower than that reported by Ono *et al.* (1965) for dihydrocytidine (6.1) and by Johns *et al.* (1965) for cytidylic acid hydrate (5.56). Irradiation at 254 nm of an aqueous solution of Cd_1 resulted in an increase in absorbance at 271 nm. Paper chromatogram (solvent A) of the irradiated solution revealed the presence of only one product. On the basis of chromatographic mobilities and ultraviolet absorption spectra in acidic and basic solutions, this product has been identified as cytidine. Thus, the ultraviolet absorption spectrum and photoreversibility indicate that Cd_1 is probably a cyclobutane-type dimer of cytidine.

In aqueous solution, Cd_1 is converted into different products, depending on the pH of the solution (Scheme I). The conversion proceeds slowly at room temperature and can be hastened by heating. As can be seen from Figure 3, in acidic solution cytidine is the major product, while in alkaline solution uridine is the major detectable product. In aqueous solution (pH 5–8), the main product is cyclobutane-type uridine dimer. Easy deamination of C_5–C_6-saturated cytosines to form the corresponding uracils has been reported by other investigators (Green and Cohen, 1957; Daniels and Grimison, 1964). We find that deamination of cyclobutane-type dimeric cytidine is strongly pH dependent, and at pH 2–3 very little deamination takes place, Cd_1 being converted into cytidine. Cyclobutane-type thymine dimers do not exhibit such monomerization in acidic solution (Herbert *et al.*, 1969).

A cyclobutane-type dimer of cytidine could have one of four possible stereoisomeric structures. From both thymidine and uridine irradiated in frozen aqueous solution, three isomeric dimers have been isolated (Fahr, 1968). Our attempts to

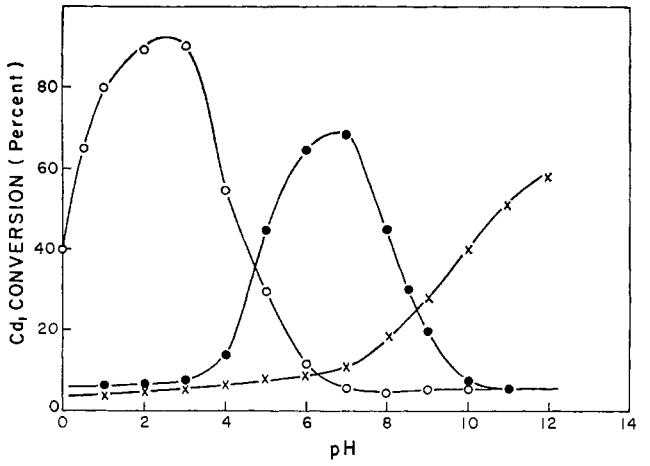


FIGURE 3: Conversion of Cd_1 to different products as a function of pH. (○) Cytidine, (●) uridine dimer, (×) uridine. Aqueous solutions of Cd_1 were heated for 30 min at 100°. The solutions were chromatographed on paper using solvent system A in which uridine dimer, cytidine, and uridine have R_F values 0.16, 0.33, and 0.42, respectively. The amounts of the individual products were determined on the basis of detectable radioactivity.

