Photochemistry of Uracil and Uridine*

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ABSTRACT: The photoproducts of uracil and uridine formed under different irradiation conditions have been isolated and their properties studied. Separation of the various products was accomplished by paper chromatography and ion-exchange column chromatography. Irradiation of uracil in frozen aqueous solution with ultraviolet light (λ 254 nm) produces cyclobutane-type dimers having the cis-syn and cis-anti configurations, and 6-(4′-pyrimidin-2′-one)uracil (PO-U). Irradiation of uridine as a thin-solid film also produces these products. Irradiation of uridine in frozen aqueous solution produces PO-U, uridine hydrate, cis-syn, cis-anti, and trans-anti dimers. Irradiation of uracil or uridine with light of wavelength >290 nm in aqueous solution in the presence of acetone produces cyclobutane-type cis-syn, cis-anti, and trans-anti dimers, the trans-anti isomer being the major product. Dihydrouracil is also formed when uracil in solution is irradiated in the presence of acetone. The results show that the amounts and types of uracil and uridine photoproducts depend on irradiation conditions.

A major fraction of the lethal and mutagenic effects of ultraviolet light on biological systems has been attributed to photochemical transformations of pyrimidine bases. The types and amounts of photoproducts formed from pyrimidine bases depends to a great extent on the irradiation conditions. In a number of cases, correlations between changes in biological function resulting from ultraviolet radiation and specific photoproducts have been possible. To achieve further progress in this direction, a detailed knowledge of the properties of the different photoproducts is essential. We have been studying the photochemistry of nucleic acid constituents with special emphasis on the isolation and chemical characterization of the photoproducts. The results of our studies on thymine (Varghese and Wang, 1968a,b), thymidine (Varghese, 1970, 1971b), and two cytosine nucleosides (Varghese and Rupert, 1971; Varghese, 1971a) have been previously reported. The present work summarizes the results of our investigations on the photochemistry of uracil and uridine.

Although the photochemistry of uracil and uridine has been studied in different laboratories (Wang, 1961; Smith, 1963; Donges and Fahr, 1966; Fahr *et al.*, 1968; Blackburn and Davies, 1966; Rosenthal and Elad, 1968; Greenstock and Johns, 1968), there is contradictory evidence concerning the homogeneity of the preparations and the properties of even the major products. To give one example: it has been reported (Blackburn and Davies, 1966) that irradiation of frozen aqueous solutions of uracil leads to a single stable cyclobutane-type dimer; however, it has also been suggested (Setlow *et al.*, 1965; Smith, 1963) that this dimeric product is probably a mixture of stable and unstable isomers. We have separated two isomeric cyclobutane-type dimers of uracil and the dehydration product of a uracil–uracil adduct from irradiation of frozen solutions of uracil. The kind of photoproducts obtained from the irradiation of uridine have been found to depend strongly on the conditions used during irradiation. For example, irradiation of uridine in frozen aqueous solution forms three isomeric cyclobutane-type dimers, a uridine–uridine adduct having an ultraviolet absorbance maximum at 309 nm, and a water addition product. On the other hand if uridine (or uracil) is irradiated (λ > 290 nm) in solution in the presence of acetone as a photosensitizer, at least three isomeric dimers are formed with the trans-anti isomer being the major product. When uridine is irradiated as a thin-solid film, the cis isomers of cyclobutane-type uridine dimer and a uridine–uridine adduct are the major products. According to Wechter and Smith (1968), irradiation of uridine in aqueous solution produces two isomers of 5,6-dihydro-6-hydroxyuridine.

Materials and Methods

Materials. Uracil and uridine were obtained from Sigma Chemical Co. and were used without further purification. [2-^{14}C]Uracil and [2-^{14}C]uridine were from Schwarz Bio-Research, Inc. Dowex 50W X-12 (H^+, 100–200 mesh) and Dowex 1-X8 (Cl^-, 200–400 mesh) were from Bio-Rad Laboratories. Baker Analyzed Reagent acetone was distilled before use for sensitized irradiations.

Irradiation. The method of irradiation in frozen aqueous solution with ultraviolet light has been previously described (Varghese, 1970). For irradiation of uridine as a thin-solid film, the same conditions as those described for the irradiation of thymidine were used (Varghese, 1971a). For sensitized irradiation a bank of 12 Rayonet photochemical reactor 3000-Å lamps were used. Uracil or uridine (2 mmoles) was dissolved in 500 ml of water and 500 ml of acetone in a 1-l. Pyrex conical flask. The solution was flushed with nitrogen for 1 hr prior to irradiation and continually during irradiation. The solution was kept air-cooled by a fan. Irradiation was continued for about 16 hr.

