Photochemistry of Polycytidylic Acid, Deoxycytidine, and Cytidine*

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ABSTRACT: Irradiation (mainly 254 nm) of poly(rC) in aqueous solution and in pH 7 and 4 buffered solutions results in absorbancy decreases in the 275-nm region with concomitant increases in the 330-nm region. The compound responsible for this increase was isolated from the acid hydrolysates of poly(rC) irradiated in water. Its quantum yield, 0.8×10^{-3}, is relatively high in double-helical poly(rC) at ultraviolet doses comparable to those used in biological studies. Its structure was characterized as a 5-[4′-pyrimidin-2′-one]cytosine (Pyo4-5Cyt) derivative, which is formed by the deamination of a cytidine-cytidine adduct (Cyd4-5Cyd) and that of a deoxycytidine-deoxycytidine adduct (dCyd4-5dCyd). The nucleosides of Pyo4-5Cyt were isolated from the irradiation in frozen state of Cyd·0.5HCl and dCyd·0.5HCl, respectively; their structures were characterized by ultraviolet, infrared, and nuclear magnetic resonance studies. Through the determination of pH and "puddle" effects on Cyd and dCyd in frozen state and the study of the absorbancy–temperature profile of irradiated poly(rC), a preliminary mechanistic understanding was realized. Apparently, it is a termolecular reaction, involving two nucleoside molecules and 1 mole equiv of protons, which is favored in solid state. This type of reaction may serve as a unique example of a photoreaction that occurs with charge-transfer complexes. It is significant not only in photochemistry but also in the photobiology of nucleic acids.

In 1961, full articles on the discovery of thymine dimer (Thy=Thy) were published by Wang (1960, 1961) and by Beukers and Berends (1960, 1961). While the Beukers and Berends paper was concerned mainly with the characterization of Thy=Thy, the Wang article (1961) reported the possible dimer formation of other pyrimidines. Among them, the biologically important uracil dimer (Ura=Ura) was characterized. Now both of these dimers are considered to be of importance in photobiology and, in particular, in the photoreactivation phenomenon, which is the recovery of a biological system from the effects of *irradiation* (190–300 nm) as a result of postirradiation treatment with *illumination* (315–500 nm) (Jagger, 1967; Small *et al.*, 1968; Smith and Hanawalt, 1969). In that same year, Rupert (1961) obtained some compelling evidence from a competition experiment for another photoreactivable lesion. Uv-irradiated poly(dC·dG) was first mixed with irradiated transforming DNA. In the presence of crude yeast photoreactivating enzyme, the transforming activity of the DNA was measured after various periods of illumination. Not only did the synthetic poly(dC·dG) compete (in that it depressed the rate of restoration of transforming activity), but the ability of the irradiated poly(dC·dG) to compete could be eliminated by preilluminating it in the presence of yeast extract. Clearly a photoproduct derived from cytosine moiety behaves as a substrate for a photoreactivating enzyme. (Guanine is believed to be relatively inert under this condition (Kland and Johnson, 1957).)

Subsequently, Ono *et al.* (1965) reported that irradiation of poly(rC) in solutions altered its template properties as a RNA in polypeptide syntheses *in vitro*. A similar uv mutagenic effect was reported by Wacker *et al.* (1962). Irradiation of poly(U) (DeBoer *et al.*, 1967) resulted in the formation of uridine photohydrates and Ura=Ura (Grossman *et al.*, 1965; Ottensmeyer and Whitmore, 1968). It was disclosed that photohydrates code like Cyt, and Ura=Ura like U-G (Ottensmeyer and Whitmore, 1968). Furthermore, template transitions as studied by the incorporation of ribonucleoside triphosphates were also observed by these workers. However, Singer and Fraenkel-Conrat (1970), in reexamining these findings, reported that poly(rC) treated with uv to 5% loss in absorbancy showed some loss in proline incorporation but no new amino acid incorporation. Its GTP incorporation was somewhat reduced, without definite effects on ATP, UTP, or CTP binding. Extensive irradiation of poly(U) (to 20% loss in absorbancy) did not affect the incorporation of any triphosphate, compared to that caused by untreated poly(U). Also, their data do not support the contention that photohydrates code like Cyt, and Ura=Ura like U-G.

This paper reports the isolation and identification of a new type of adduct of cytosine derivatives. In poly(rC), the adduct forms in about the same amount as "cytosine photohydrate" (DeBoer and Johns, 1970, and references therein). A third product with unknown properties, which may be cyclobutyl dimer of cytosine (Cyt=Cyt) (Setlow *et al.*, 1965), is also formed in similar proportion. Since the photochemical changes in ribonucleotides or ribonucleosides compare to those in deoxyribonucleotides or deoxyribonucleosides (see below), similar chemical changes are not unexpected in poly(dC) or poly(dC·dG) (unpublished results). Thus, the formation of the cytosine photoadduct should be of interest in photochemistry in general, and, in particular, in the photobiological study of the chemical nature of the substrates for photoreactivating enzyme and its possible mutagenic effects in uv-irradiated DNA.

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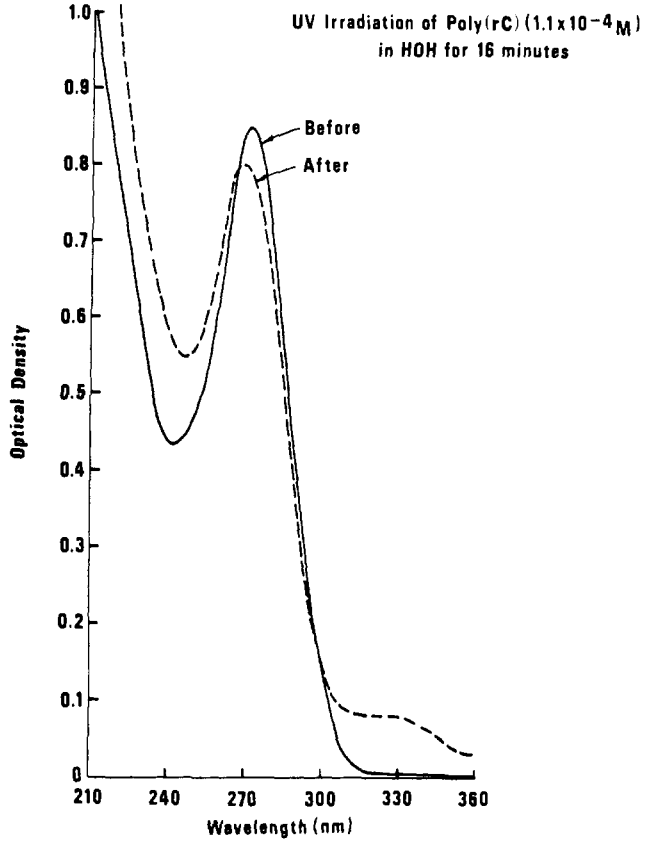


FIGURE 1: Ultraviolet spectra of poly(rC) in HOH before and after irradiation.