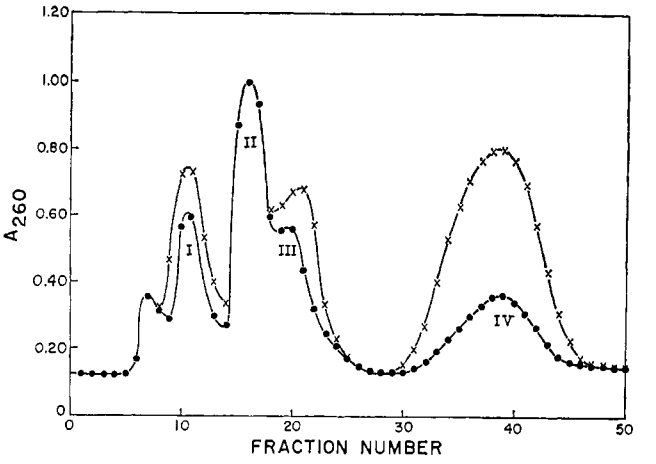


FIGURE 4: Elution profile of Cd_1, after heating at 100° for 2 hr, from a Dowex 1X-8 (formate) column. Elution was performed with a linear ammonium formate gradient, as described in Methods. (●) Absorbance at 260 nm of the eluent; (×) absorbance after exposure to 254-nm radiation. The individual peaks are: I cytidine dimer; II cytidine; III cytidine–uridine dimer; and IV uridine dimer.

establish whether Cd_1 is a mixture of isomeric dimers were unsuccessful.

Cyclobutane-Type cis-syn-Uracil Dimer from Cd_1. As shown above, Cd_1 on heating in aqueous solution is converted into different products. These products were separated by Dowex 1X-8 column chromatography with a linear formate gradient. The elution pattern is shown in Figure 4. Fractions 9–14 contained cytidine dimer, 15–18 contained mainly cytidine, 18–24 contained cytidine–uridine dimer, probably derived from deamination of one cytosine residue, and fractions 30–45 contained uridine dimer. Prolonged heating of Cd_1 resulted in lesser amounts of cytidine dimer and cytidine–uridine dimer and a higher amount of uridine dimer. Beyond fractions 70 there was a small peak which, on the basis of photoreversibility, appeared to be a cyclobutane-type uridine dimer. However, the amount was extremely small and further studies were not carried out. A typical experiment in which a solution of Cd_1 (50 mg/10 ml) was heated for 2 hr in a boiling-water bath, yielded cytidine dimer (5 mg), cytidine (11 mg), cytidine–uridine dimer (4 mg), and uridine dimer (16 mg).

The uridine–dimer containing fractions 30–45 were combined, evaporated, desalted, and subjected to mild acid hydrolysis. The hydrolysate was evaporated to dryness and was washed several times with warm methanol. The residue (3.5 mg) was dissolved in hot water and filtered. The filtrate was concentrated to crystallization and left overnight. The ultraviolet and infrared (Figure 6A) absorption spectra of the crystalline product were found to be identical with those of cyclobutane-type uracil cis-syn dimer (V) isolated from uracil. These results suggest that Cd_1 is mainly *cis-syn*-cytidine dimer.

Characterization of Cd_2. When eluted from the chromatograms, Cd_1 was found to be the major impurity associated with Cd_2. By rechromatography in the same solvent system, followed by Bio-Gel P-2 column chromatography, pure Cd_2 was obtained. Cd_2 in aqueous solution exhibited an ultraviolet absorbance maximum at 314 nm. Attempts to crystallize Cd_2 were unsuccessful.

Cd_2 was hydrolyzed with trifluoroacetic acid and chromatographed on paper using solvent system B. From the chromatograms, strips (R_F 0.12–0.22) were cut out and extracted with water. The extract was evaporated and subjected to column chromatography in the same manner as that described for the isolation of PO-U from irradiated uracil. Fractions 20–35 having an ultraviolet absorbance maximum at 305 nm were combined and evaporated to dryness. The residue was crystallized from water. The ultraviolet (Figure 5) and infrared (Figure 6B) absorption spectra of the crystalline product were found to be identical with those of PO-U (VI) isolated from uracil.