Paper and Thin-Layer Chromatography. For 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. For ascending thin-layer chromatography, Eastman chromatogram sheets (6065 cellulose coated) were used. The solvent systems used were: (A) 2-butanol saturated with water; (B) 1-butanol–acetic acid–water (80:12:30); (C) 2-propanol–water (70:30); (D) 2-propanol–NH_4OH–water (70:10:20); (E) *tert*-butyl alcohol–methyl ethyl ketone–NH_4OH–water (40:30:10:20); and (F) 1-propanol–water (70:30).

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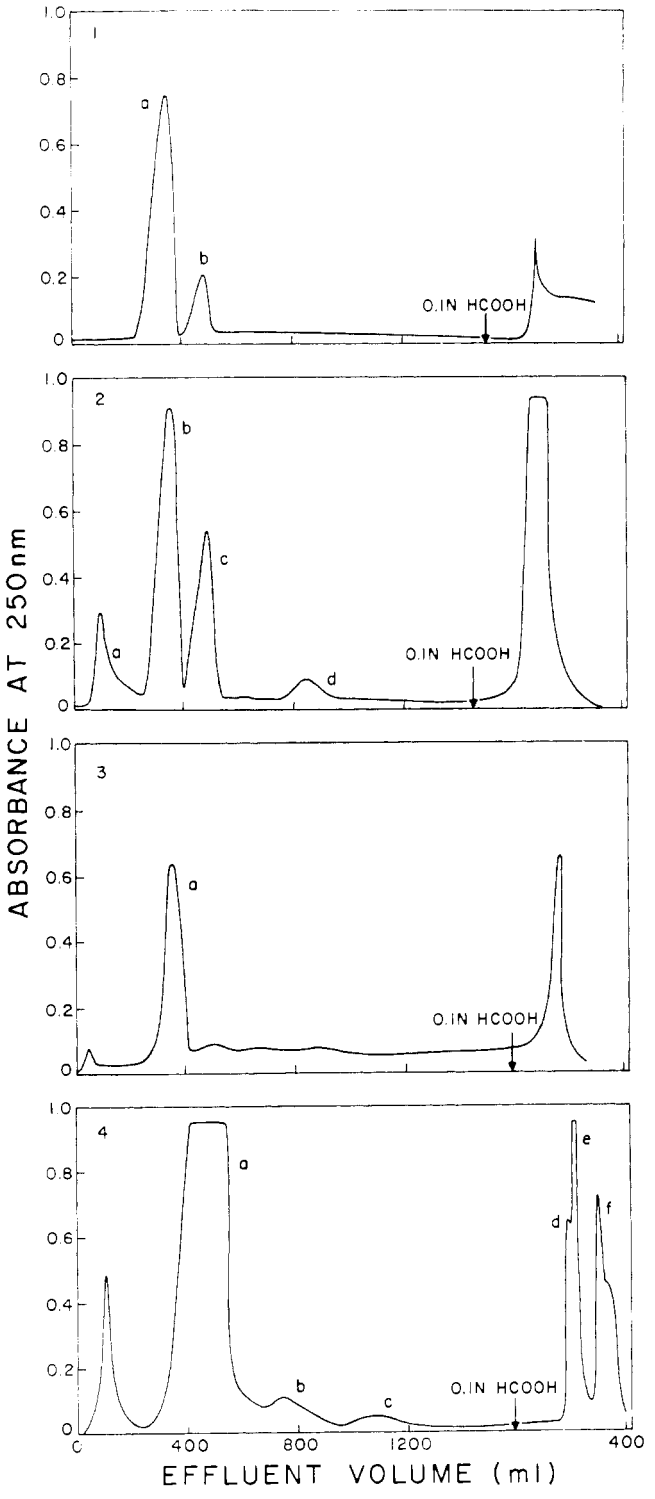


FIGURE 1-4: Elution profiles of cyclobutane-type uracil and uridine dimers on Dowex 1-X8 column. Conditions were as described in Methods. The effluent was continuously monitored by an LKB 4701A Uvicord ultraviolet absorption meter equipped with an LKB 6520A chopper bar recorder. (1) Uracil dimer from frozen solution irradiation of uracil; (2) uracil irradiated in solution in the presence of acetone; (3) uracil dimer obtained from Ud_1 after acid hydrolysis; (4) Ud_1 eluted from paper chromatograms of acetone-sensitized irradiation of uridine.

Column Chromatography. For the separation of cyclobutane-type dimers, a Dowex 1-X8 (formate form) column (2 × 32 cm) was used. For uridine dimers the sample (about 200 mg) was dissolved in 20 ml of 0.1 N NH_4OH and applied on the column. For uracil dimers about 100 mg of the sample was dissolved in about 75 ml of 0.1 N NH_4OH. Elution was performed with a linear ammonium formate gradient (Weinblum and Johns, 1966). The mixing chamber contained 1.25 ml of formic acid, 5 ml of NH_4OH, and 1 l. of water while the reservoir contained 2 ml of formic acid, 7.5 ml of NH_4OH, and 1 l. of water. After 180 10-ml fractions were collected, the column was eluted with 0.1 N formic acid. The effluent was continuously monitored by an LKB 4701A Uvicord ultraviolet absorptiometer equipped with an LKB 6520A chopper bar recorder.