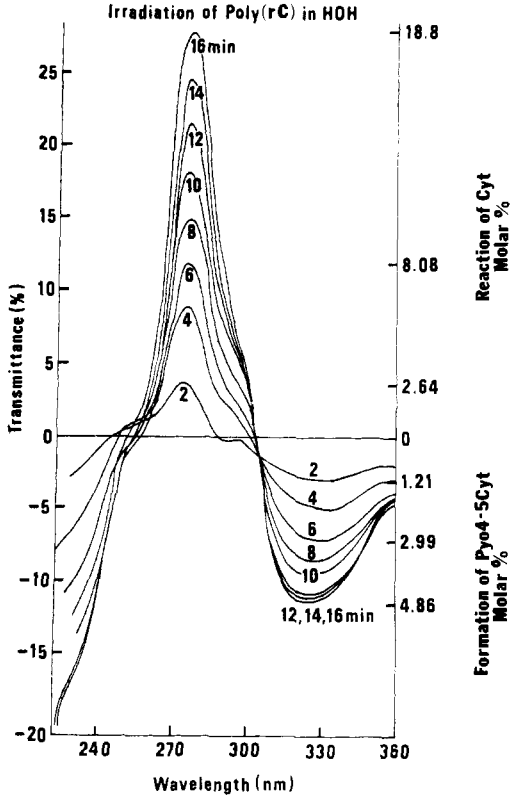


FIGURE 2: Differential transmittance spectra of poly(rC) irradiated in HOH at 2-min intervals.

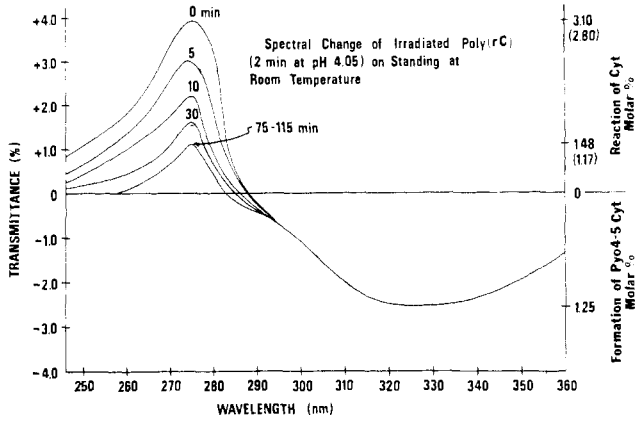


FIGURE 3: Differential transmittance spectra of 2-min irradiated poly(rC) on standing at room temperature at pH 4.

Experimental Section

Light Source. G. E. germicidal lamps were used (mainly 254 nm).

Actinometry and Quantum Yields. Incident dose rates were determined by the method of Hatchard and Parker (1956) using Corning uv filter no. 9863. (The wavelength region transmitted is 230–430 nm; 90% of this transmitted light is 254 nm and transmittancy at 254 nm is 29%.) $I_0 = 2.64 \times 10^4$ ergs/(mm² per min) or $I_0 = 5.60 \times 10^{-3}$ μE per (mm² per min). For poly(rC) solutions, $OD_{254} = 0.52$. $I_a = 0.7$ and $I_0 = 3.90 \times 10^{-3}$ μE/(mm² per min), or $I_a = 1.17$ μE/3 ml per min.

The rates of uv spectral change in poly(rC) solutions with and without the filter were found to be proportional to the above measured dose rates; therefore, light with wavelengths shorter than 254 nm are unimportant in this reaction.

Since the change in optical density at 254 nm was slight for poly(rC) solutions irradiated for 16 min, the absorbancy changes in samples irradiated for 2 min should be insignificant. Thus, the total dose used to calculate the quantum yield was $I_a \times$ time of irradiation.

Irradiation of Poly(rC) in H_2O (pH 6.2), pH 7 Phosphate Buffered Solution, and pH 4 Acetate Buffered Solution. A 0.11 mM solution of poly(rC) ($OD_{274} = 0.765$ for 0.1 mM according to Akinrimisi *et al.* (1963)) in water was irradiated in a cuvet for a total of 16 min. The absorption spectra of poly(rC) before and after irradiation are shown in Figure 1. Also shown (Figure 2) are the differential transmittance spectra of this solution irradiated at 2-min intervals. Similar studies were done in pH 7 and in pH 4 buffered solution. While the same spectral decreases at 274 nm were observed, the maximal absorption increase shifted to ~328 nm for pH 4 as compared to ~323 nm for the others.

Spectral Reversal in the Irradiated Poly(rC) Solution. A solution of poly(rC) in 0.1 M NaOAc was brought to pH 4.05 with HOAc to give a solution of $OD_{274} = 0.765$. A portion of the solution was irradiated in a cuvet for 2 min. The uv differential transmittance spectrum was taken immediately (Figure 3). The solution was then allowed to stand at room temperature in the dark. The spectral changes were recorded periodically as shown. The decrease in transmittancy (or increase in absorbancy) at 328 nm remains unchanged, while the increase in transmittancy at 274 nm decays to a stationary

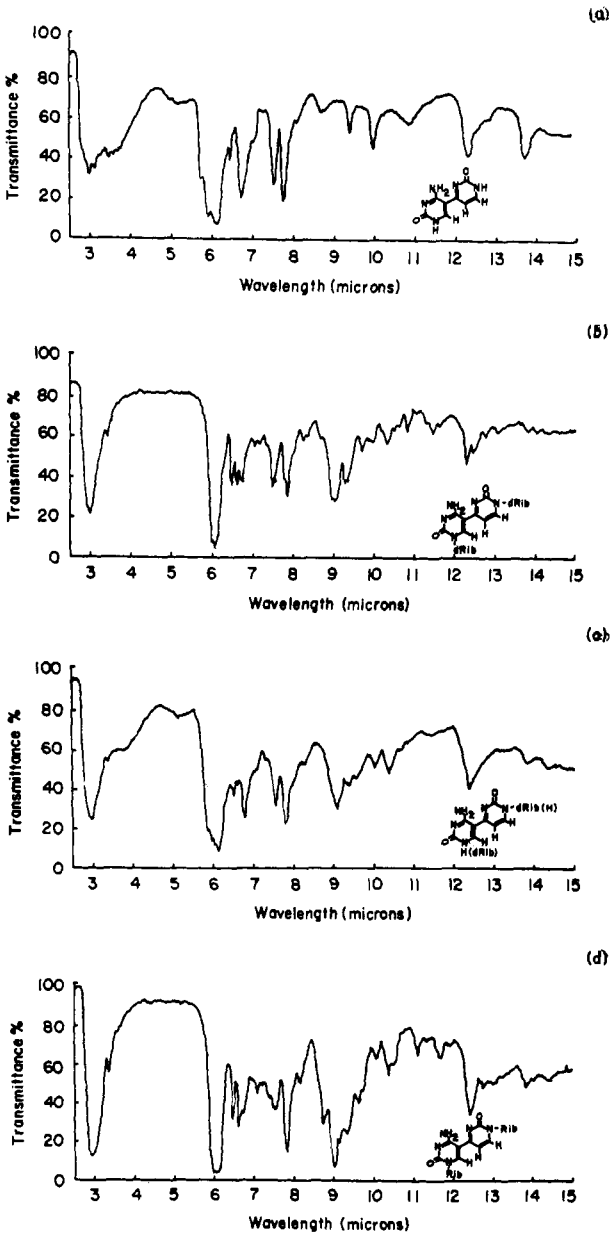


FIGURE 4: Infrared spectra of (a) Pyo4-5Cyt, (b) dPyo4-5dCyd, (c) dPyo4-5Cyt, and (d) rPyo4-5Cyd in KBr pellets.

state in ~75 min. The total decay in transmittancy is shown to be 2.8% or from 3.9 to 1.1%.