Isolation of VI from Irradiated DNA. From the chromatograms, developed in solvent system B, of acid hydrolysates, of irradiated DNA, strips (R_F 0.05–0.22) were cut out and extracted with water. The combined extract from 3 g of DNA was concentrated and chromatographed on a Dowex 50W X-

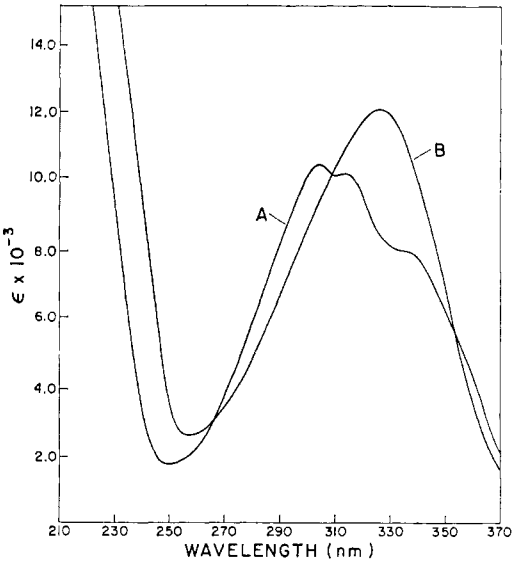


FIGURE 5: Ultraviolet absorption spectra of PO-U isolated from uracil. (A) In water and in 0.1 N HCl; (B) in 0.1 N NaOH. Identical spectra were obtained for PO-U isolated from cytidine and from DNA.