For the purification of 6-(4'pyrimidin-2'-one)uracil and for desalting a Dowex 50W X-12 (H^+) column (30 × 2 cm) was used. An aqueous solution of the sample was applied on the column and was eluted with water. Fractions of 10 ml each were collected.

Detection of Products. From paper chromatograms strips (1-cm wide) were cut out and eluted with water. The ultraviolet absorption spectrum of each fraction was taken. Aliquots from each of these fractions were irradiated for about 2 min at a distance of 4 cm from two germicidal lamps and their ultraviolet absorbance spectra were again taken. If cyclobutane-type dimers were present the absorbance at 260 nm increased after irradiation. To detect uridine hydrate a solution of the sample in 0.1 N HCl was heated in a boiling-water bath for 5 min; an increase in absorbance at 260 nm indicated the presence of uridine hydrate. To detect dihydrouracil and dihydrouridine, chromatograms were sprayed with 0.5 N NaOH, dried at room temperature, and again sprayed with a solution containing 1 g of *p*-dimethylaminobenzaldehyde, 10 ml of concentrated HCl, and 100 ml of ethanol. A yellow color develops almost immediately for dihydrouracil and dihydrouridine (Cline and Fink, 1956). The fractions eluted from the column were also analyzed in an analogous manner.

Uracil Dimers from Uridine Dimers. To convert cyclobutane-type uridine dimers to uracil dimers the samples were heated in 4 N hydrochloric acid for 30 min at 100°. The hydrochloric acid was then removed by repeated flash evaporation. The residue, after extraction with warm methanol to remove any uracil also formed during hydrolysis, was crystallized from water.

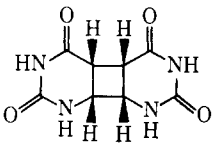
6-(4'-Pyrimidin-2'-one)uracil (V). The methanol extract of uracil irradiated in frozen aqueous solution was evaporated to dryness. The residue dissolved in trifluoroacetic was chromatographed on paper using solvent system A. To isolate V from irradiated uridine, samples containing the uridine–uridine adduct were evaporated to dryness and the residue was hydrolyzed in trifluoroacetic acid at 165° for 60 min in sealed tubes. The hydrolysate was chromatographed on paper as before. From the chromatograms strips (R_F 0.15–0.25) were cut out and extracted with water. The extract was concentrated and chromatographed on the Dowex 50W X-12 column. Those fractions having an absorbance maximum at 305 nm were combined and evaporated to dryness. The residue on recrystallization from hot water deposited yellow needle-like crystals of product V (Khattak and Wang, 1968).

Results

Frozen-State Irradiation of Uracil. When uracil is irradiated in frozen aqueous solution about 65% of uracil is converted into photoproducts. When the irradiated solution was evaporated to dryness and the residue extracted with warm

methanol until the extract showed no absorbance above 250 nm, the methanol-insoluble residue (540 mg from 1 g of uracil) was found to be a cyclobutane-type uracil dimer by its ultraviolet absorption spectrum and photoreversal to uracil. The methanol fraction was evaporated to dryness, the residue was dissolved in trifluoroacetic acid, and was chromatographed on paper using solvent system B. Analysis of the paper chromatogram, as described in Methods, revealed the presence of uracil (R_F 0.50), 6-(4′-pyrimidin-2′-one)uracil (R_F 0.18), and trace amounts of uracil dimer (R_F 0.12). 6-(4′Pyrimidin-2′-one)uracil after further purification by column chromatography and crystallization from water has ultraviolet absorbance maxima at 305 nm (ϵ 10,800) and at 325 (ϵ 12,000) in aqueous solutions at pH 2 and pH 12, respectively. From 1 g of irradiated uracil about 27 mg of V was obtained.

The uracil dimer fraction was resolved into two components, **a** and **b** (Figure 1), by column chromatography as described in Methods. The fractions constituting the individual peaks were pooled and desalted, and the products were recrystallized from hot water. The major product a amounted to about 90% of the uracil dimer and its infrared absorption spectrum was identical with that of cis-syn uracil dimer (I) (Adman *et al.*, 1969; Blackburn and Davies, 1966; Khattak and Wang, 1968).