Isolation of 5-[4'-Pyrimidin-2'-one]cytosine-(Pyo4-5Cyt) from Poly(rC) Irradiated in Water. Poly(rC) (16.0 mg) was dissolved in water at ~70°, cooled to room temperature, and allowed to stand for 30 min. This solution (pH 6.2, 0.105 mM) was irradiated in quartz tubes (Wang, 1958) for 20 min and then evaporated until dry. The residue was hydrolyzed with ~0.2 ml of 70% $HClO_4$ at 100° for 45 min. The black hydrolysate was diluted to 15 ml with water, and the carbonaceous material (decomposition of sugar moieties) was removed by filtration. The filtrate was neutralized to pH 7 with dilute NaOH, concentrated to ~1 ml, applied on Whatman No. 3MM paper, and developed with 0.1 N HCl. Strips were cut from the dried chromatogram and were eluted with 0.1 N HCl. Spectral examination of the eluates revealed the presence of two bands. The band with R_F 0.50, having an absorbancy maximum at ~328 nm, is the product, while the one with R_F 0.83 is the unreacted cytosine. The product was eluted with 0.1 N HCl and the eluates were neutralized with dilute NaOH to give 80 ml with OD_{328} = 0.206. This corresponds to 0.245 mg (4.55% yield of Pyo4-5Cyt) (see below).

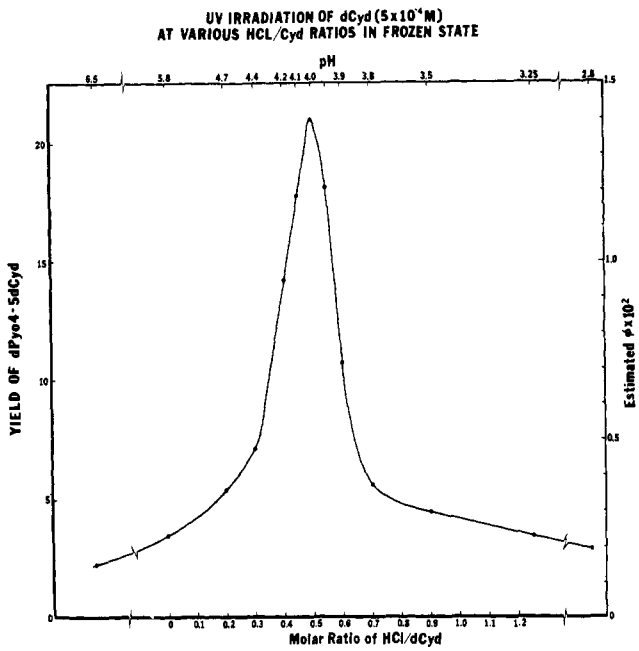


FIGURE 5: Yields and quantum yields of dPyo4-5dCyd from dCyd irradiated in frozen state at various pH or molar ratios of HCl: dCyd.

When the solution was concentrated to ~5 ml, a small amount of amorphous precipitate formed. Since it had no uv absorption, it was discarded. Pyo4-5Cyt, normally quite insoluble in water, remained in solution because of the high concentration of inorganic salts. The solution was further concentrated, applied on paper, and developed with water. The band with R_F 0.76 corresponds to uracil, and the material has the uv spectral charactertistics of uracil. The adduct, R_F 0.44, was eluted with 0.02 N HCl, neutralized with dilute NaOH, and concentrated to ~1 ml. This resulted in the appearance of an amorphous precipitate, which was collected by filtration and washed with water, alcohol, and ether. The dried material weighed 0.15 mg and had an ir spectrum identical with that of Pyo4-5Cyt (Figure 4a).

Effect of pH on the Yields of rPyo4-5Cyd and dPyo4-5dCyd. Cyd (1 mmole) was dissolved in distilled water (200 ml). The solution was titrated to the desired pH with dilute HCl. A series of 15 solutions were prepared from pH 2.4 to 5.2, each with an increment of pH 0.2. Portions of 20 ml of these solutions were placed in petri dishes (i.d. 8.6 cm) and irradiated in a frozen state for 2 hr. The OD_{325} readings for the thawed solutions were determined as follows:

pH	2.4	2.6	2.8	3.0	3.2	3.4	3.6	
OD	0.41	0.44	0.49	0.50	0.63	0.88	0.88	
% yield	0.9							
pH	3.8	4.0	4.2	4.4	4.6	4.8	5.0	5.2
OD	2.68	1.80	0.51	0.40	0.39	0.31	0.41	0.42
% yield	5.9							0.9

Similar study using varying ratios of dCyd:HCl gave the results presented in Figure 5.

Isolation of rPyo4-5Cyd and dPyo4-5dCyd from the Irradia-

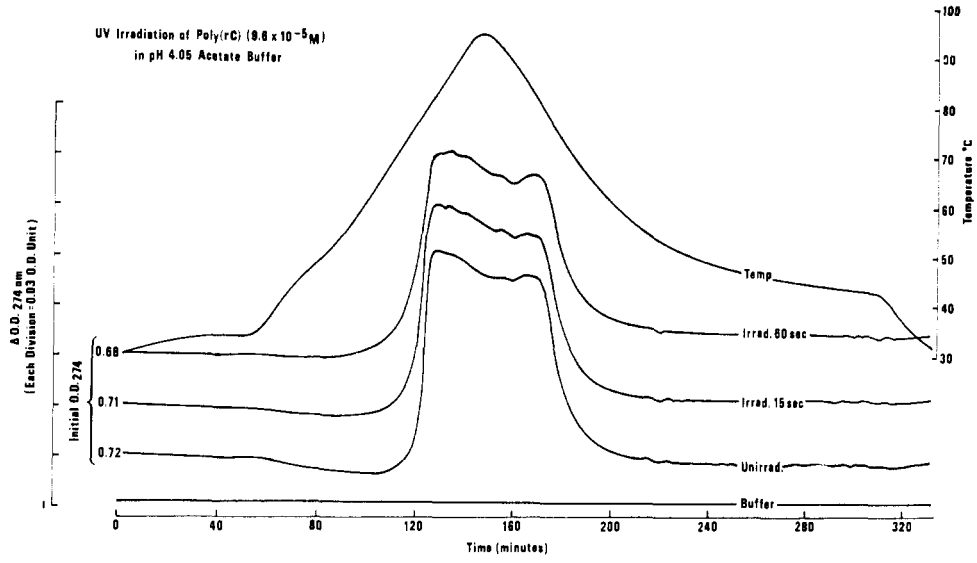


FIGURE 6: Absorbancy–temperature profiles of poly(rC) and irradiated poly(rC) at pH 4.