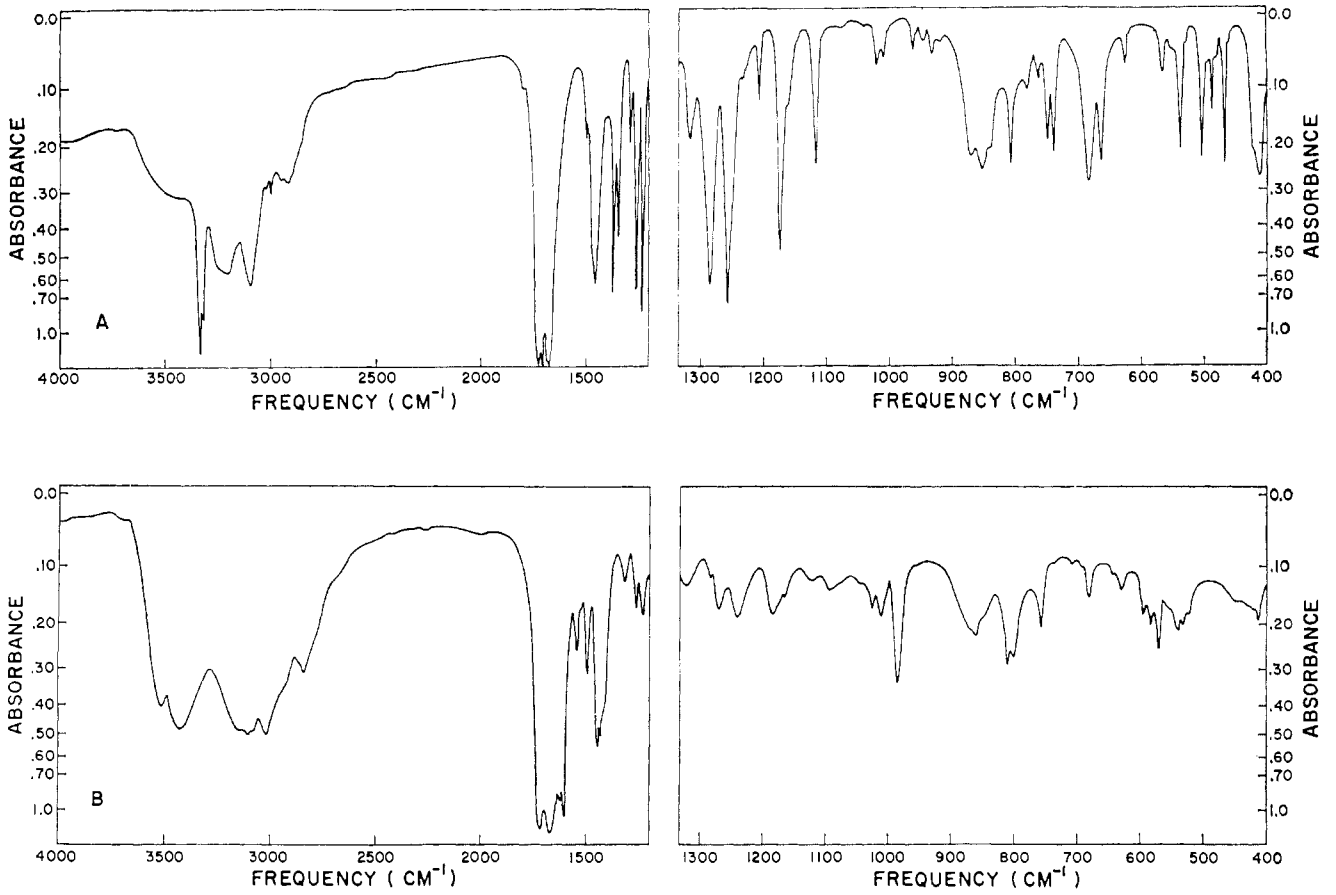


FIGURE 6: Infrared absorption spectra of (A) *cis-syn*-uracil dimer; (B) PO-U isolated from uracil. Identical spectra were obtained for PO-U and uracil dimer isolated from cytidine.

12 column, as described in Methods. Cyclobutane-type uracil dimer and uracil–thymine dimer, which have R_F's of 0.10 and 0.18, respectively, in solvent B (Smith, 1963), appeared in fractions 3–8. Fractions 20–32, contained a product with ultraviolet absorbance maximum at 305 nm, were combined and evaporated to dryness. The residue was crystallized from water. The ultraviolet (Figure 5) and infrared (Figure 6B) absorbance spectra of the product were found to be identical with those of PO-U isolated from uracil.

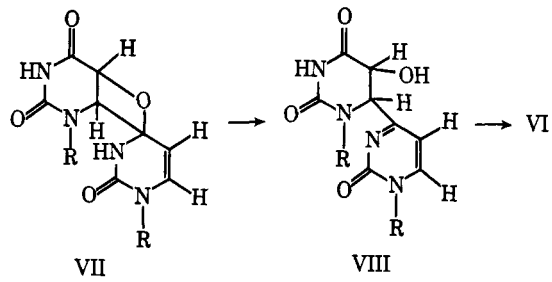
Fractions 3–7, containing the cyclobutane-type dimers, were combined and evaporated to dryness. The residue was dissolved in 0.1 N ammonium hydroxide and chromatographed on a Dowex 1X-8 column, as described in Methods. Two peaks were obtained. On the basis of photoreversibility, the first peak was found to be due to uracil–thymine dimer and the second one to uracil dimer. Similar elution patterns were also observed by Weinblum (1967) for cyclobutane-type thymine–uracil dimer and uracil–uracil dimer isolated from DNA. Since the amounts were small, further studies to establish the isomeric configurations of these dimers were not made.

Irradiation of Thymidine–Cytidine Mixture. In a previous paper (Varghese, 1970), we showed that thymine dimer, 5-thyminyl-5,6-dihydrothymine (IV), and 5-hydroxy-6-(4′-pyrimidin-2′-one)dihydrothymine (thymine analog of VII) can be isolated from thymidine irradiated in frozen aqueous solution with ultraviolet light. Irradiation of a mixture of thymidine and cytidine or thymidine and deoxycytidine produce not only the products derived from the individual bases but also products formed from both bases. Thus, following the same procedures as used for the isolation of DNA photoproducts thymine dimer (Varghese and Wang, 1967a) thymine–uracil dimer, uracil dimer (Weinblum, 1967), and 6-(4′-pyrimidin-2′-one)thymine (Varghese and Patrick, 1969) were found to be present in the acid hydrolysates of the irradiated mixture.