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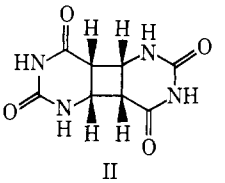

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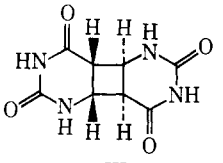

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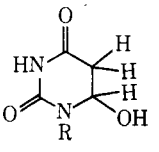

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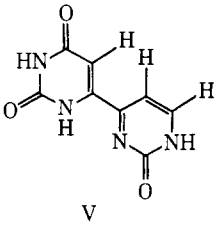

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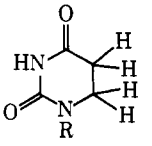

VI

The absence of a maximum above 250 nm in the ultraviolet absorbance spectrum of **1b** and its photoreversibility to uracil indicated that it also is a cyclobutane-type uracil dimer. Recently Konnert *et al.* (1970) have shown that a uracil dimer isolated from frozen-state irradiation of uracil has the cis–anti configuration. Like the cis–anti thymine dimer (Witkop, 1968; Herbert *et al.*, 1969), **b** is unstable to acid. Therefore we conclude that **b** is the cis–anti uracil dimer (II). The cis–anti isomer is expected to occur in 2 optically active forms. The absence of any rotation in optical rotatory dispersion measurements suggested that **1b** is probably a mixture of the two enantiomers in equimolar proportions.

Acetone-Sensitized Irradiation of Uracil. When uracil in solution in the presence of acetone irradiated with light of wavelength > 290 nm, about 85% of the uracil was converted to photoproducts. The irradiated solution was concentrated to about 80 ml on a rotary evaporator at about 40° and filtered.

NH_4OH was added to the filtrate to a final concentration of 0.1 N, which was then chromatographed on a Dowex 1-X8 column, as described in Methods. The elution profile shown in Figure 2 was obtained. The first few fractions contained a product or products with an absorbance maximum at 305 nm. Since the amount of this product was extremely small, characterization was not attempted. The second product (**2a**) has no absorbance maximum above 250 nm and was not photoreversible. It showed identical chromatographic mobilities with an authentic sample of dihydrouracil and produced an immediate yellow color when sprayed with *p*-dimethylaminobenzaldehyde reagent. Therefore we conclude that **2a** is 5,6-dihydrouracil (VI). Analysis showed that fractions **2b**, **2c**, and **2d** are all cyclobutane-type uracil dimers; they were desalted and crystallized from water. The chromatographic mobilities and infrared absorption spectra of **2b** and **2c** were identical with those of cis–syn and cis-anti-uracil dimers obtained from frozen-solution irradiation of uracil.

An infrared absorbance spectrum different from those of I and II suggested **2d** is probably one of the trans isomers (Wulff and Fraenkel, 1961). According to Richter and Fahr (1969), who have synthesized the trans isomers, the trans-syn isomer is quite stable to acid and alkali. Since **2d** was unstable to acid and alkali (half-life less than 1 min in 2 N KOH) we concluded that it is the trans–anti isomer (III). This conclusion was confirmed by direct comparison of this photoproduct with the trans–anti isomer of uracil dimer synthesized by the procedure of Richter and Fahr (1969). The infrared spectrum of the synthesized dimer and the dimer from **2d** were identical. From a typical experiment, about 40 mg of cis-syn, 17 mg of cis–anti, and about 2 mg of the trans–anti isomers were obtained. On elution of the column with formic acid, uracil was the only identifiable product.

The precipitate (310 mg) was washed with hot methanol (to remove any uracil) and was crystallized from water. The ultraviolet absorbance spectrum and photoreversibility to uracil indicated that the crystalline product is uracil dimer, and its infrared absorption spectrum showed that it is mainly the trans–anti isomer. Upon column chromatography, as described above, no cis-syn or cis–anti isomer was detected and less than 10% of the dimeric uracil was recovered. This is in agreement with the fact that the trans–anti isomer is very unstable in alkaline solutions. In 0.1 N alkali, it is probably converted to the corresponding cyclobutane dicarboxylic acid (Witkop, 1968). The properties of the three isomeric uracil dimers are summarized in Table I. When a cyclobutane-type uracil dimer was irradiated (λ 254 nm) in aqueous solution, the maximum conversion of the dimer to uracil was about 60%.

Frozen-Solution Irradiation of Uridine. Analysis of paper chromatograms of uridine irradiated in frozen aqueous solution and developed in solvent B revealed the presence of four products. In the order of chromatographic mobilities in solvent A, they are referred to as Ud_1, Ud_2, Ud_3, and Ud_4 and their properties are summarized in Table II.

Under our irradiation conditions only about 18% of uridine was converted to photoproducts.