tion of Cyd·0.5HCl and dCyd·0.5HCl in the Frozen State. A 5 mM aqueous solution (300 ml) of dCyd containing 2.5 mM of hydrochloric acid was irradiated in the frozen state (8 mm thick) over crushed Dry Ice at a distance of 7 cm from the G. E. germicidal lamps of an irradiator (Wang, 1961) for a total of 8 hr with thawing and refreezing every 2 hr. Afterward the irradiated solution was thawed, concentrated, applied on Whatman No. 3MM paper, and developed with eluent A (80% ethanol). The dried chromatograms were cut into sections and the materials were eluted with water. Examination of the uv spectra of the eluates revealed the presence of two bands in addition to that of dCyd (R_F 0.71). The major band (R_F 0.5) was well separated from dCyd, and the material was extracted with warm water (70–80°). Upon concentration of the solution to 5 ml, a white crystalline precipitate was obtained and was redissolved in hot water for recrystallization. After it was allowed to stand at 5° overnight, 60 mg (~18%) of purified product was isolated. It was identified as dPyo4-5dCyd (see below).

The minor product (R_F 0.32) exhibited a uv spectrum identical with that of the major product. It is probably dPyo4-5Cyt or Pyo4-5dCyd formed from dPyo4-5dCyd by the cleavage of one deoxyribose moiety during the isolation procedure.

Under conditions identical with those described above, 1.5 mmoles of Cyd·0.5 HCl in 300 ml of water was irradiated for a total of 6 hr. The thawed solution appeared cloudy because of the limited solubility of the product. Therefore, the solution was neutralized with 0.75 ml of 1 N NaOH and was then concentrated to 10 ml. The crude product, which appeared as pale yellow microcrystals, was collected by filtration. The product was redissolved in boiling water for recrystallization. After it was allowed to stand at 5° overnight, 50 mg (15%) of purified product was obtained. This was characterized as rPyo4-5Cyd (see below). It appeared as a single band, R_F 0.21, when chromatographed with eluent A on Whatman No. 3MM paper.

The ir spectra of dPyo4-5dCyd, dPyo4-5Cyt, and rPyo4-5Cyd are shown in Figure 4b–d, respectively.

Water of Crystallization of dPyo4-5dCyd and rPyo4-5Cyd. Freshly prepared samples of dPyo4-5dCyd and rPyo4-5Cyd contain water of crystallization. The loss of this water occurs slowly when the samples are allowed to stand at room temperature; it can be removed quantitatively by heating at 138° (*p*-xylene) *in vacuo* for 2 hr. The molecular formulae are dPyo4-5dCyd·$5H_2O$ and rPyo4-5Cyd·$7H_2O$ as estimated by the weight loss upon drying and by molecular extinction coefficient measurements. (Nmr spectra of these compounds were taken with undried samples, as the anhydrous materials were found to be quite insoluble in neutral solvents.)

"Puddle Effect" (Wang, 1961) on the Formation of dPyo4-5dCyd. A 5 mM aqueous solution of dCyd·HCl was divided into two portions. One portion was neutralized by the addition of a calculated volume of NaOH to give a solution of 5 mM dCyd·0.5HCl·0.5NaCl. The other portion was neutralized with Dowex 1-X8 (OH^- form) and was mixed with an equal volume of the original solution to give a solution of 5 mM dCyd·0.5HCl. Both solutions were then irradiated in a frozen state for 2 hr under the conditions described above. Examination of the uv spectra of the irradiated solutions showed that the yield of dPyo4-5dCyd was 9% in the absence of NaCl and decreased to 4% in the presence of NaCl.

A solution of 5 mM dCyd·0.5HCl containing 10 mM of methanol was used in another experiment. No dPyo4-5dCyd was detected after this solution was irradiated in a similar manner.

Isolation of Pyo4-5Cyt from Acid Hydrolysis of dPyo4-5dCyd, dPyo4-5Cyt, and rPyo4-5Cyd. Hydrolysis of both dPyo4-5dCyd and dPyo4-5Cyt proceeded smoothly in CF_3COOH to give almost quantitative yields of Pyo4-5Cyt. dPyo4-5dCyd (35 mg) was hydrolyzed in 0.5 ml of CF_3COOH for 20 min at 80° in a sealed tube. After evaporation, the residue had the appearance of black tar. The hydrolyzed product was extracted twice with 10 ml of boiling water. Neutralization of the colorless aqueous extract with dilute NaOH to pH 7 gave a white amorphous precipitate of Pyo4-5Cyt (15 mg, 95%). Although the product is quite pure, as indicated by the ir and uv spectra, it can be further purified by redissolution in 1 N HCl and neutralization.

rPyo4-5Cyd (10 mg) was hydrolyzed in 1 ml of 70% $HClO_4$ at 100° for 15 min. The black reaction mixture was treated with 15 ml of boiling water. After filtration, the pale yellow extract was neutralized to give 4 mg (95%) of a white amorphous precipitate of Pyo4-5Cyt. This was shown to be identi-

TABLE I

$OD^{A}_{329} = \epsilon^{329}_{A}[A]$; $OD^{H}_{275} = \epsilon^{275}_{C_{hypo}}[H]$; $-OD^{Irv}_{275} = \epsilon^{275}_{A}[A] - 2\epsilon^{275}_{C_{hypo}}[D]$; or $2\epsilon^{275}_{C_{hypo}}[D] = OD^{Irv}_{275} + (\epsilon^{275}_{A} - 2\epsilon^{275}_{C_{hypo}})[A]$

	λ (nm)	ΔT	ΔOD	Concn (Moles/l.)	ϕ (Moles/E)
Adduct	329	0.025	0.0114	6.30×10^{-7}	0.81[c] (1.62)[d]
"Photohydrate"	275	0.031[a]	−0.0703[a,b]	91.3×10^{-7}	11.7 (11.7)
"Dimer"^{Irv}	275	0.011	−0.0219[b]	14.2×10^{-7}	1.82 (3.64)
"Dimer"^{Add}	275				

[a] Value corrected to account for spectral reversal during irradiation. [b] Refers to loss of monomer absorption. [c] Quantum yield $\times 10^{-3}$. [d] Values in parentheses are the quantum yields for monomer loss due to product formation.

cal with that obtained from dPyo4-5dCyd by ir and uv spectra (see below).