Mechanism of Formation. Good discussions concerning the mechanism of formation of cyclobutane-type dimers of thymine and uracil derivatives have appeared in the literature (Eisinger and Shulman, 1968; Lamola and Eisinger, 1968). For thymine it has been demonstrated that photodimerization can proceed by way of a triplet-state precursor in dilute aqueous solution, while a singlet is directly involved in dimerization in frozen aqueous solution. Since cyclobutane-type dimer formation of cytidine can be photosensitized by acetone in a manner analogous to that of thymidine (A. J. Varghese, manuscript in preparation), it is reasonable to assume that photodimerization of cytosine nucleosides and thymidine occurs by the same mechanism.

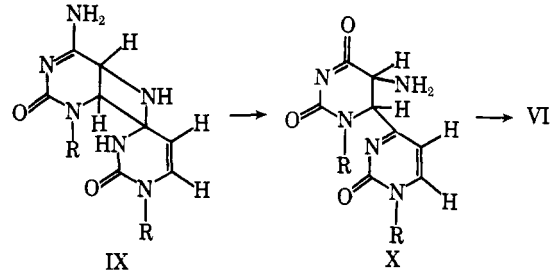
The structure of the dehydration product of a uracil–uracil adduct was established as 6-(4′-(pyrimidin-2′-one)uracil (VI) (Khattak and Wang, 1968). We have isolated the same product from uridine irradiated in frozen aqueous solution with ultraviolet light (A. J. Varghese, manuscript in preparation). The mechanism of formation of uracil–uracil adduct VIII probably involves an oxetane intermediate VII similar to the one proposed for the formation of a thymine–thymine adduct (Varghese and Wang, 1968).

Even though the exact structure of Cd_2 is not known, the formation of PO-U from Cd_2 by acid hydrolysis suggests that Cd_2 may be a cytidine–cytidine adduct X. The formation of



VII VIII

X can be explained by an initially formed azetidine intermediate IX. Such an azetidine intermediate, formed from cytosine and thymine residues in DNA, has been proposed to explain the formation of 6-(4′-pyrimidin-2′-one)thymine in irradiated DNA (Wang and Varghese, 1967). Thus, VI constitutes the second such product isolated from irradiated DNA. The



IX X

possible biological significance of this class of products, having characteristic absorption maxima above 300 nm, is not yet clear. Jagger *et al.* (1970) have shown that an unusual kind of photoreactivation, which these authors refer to as type III photoreactivation, occurs at 313 nm in *Streptomyces griseus* and *Streptomyces coelicolor*, inactivated with ultraviolet light. A noteworthy feature of these organisms is the high guanine–cytosine content of its DNA (74% G + C, Belozersky and Spirin, 1960). It is possible that photoproducts related to PO-U might play a significant role in the photoreactivation of these organisms.

In TMV RNA, Small *et al.* (1968) have shown that irradiation at high ionic strength results in the formation of two dimeric products, referred to as photoproducts I and II, which are not cyclobutane-type dimeric uracils. These investigators have shown that these unidentified products and two cyclobutane-type dimeric products are responsible for the inactivation by ultraviolet light of TMV RNA at high ionic strength. It is possible that photoproducts I and II are derivatives or precursors of PO-U.

The procedure for determining the amount of cyclobutane-type dimers of cytosine in DNA is based on the amount of detectable uracil dimer in acid hydrolysates of irradiated DNA. [The procedure also involves heating the irradiated DNA prior to hydrolysis to convert the cytosine dimers to uracil dimers (Setlow *et al.*, 1965).] Our results show that cytidine dimer is not converted completely into uracil dimer under any condition. Moreover, acid hydrolysis of *cis-syn*-uridine dimer produces a mixture of uracil and *cis-syn*-uracil dimer. Therefore, it is possible that the contribution of cyclobutane-type cytosine dimers in the inactivation of biological systems might be much greater than could be accounted for by the detectable uracil dimer in acid hydrolysates of irradiated DNA.

Acknowledgments

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