On the basis of ultraviolet absorption spectrum, photoreversal to uridine, and conversion to uracil dimer on acid hydrolysis, Ud_1 was identified as a cyclobutane-type uridine dimer. A characteristic property of Ud_2 was its ultraviolet

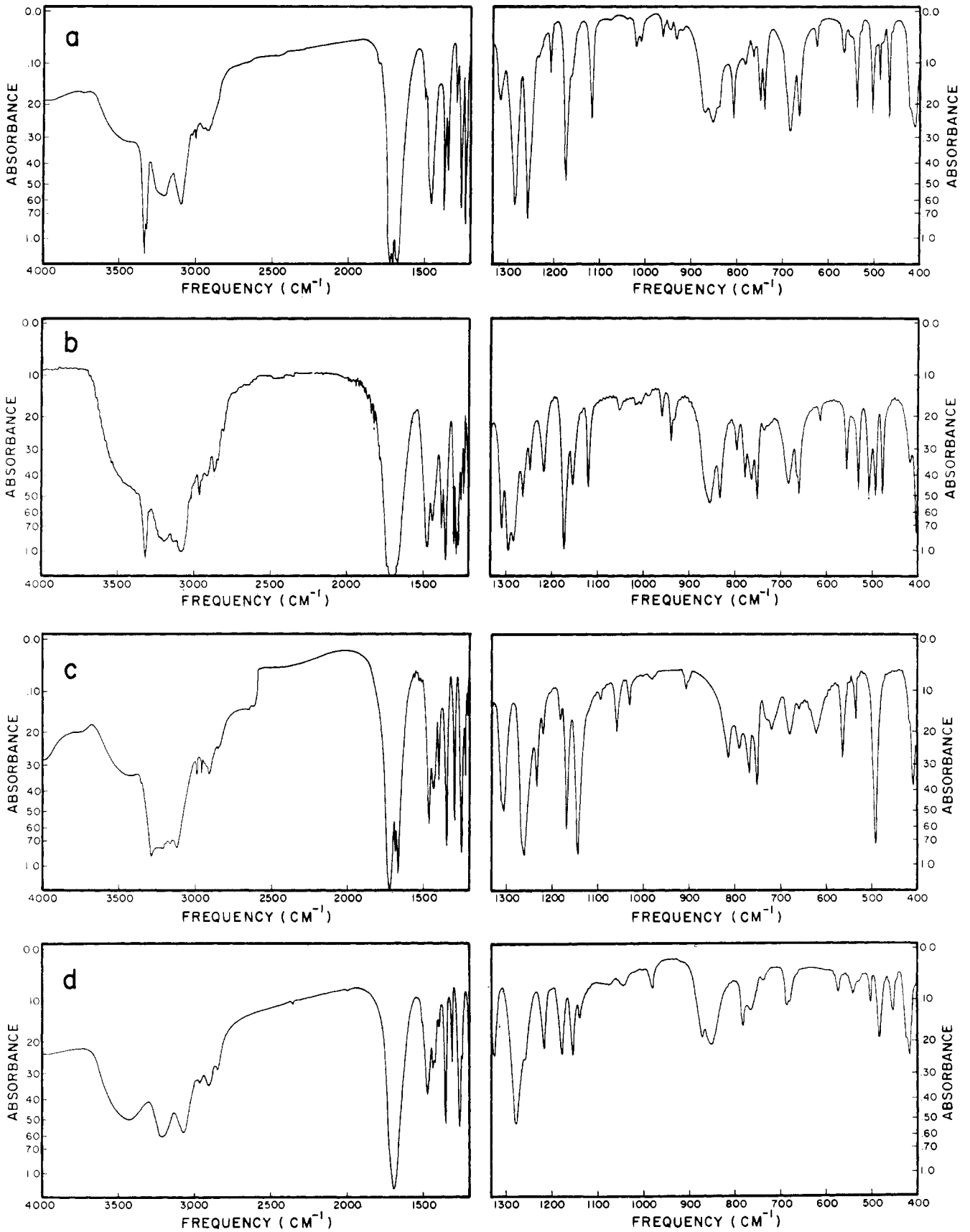

FIGURE 5: Infrared absorption spectra in KBr of (a) stable α form of cis-syn uracil dimer, (b) β form of cis-syn uracil dimer which was obtained from uridine dimer after acid hydrolysis, (c) cis-anti uracil dimer, (d) trans-anti uracil dimer.

TABLE I: Properties of Uracil Dimers.

Uracil dimer isomers	R_F in different solvent systems					Effect of heating at 100%		
	B	C	D	E	F	In water (15 min)	In 4 N HCl (20 min)	In 0.4 N NaOH (1 min)
Cis-syn	0.22	0.24	0.21	0.24	0.31	No change	>5% uracil	30% destroyed
Cis-anti	0.24	0.29	0.25	0.28	0.34	30% uracil	100% uracil	85% uracil
Trans-anti	0.20	0.20	0.20	0.21	0.25	No change	10% uracil	100% destroyed
Uracil	0.52	0.62	0.54	0.57	0.65			

TABLE II: Properties of Uridine Photoproducts.