Absorbancy–Temperature Profile (Mandel and Marmur, 1968) of Poly(rC) and Uv-Irradiated Poly(rC) in pH 4 Buffered Solutions. Changes at 260 nm in the absorbancy of these solutions were followed by a Beckman DU spectrophotometer coupled with a Gilford 2000 recorder. The temperature of the cell compartment was regulated by a Tamson constant-temperature circulator equipped with a Neslab temperature programmer. Quartz cuvets with 1.0-cm light path, 3-ml capacity, and ground-glass stoppers, were used for these measurements. These solutions were freshly prepared and were degassed before use. The change in hyperchromicity was recorded by increasing the temperature 1.0°/min. The concentrations of the solutions correspond to the optical density readings given in Figure 6.

Results and Discussion

Irradiation of Poly(rC). The changes seen in a 0.11 mM solution in water irradiated with a total dose of 6.24 μE/ml (Figure 1) are similar to those observed in a pH 7 buffered solution. At pH 4, the maximal absorption increases shifted toward longer wavelengths; this shift indicates that the photoproducts formed under neutral and acidic conditions are different (see below). In either case, the absorbancy decrease in the 275-nm region is accompanied by a spectral decrease in the 300- to 340-nm region. These changes are more obvious when recorded as differential transmittancy spectra (Figure 2). Apparently, there is a continued decrease in transmittancy in the 329-nm (T_{329}) region. However, the rate of decrease diminishes greatly after an absorbed dose of 4.68 μE/ml. In contrast, the increase in the T_{275} region continues at a similar rate. It is assumed that the decrease in T_{329} is caused by the formation of Pyo4-5Cyt (see below) and that the increase in T_{275} is due to the combined effects of the formation of adduct (A), of "photohydrate" (H) (Fahr, 1969; DeBoer and Johns, 1970) and of an unknown product(s), possibly "cytosine dimer" (D). The characteristic spectral changes associated with each of these compounds are known and can be easily recognized and calculated. The adduct formation not only causes an increase in absorbancy in the 329-nm region (OD^{A}_{329}) but also in the 275-nm region (OD^{A}_{275}). The value can be estimated from the change observed in OD^{A}_{329}. The "photohydrate" formation by cytosine derivatives is characterized by the decrease in absorption in the 275-nm region and this decrease is known to reverse upon standing at room temperature, especially in acidic and alkaline pH (Fahr, 1969; DeBoer and Johns, 1970). Thus the spectral reversal at 275 nm (OD^{H}_{275}) may be used as a criterion for estimating the concentration of "photohydrate." The remaining irreversible change in the 275-nm region ($-OD^{Irv}_{275}$) may be due to cyclobutyl dimerization. This assumption is based on the observation that the formation of various photoproducts causes little hyperchromism in the double-helical poly(rC) ($\epsilon^{275}_{C_{hypo}} = 7.7 \times 10^{3}$ due to hypochromism (Akinrimisi *et al.*, 1963)) irradiated with low uv doses. This is indicated by the unaltered hyperchromic effect observed in the thermal denaturation of irradiated and unirradiated poly(rC) samples (Figure 6).

Thus, when poly(rC), irradiated for 2 min in a pH 4.1 solution, was allowed to stand at room temperature in the dark (Figure 3), the decrease in T_{329} remained constant, whereas the increase in T_{275} decayed to a stationary state in ~75 min. Therefore, the relationships given in Table I exist and permit various factors to be calculated. Therefore, the various products, adduct, hydrate, and possibly "dimer," form in a ratio of 1:14.5:2.2, respectively.

Interestingly, the rate of formation of Pyo4-5Cyt levels off at ~5% (Figure 2). It is possible that, at this level, the configuration of poly(rC) no longer favors the formation of dimeric photoproducts, and that "photohydrate" is the only photoproduct. In studies using large uv doses, high percentages of photohydrate formation have been reported. Thus, the adduct formation has, so far, escaped detection.

Isolation of Pyo4-5Cyt from Irradiated Poly(rC). The importance of Pyo4-5Cyt to photobiology is indicated by its formation in significant quantities at uv doses comparable to those used in biological studies. Also, Pyo4-5Cyt formation is irreversible under reaction conditions and should have permanent biological effects.

Chromatographic separation of Pyo4-5Cyt from the acid hydrolysates of poly(rC) solutions (water, pH 6.2) irradiated with biological doses yielded sufficient material for the determination of uv and ir spectra. These spectra were subsequently found to be identical with those of Pyo4-5Cyt obtained by acid hydrolysis of dPyo4-5dCyd and rPyo4-5Cyd, which were isolated by irradiation of the corresponding nucleoside hemihydrochloride in frozen aqueous solutions.

Effect of pH on the Formation of Adducts of Cytosine Derivatives. Isolation of Pyo4-5Cyt from irradiated poly(rC) in quantities sufficient for its structural elucidation would be ideal. However, since this presents an almost impossible task, other approaches must be found. The most logical step would be the irradiation of Cyt. Unfortunately, numerous attempts were not very successful. On the other hand, preliminary observations indicated the possible formation of reasonable yields of adducts of dCyd and Cyd. Subsequent study provided a most interesting finding, *i.e.*, the effect of pH on

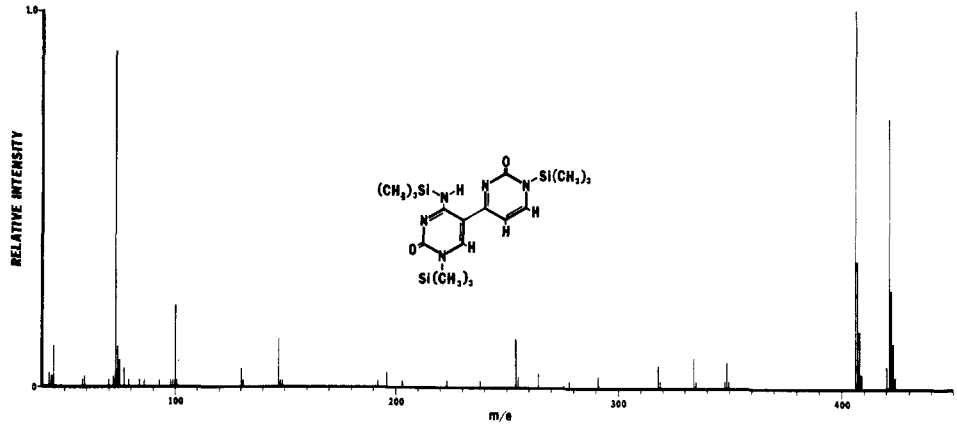


FIGURE 7: Mass spectrum of $(Me_3Si)_3$-Pyo4-5Cyt.

these bimolecular photoreactions. Apparently, the pH of the medium plays a critical role. In the formation of rPyo4-5Cyd, the yield or the increase of OD_{325} rose slightly with the pH from 2.4 to 3.4, and was followed by a sharp rise in yield with a maximum at pH 3.8. Further increase in pH sharply reduced the yield of rPyo4-5Cyd to the level observed at pH 2–3. The same dramatic pH effect was observed with the formation of dPyo4-5dCyd from the irradiation of dCyd in frozen state. It is best described by Figure 5. In both cases, the pH for the maximal yields are calculated to be 0.5 molar equivalent in HCl; the formation of each rPyo4-5Cyd or dPyo4-5dCyd molecule requires one molecule of HCl and two molecules of the nucleoside.

Isolation of rPyo4-5Cyd and dPyo4-5dCyd. On the basis of the above information, rPyo4-5Cyd and dPyo4-5dCyd were isolated from the irradiation of Cyd·0.5 HCl and dCyd·0.5 HCl, respectively, in the frozen state. Under the experimental conditions, yields of 15% of rPyo4-5Cyd and 18% of dPyo4-5dCyd were obtained. Also, these adducts were found to contain water of crystallization; their molecular formulae are estimated as dPyo4-5dCyd·H_2O and rPyo4-5Cyd·$7H_2O$.

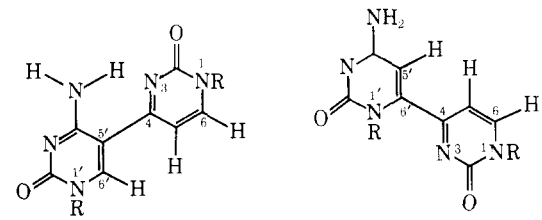
Preliminary Mechanistic Study. The molecular aggregation–puddle formation hypothesis (Wang, 1961, 1965) has been used to explain certain phenomena of frozen aqueous solutions. During freezing, water forms ice crystals, which force solute molecules into solid aggregates. Thus, irradiation of frozen solution would be analogous to that of solid state. In an aqueous solution containing a small amount of low melting point organic solvents or electrolytes, one may visualize that as the solution freezes, the solute molecules together with some organic solvent or electrolyte would be excluded from the water crystals and form puddles (Wang, 1961, 1965). Thus, irradiation of puddles would be analogous to that of concentrated solutions. In order to have an understanding of the photochemical mechanism of the formation of these adducts, "puddle effect" was studied. In "electrolyte (NaCl) puddles" (Bruice and Butler, 1965), the adduct yields were found to be less than in the absence of the "puddles." Under identical conditions, no adduct formation was detected after irradiation in "methanol puddles." These results strongly suggest that formation of rPyo4-5Cyd is greatly favored in solid state. These data serve as a basis for our understanding of the mechanism (see below).

Acid Hydrolysis of dPyo4-5dCyd and rPyo4-5Cyd. dPyo4-5dCyd and minor product dPyo4-5Cyt were easily hydrolyzed in CF_3COOH to give Pyo4-5Cyt in a yield over 95%. Similarly, almost quantitative yields of Pyo4-5Cyt were obtained from rPyo4-5Cyd hydrolyzed in 70% $HClO_4$. The ir spectra of Pyo4-5Cyt from these two sources and from irradiated poly(rC) were found to be identical in every respect.

Structure Elucidation

The mass spectrum determinations (Figure 7) were obtained for the Pyo4-5Cyt adduct after silylation with Regisil No. 27002 in pyridine; this method has been used to determine other photoproducts (Fenselau and Wang, 1969). The molecular ion peak at *m/e* 421 corresponds to a tritrimethylsilyl $(Me_3Si^-)_3$ derivative of a dimeric cytosine compound with the loss of one molecule of ammonia, *i.e.*, $3 \times (73 - 1) + 2 \times 111 - 17 = 421$.

On the basis of the ultraviolet absorption spectra of dPyo4-5dCyd, rPyo4-5Cyd, and Pyo4-5Cyt (Figure 8), it may be said that these compounds have the same chromophores as substituted pyrimidinones and are similar to the dehydrated adducts of (Pyo4-6Ura) (Khattak and Wang, 1969), uracil-thymine (Pyo4-6Thy) (Varghese and Wang, 1967; Wang and Varghese, 1967; Rhoades and Wang, 1970), and thymine-thymine (5MePyo4-6Thy) (Varghese and Wang, 1968). Thus, there are two structural isomers possible. One is 4′-amino-



I II

[4,5′-bipyrimidine]-2,2′(1*H*,1′*H*)-dione (I), and the other is 4′-amino[4,6′-bipyrimidine]-2,2′(1*H*,1′*H*)-dione (II). Both types of linkage, *i.e.*, 4,5′ and 4,6′, are possible for these pyrimidine adducts.

The nuclear magnetic resonance spectrum of the dPyo4-5dCyd·$5H_2O$ in $(CD_3)_2SO$ at 100 MHz appears to be very complex (Figure 9); (for clarity, the numbering system used in nmr differs from that used in the naming of these compounds). For the two *deoxyribose* moieties, the two methylene protons of both C′(2) and both C′(5) show as multiplets at δ 2.26 (4 H) and 3.62 (4 H), respectively. The methine protons

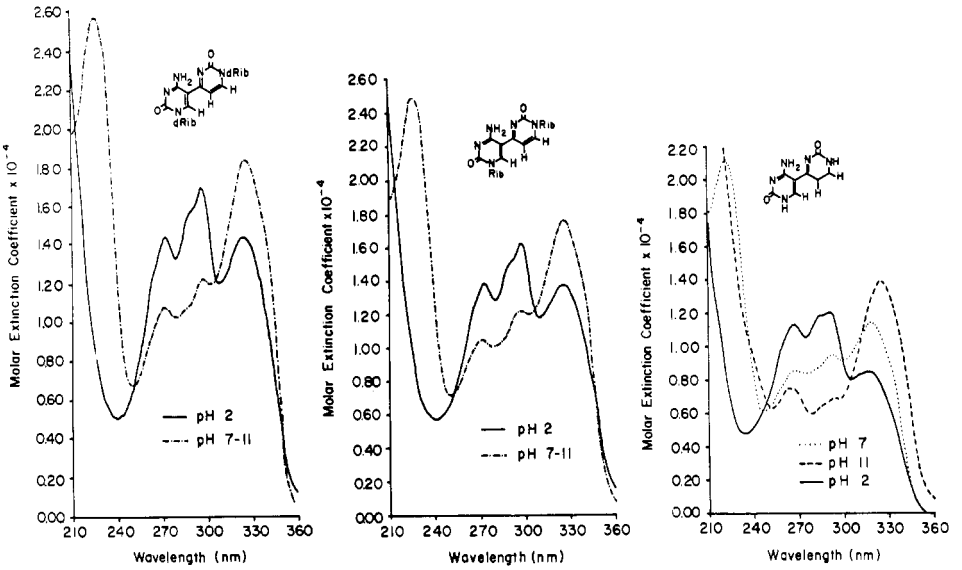


FIGURE 8: Ultraviolet absorption spectra of dPyo4-5dCyd, rPyo4-5Cyd, and Pyo4-5Cyt.