Photo-product	R_F in solvent A	Ultraviolet absorbance maximum (nm)	Per cent yield under different irradiation conditions			Assigned structure	Method of identification
			Frozen	Solid	Sensi-tized		
Ud_1	0.13	No maximum above 250	15	6	85	Cyclobutane-type uridine dimer	Photoreversibility
Ud_2	0.15	309	1.5	2		Uridine–uridine adduct	PO-U on acid hydrolysis
Ud_3	0.18	260	<0.5	4		Unidentified	
Ud_4	0.30	No maximum above 250	0.6	<0.5		5,6-Dihydro-6-hydroxy-uridine	Heat and acid reversibility, chromatographic mobility

absorbance maximum at 309 nm. Since Ud_2 and Ud_1 showed identical chromatographic mobilities in a number of solvent systems we were unable to isolate a pure sample of Ud_2. Therefore, characterization of Ud_2 was accomplished by identifying its acid hydrolysis product. From paper chromatograms, strips containing Ud_2 were cut and extracted with water. The extract was evaporated to dryness, and the residue was hydrolyzed in trifluoroacetic acid, the hydrolysate being chromatographed on paper using solvent system B. From the chromatograms a product (R_F 0.18) having an absorbance maximum at 305 nm was extracted with water, purified by Dowex 50W X-12 column chromatography, and crystallized from hot water. On the basis of ultraviolet and infrared absorbance spectra (Khattak and Wang, 1968; Varghese, 1971b) the crystalline product was identified as V. Thus the isolation of V from Ud_2 suggest that the latter is a uridine–uridine adduct. The uracil–uracil adduct appears to undergo dehydration more easily than thymine–thymine adduct since uracil–uracil adduct could not be isolated either from frozen-solution irradiation of uracil or from Ud_2 by mild acid hydrolysis.

Ud_3 has an ultraviolet absorbance maximum at 260 nm. Since the amounts obtained were extremely small, characterization was not possible. Ud_4 (R_F 0.30) has no absorbance maximum above 250 nm and was not photoreversible, however, it was converted to uridine when heated in 0.1 N HCl for 5 min. These properties are characteristic of 5,6-dihydro-6-hydroxyuridine (IV), the major product of uridine irradiated in solution. It was also found that IV has an R_F of 0.30 in solvent system A. Hence it was concluded that Ud_4 is IV.

Irradiation of Uridine as a Thin-Solid Film. Irradiation of uridine as a thin-solid film produced the same type of products of frozen-state irradiation. However, the relative amounts differed considerably (Table II). Ud_2 and Ud_3 were formed in larger amounts than in the frozen-solution irradiations. Acid hydrolysis of Ud_3 yielded uracil and two other products. Unlike Ud_1 and Ud_2, the sugar moiety was not completely removed from Ud_3 when it was subjected to trifluoroacetic acid hydrolysis, since even after 2 hr at 165°, Ud_3 eluted from paper chromatograms gave a positive orcinol reaction. Neutral aqueous solutions of Ud_3 showed an absorbance maximum at 260 nm. In the case of labeled samples, the radioactivity per OD at 260 nm was about 3.5 times that of uridine, suggesting that Ud_3 is probably a polymeric uridine photoproduct. However, no definite conclusions could be reached concerning its structure.

Acetone-Sensitized Irradiation of Uridine. Irradiation of uridine in aqueous acetone with light of wavelength > 290 nm resulted in almost 85% conversion to photoproducts. Paper chromatographic analysis (solvent A) showed that the major product was Ud_1.

Analysis of Cyclobutane-Type Uridine Dimers (Ud_1). From paper chromatograms of irradiated uridine developed in solvent B, strips (R_F 0.13) containing the cyclobutane-type dimers (Ud_1) were cut out and extracted with water. The extracts were evaporated to dryness, and the residue was taken up in 0.1 N NH_4OH and chromatographed on a column, as described in Methods. The elution profile for Ud_1 from acetone-sensitized irradiation of uridine is shown in Figure 4.

TABLE III: Analysis of Cyclobutane-Type Uridine Dimers from Different Irradiation Conditions.

Peaks	Percentage under different irradiation conditions			Recoverable uracil dimer after heating in (%) 4 N HCl (30 min)	Assigned structure	Method of identification
	Sensitized	Frozen	Solid			
a	28	64	72	Earlier fractions 20	Cis–anti	Acid stability
				Later fractions 85	Cis-syn	
b	>2	4	8	70		Ir spectrum
c	>2	3	8	75	Cis-syn	Ir spectrum
d	10	9	12	15	Cis–anti(−)	Ir, ORD studies, and chromatographic mobilities
e					Uridine	
f	55	20	None	58	Trans–anti	Chemical synthesis

Analysis, as described in Methods, of the effluent fractions showed that all peaks except **4e**, which was identified as uridine, were cyclobutane-type uridine dimers. The fractions constituting the individual peaks were pooled, concentrated, and desalted. Whenever possible, the uridine dimers were identified by converting them to the corresponding uracil dimers by acid hydrolysis. When the purity of the uracil dimers was examined by thin-layer chromatography, each product developed only one detectable spot. The results are summarized in Table III.