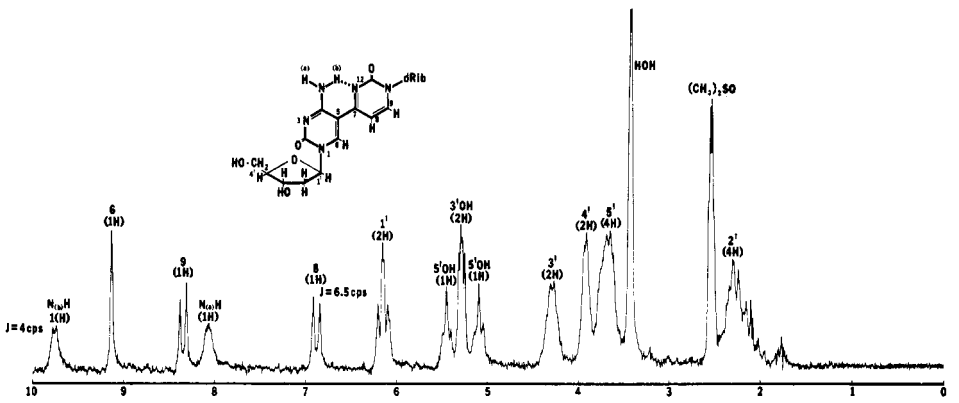


FIGURE 9: Nuclear magnetic resonance spectrum of dPyo4-5dCyd in $(CD_3)_2SO$ at 100 MHz with internal standard tetramethylsilane.

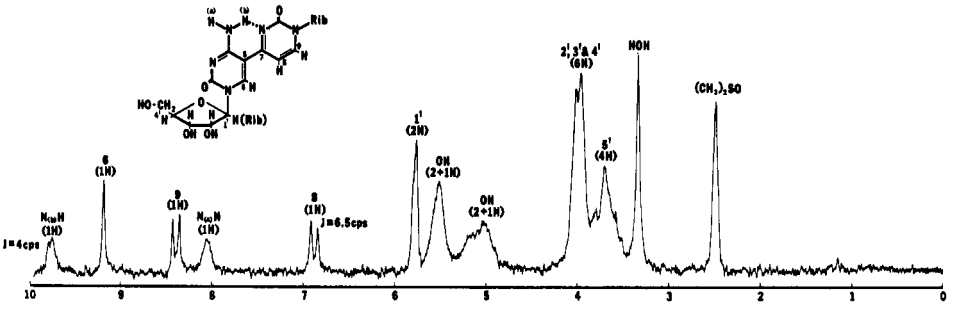


FIGURE 10: Nuclear magnetic resonance spectrum of rPyo4-5Cyd in $(CD_3)_2SO$ at 100 MHz with internal standard tetramethylsilane.

of both C′(4), both C′(3), and both C′(1) display a multiplet (δ 3.90, 2 H), a multiplet (δ 4.26, 2 H), and two triplets (δ 6.14, 2 H), respectively, as expected. The two C′(5)-OH protons manifest as two pairs of triplets (δ 5.08, 1 H and δ 6.14, 1 H). The signals of the two C′(3)-OH protons appear as two doublets centered at δ 5.26 (2 H). For the pyrimidine rings, there are three vinyl protons and two amino protons. The former protons show as two doublets (δ 6.86, 1 H; δ 8.33, 1 H; J = 6.5 cps) for C(8) and C(9) and a singlet (1 H) at δ 9.11 for C-(6) (see below). The latter two protons occur with markedly different chemical shifts (δ 8.05, 1 H; δ 9.74, 1 H; J = 4 cps) indicating two nonequivalent amino hydrogens. The low-field signal is most likely the one forming an H bond with N-(12) as depicted (Figure 9). Addition of D_2O to the sample resulted in the disappearance of the signals assigned to NH and OH protons.

A similar nmr spectrum was obtained for rPyo4-5Cyd·$7H_2O$ (Figure 10). For the two *ribose* moieties, two pairs of

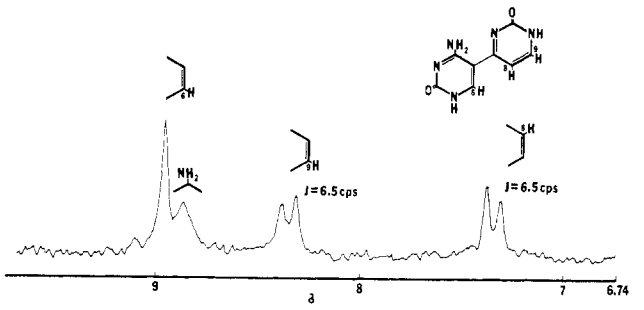
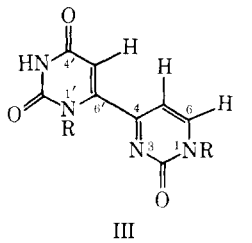


FIGURE 11: Nuclear magnetic resonance spectrum of Pyo4-5Cyt trifluoroacetic acid at 100 MHz with internal standard tetramethylsilane.

methylene protons of C′(5) appear as multiplets at δ 3.69 (4 H). Both sets of the C′(2), C′(3), and C′(4) methine protons are seen as a complex signal centered at δ 3.97 (6 H). The resonance at δ 5.7 (2 H) is the signal for the two remaining C′(1) methine protons. The OH protons appear as two group signals at δ 5.06 (2 + 1 H) and 5.52 (2 + 1 H) with probably one C′(5)-OH proton appearing at the position with the two C′(3)-OH and the other with the two C′(2)-OH protons. For the pyrimidine moiety, two doublets (δ 6.86, 1 H; δ 8.38, 1 H; J = 6.5 cps) are assigned to C(8) and C(9) vinylic protons and a singlet (δ 9.17, 1H) to the other vinylic C(6) proton (see below). As in the case of dPyo4-5dCyd, a pair of nonequivalent amino proton signals are present at δ 8.04 (1 H) and δ 9.76 (1 H) with discernible coupling (J = 4 cps). The low-field resonance again indicates the possible intramolecular H bonding with N(12).

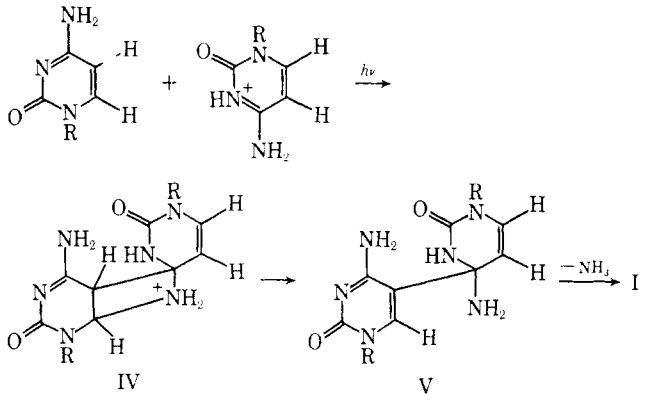
Since Pyo4-5Cyt is insoluble in $(CD_3)_2SO$, its spectrum was measured in CF_3COOH (Figure 11). It exhibits two doublets (δ 7.34, 1 H; δ 8.35, 1 H; J = 6.5 cps) for C(8) and C(9) vinylic protons and a singlet (δ 8.95, 1 H) for the other vinylic proton (see below). The latter signal partially coincides with a broad NH signal at δ 8.86. In CF_3COOH, unlike $(CD_3)_2SO$, intramolecular H bonding is unlikely and nonequivalent amino proton signals are no longer observed. In CF_3COOD, the spectrum displays only the three vinylic proton resonances.

The selection between the 4,5′- and 4,6′-bipyrimidine linkages may be made by comparing the nmr spectra. The vinyl singlets of dPyo4-5dCyd, rPyo4-5Cyd, and Pyo4-5Cyt occur at δ 9.11, 9.17, and 8.95, respectively. The appearance of these signals at such a low field suggests that they may be characteristic of C(6)-H. For C(5)-H, the signals should be somewhat upfield. For example, (see structure III) the vinylic singlet for C(5′)-H is at δ 6.74 and the coupled vinylic doublets for C(5)-H and C(6)-H are at δ 7.37 and 8.52 in the nmr spectrum of Pyo4-6Ura (Khattak and Wang, 1969), which has basically the same ring system as structure (II). The latter signals correspond well to C(5)-H and C(6)-H (or C(8) and C(9) as indicated in the nmr figure) of dPyo-4-5dCyd, rPyo4-Cyd, and Pyo4-5Cyt. Therefore, structure I is most probably the fundamental ring system for these compounds. Furthermore, if these adducts of cytosine derivatives have structure II, then the presence of intramolecular H bonding of the amino group is unlikely. Therefore, the observed signals for nonequivalent amino hydrogens in the nmr, in turn, again favor structure I.



III

SCHEME I



We may conclude that dPyo4-5dCyd is a 1,1′-bis(2-deoxy-β-D-*erythro*-pentofuranosyl), and rPyo4-5Cyd is a 1,1′-di-β-D-ribofuranosyl derivative of Pyo4-5Cyt, which is 4′-amino[4,5′-bipyrimidine]-2,2′(1*H*,1′*H*)-dione.

In regard to the uv spectra, derivatives of cytosine and pyrimidin-2-one generally exhibit absorbancy maxima in the 270-nm (~10 × 10^3) and 305-nm (~5 × 10^3) regions, respectively. Although the Pyo4-5Cyt derivatives consist of these two chromophores, their absorbancy maxima are in the 326- to 329-nm region with high molar absorbancy (~14–18 × 10^3). This observation suggests that there is strong interaction between the two chromophores and that the ring system should be coplanar. This conforms with the ring system of Pyo4-6Ura (Khattak and Wang, 1969) and contrasts with that of 5MePyo4-6Thy, of which two rings are in a skew conformation (Varghese and Wang, 1968; Karle, 1969).

Mechanism of Adduct Formation

With the knowledge of the structures of the adducts and the probable compositions of the activated complexes, *i.e.*, one neutral and one protonated cytosine moiety, a possible mechanism for their formation is Scheme I. The formation of azetidine derivatives IV is analogous to the oxetane ring formation in irradiated thymine and uracil derivatives. Subsequently, this four-membered ring may be opened in solutions to give the intermediate V. Again, these steps are analogous to those for oxetane derivatives of thymine and uracil adducts (Rhoades and Wang, 1970). Under acidic conditions, V is deaminated to yield the final product I. Fluorescent study of poly(rC) (W. Hauswirth and S. Y. Wang, unpublished results, 1971) in water (pH 6) and in pH 4 acetate buffer indicated that product I is formed directly in pH 4 solution and product V is the possible intermediate in neutral solution. Upon acidification, V is converted to I. In view of the molecular structure, the adducts may be formed by either *inter-* or *intrastrand* reactions in polynucleotides. Thus, it would be interesting to investigate how Pyo4-5Cyt is formed in the poly(rC) double helices. At pH 4, poly(rC) apparently exists as double-stranded helices (Akinrimisi *et al.*, 1963; Lang-

ridge and Rich, 1963). Therefore, thermal denaturation–renaturation profiles were studied at pH 4.05. With very low irradiation doses, the number of Pyo4-5Cyt formed would be relatively few. Thus, the thermal denaturation, as measured by the increase in uv absorbancy, of irradiated and unirradiated samples may not be appreciable even if a few interstrand Pyo4-5Cyt are formed. On the other hand, one or two cross-links would cause spontaneous renaturation on annealing resulting in a precipitous decrease in uv absorbancy. Our data (Figure 6) indicated no apparent change in the rate of absorbancy increases and decreases. Thus, the formation of Pyo4-5Cyt may occur between neighboring intrastrand cytosin moieties. It should also be noted that decreases in absorbancy were observed prior to denaturation in all samples as the temperature rose from 35 to 70°. This hypochromicity was due to the modification of the absorption spectrum of the cytidine residues caused by the change of pK with temperature (Akinrimisi *et al.*, 1963). Apparently, the degree of hypochromicity decreases with increasing irradiation doses and suggests that the formation of adducts causes localized denaturation. This is to be expected considering the molecular structure of Pyo4-5Cyt. Further increase in temperature resulted in the sharp hyperchromic transition with T_m at 79° for the denaturation process (Sober, 1968). Upon cooling, the hypochromic effects were observed as the renaturation process progressed. For unirradiated samples, the base-line readings returned to the initial level. For irradiated samples, however, the base-line readings are somewhat higher than the originals. These differences again suggest that the formation of Pyo4-5Cyt causes localized denaturation.

It may be concluded, then, that the formation of Pyo4-5Cyt in poly(rC) is similar to that in frozen state or solid state irradiation of dCyd·0.5HCl or Cyd·0.5HCl. The former cases are the results of polymeric structure and the latter requires a neutral cytosine moiety and a protonated cytosine moiety for this bimolecular photoreaction. While it is understandable that there must be some interaction or energy transfer between the two bases for this photochemical process to occur, its specific nature was not obvious. However, recent study, by Montenay-Garestier and Hélène (1970), of fluorescence and phosphorescence titration curves of cytidine molecules suggested that charge-transfer interactions occur between Cyd and Cyd·H^+ in stacked structures where Cyd·H^+ would behave as the acceptor and Cyd as the electron donor. Our results strongly support the above suggestion and serve as a unique example for a photochemical reaction occurring with charge-transfer complexes. Therefore, these findings are significant not only in photobiology but also in photochemistry.

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