Acid hydrolysis of **4a** yielded a mixture of uracil and cyclobutane-type uracil dimers. When the uracil dimer fraction was chromatographed as described for the separation of isomeric uracil dimers, a main peak corresponding to the cis-syn uracil dimer and a barely observable peak corresponding to the cis–anti isomer were obtained. The identity of the major product was further established by its infrared absorption spectrum. Even though the amount of the minor product was extremely small for positive identification, based on the additional evidence mentioned below, we conclude that it is the cis–anti isomer. When each of the fractions of **4a** was subjected to acid hydrolysis separately, the earlier fractions yielded mainly uracil (about 70%) and the later fractions yielded mainly uracil dimer (about 90%). Since the trans–syn isomer is presumably as stable as the cis-syn (Fahr, 1969), and about 50% trans–anti uridine dimer can be converted to uracil dimer under our hydrolysis conditions, we conclude that **4a** is a mixture of cis-syn and cis–anti uridine dimers.

When **4b** and **4c** were hydrolyzed, about 70% of each were converted to uracil dimer, the chromatographic mobilities and infrared spectrum of which were identical with those of the cis-syn isomer I. One explanation for the presence of three cis-syn uridine dimers is the possibility that such a dimer can exist in three isomeric forms (Fahr, 1969).

When **4d** was subjected to acid hydrolysis only about 10% of it was converted to uracil dimer, the rest being converted to uracil. The stability to acid, alkali, and heat as well as its chromatographic mobilities in different solvent systems indicated that the uracil dimer from **4d** is the cis–anti dimer and optical rotatory dispersion studies (A. J. Varghese and D. M. Gray, unpublished data) showed that it is the levorotatory isomer. However, there were minor differences in the infrared absorption spectra of **1b** and the dimer from **4d**. It should be noted that similar differences between the infrared absorbance spectra, of the cis–anti thymine dimer obtained from sensitized irradiation of thymine and the cis–anti thymine dimer from acid hydrolysate of thymidine dimer, have been observed by Jennings *et al.* (1970).

4f is the major product of sensitized irradiation of uridine. Upon acid hydrolysis about 50% of **4f** was converted to uracil dimer, the infrared absorbance spectrum of which was identical with that of the trans–anti isomer.

Column chromatographic analysis of Ud_1 from frozen-state and solid-state irradiation of uridine gave almost the same results as sensitized irradiation except for the fact that no trans–anti isomer was detected in the case of solid-state irradiation.

Discussion

There are conflicting reports (Smith, 1963) about the stability of uracil dimers isolated from frozen-solution irradiation of uracil. From our studies it is quite clear that most of the published accounts concerning the properties of uracil dimer deal not with a single isomer but with a mixture of the stable cis-syn isomer and the unstable cis–anti isomer. According to Smith (1963) the major dimeric product of uracil obtained from frozen-state irradiation may exist in two physical forms having different chromatographic mobilities and interconvertible by heat, acid, and alkali. We find that the infrared absorption spectrum of the uracil dimer (from frozen-solution irradiation) changes substantially by heating in aqueous solution, and by treatment with alkali. The same property is also exhibited by the uracil cis-syn dimer isolated from uridine irradiation. The infrared absorption spectra of the two forms are shown in Figures 1 and 2. The less stable β form can be converted to the stable α form by heat or alkali. When the α form is subjected to column chromatography, as used for the separation of isomeric uracil dimers, the only detectable product is the stable β form.

According to Konnert *et al.* (1970), a polymeric uracil photoproduct, obtained from frozen-solution irradiation of uracil, has properties quite similar to that of a cyclobutane-type uracil dimer and on recrystallization from hot water yields the cis–anti isomer. We find that the infrared absorption spectrum of **1b** does not change on repeated recrystalliza-

tion, except for the fact that it is gradually converted to uracil on heating. Moreover, since **1b** can also be isolated from acetone-sensitized irradiation of uracil and frozen-solution irradiation of a mixture of thymine and uracil (A. J. Varghese unpublished data), we conclude that it is not a polymeric uracil photoproduct other than cyclobutane-type dimer.

From uracil irradiated insolution in the presence of acetone, Greenstock and Johns (1968) have separated four uracil photoproducts which they have identified as cyclobutane-type dimers. However, these authors have not accomplished the chemical characterization of the four products. From our studies we were able to separate only three uracil dimers in addition to dihydrouracil. It is possible that the discrepancy is due to the differences in the irradiation conditions used. The identification of dihydrouracil as a uracil photoproduct from acetone-sensitized irradiation has been previously reported (Jellinek and Johns, 1970).

Fahr *et al.* (1968) have reported the separation of four uridine dimer fractions from uridine irradiated in frozen aqueous solution. According to these authors, two fractions gave cis-syn uracil dimer on acid hydrolysis while a third one yielded the cis–anti isomer. Our studies show that in addition to these three products a uridine–uridine adduct, uridine hydrate, and trans–anti uridine dimer are also formed.

According to Rosenthal and Elad (1968), irradiation of uridine in acetone produces only one uridine dimer isomer, which on acid hydrolysis gives a uracil dimer different from the cis-syn isomer. We find that at least three isomeric uridine dimers are formed on irradiation of uridine in aqueous acetone.

It has been known for some time that irradiation of pyrimidine derivatives forms product(s) having absorbance maxima above 300 nm (Smith, 1963; Pearson *et al.*, 1965; Haug, 1964). A thymine–thymine adduct isolated from frozen aqueous-solution irradiation of thymine is one product, which has an absorbance maximum at 316 nm (Varghese and Wang, 1968b). The mechanism of formation of this adduct is probably through an oxetane intermediate involving the C=O group of one pyrimidine moiety and the C_5–C_6 double bond of a second one. Recently it has been shown that thymidine forms two such adducts (Varghese, 1970), and irradiation of a mixture of thymine and uracil forms a thymine–uracil adduct (Varghese and Patrick, 1969; Rhoades and Wang, 1970). The isolation of V, the dehydration product of a uracil–uracil adduct from uracil and uridine, suggests that the formation of such biomolecular addition products is as general a photochemical reaction of pyrimidines as dimerization to cyclobutane-type products. In rigid systems, such as solid films, these adducts constitute an important type of product. It is possible that these adducts may have significance in the ultraviolet inactivation of biological systems where the arrangement of pyrimidine bases is quite restricted.

An important aspect of the study of the photochemistry of pyrimidine derivatives is that results from these studies might be useful in understanding the chemical mechanisms involved in the inactivation of biological systems by ultraviolet light. All of the known thymine- and cytosine-derived photoproducts that have been isolated from irradiated DNA have also been isolated from frozen-solution irradiation of thymidine and cytidine or mixtures of the two nucleosides (Varghese, 1970; Varghese, 1971b). Similarly, cyclobutane-type uridine dimers and uridine hydrate, both of which have been identified as photoproducts in irradiated RNA (Small *et al.*, 1968; Merriam and Gordon, 1967), can also be isolated from frozen-solution irradiation of uridine. These facts suggest that irradiation of nucleosides in frozen aqueous solution provides a better model for studying the photochemistry of pyrimidine residues in biological systems than irradiation of the free bases under any conditions.

Since sensitized irradiation is used for the specific production of cyclobutane-type dimers in biological systems (Lamola, 1968; Meistrich *et al.*, 1970), it is of considerable significance to know which isomers are formed. Even though acetone-sensitized irradiation of thymine (Jennings *et al.*, 1970) and thymidine (Ben-Hur *et al.*, 1967) gave rise to all four isomeric dimers, the trans–anti isomer is the major product. Recently we have shown that the trans–anti isomer is the major product of acetone-sensitized irradiation of cytidine (Varghese, 1972). The present study shows that the trans–anti isomer is again the major product when uracil and uridine are irradiated in the presence of acetone. Almost all reports (Mennigmann and Wacker, 1970; Ben-Ishai *et al.*, 1968) on the products of acetone-sensitized irradiation of biological systems are of a qualitative nature and imply that the cis isomer is the major product. In these cases failure to detect the trans–anti isomer might be due to its instability.

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Intracellular Site of Proline Hydroxylation in Plant Cells*

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ABSTRACT: In animal and plant tissues, hydroxyproline is synthesized by the enzymatic hydroxylation of peptidylproline. However, data from several studies on collagen synthesis have not resolved the question of whether this hydroxylation occurs before or after release of the polypeptide from a ribosomal complex. We examined this problem using disks of carrot phloem parenchyma where hydroxyproline-containing proteins are synthesized abundantly. Disks which had incorporated [^{14}C]proline into proteins began to hydroxylate bound [^{14}C]proline 3–4 min after incorporation. The proline-rich polypeptides made during this lag period could be maximally hydroxylated during a 10-min incubation in nonradioactive proline; this is well beyond the time required for release of the chains from a ribosomal complex. Isolation of polyribosomes from [^{14}C]proline-incubated tissue and analysis of labeled polypeptides attached to them showed no significant [^{14}C]hydroxyproline, even though 10% of the cytoplasmic [^{14}C]proline residues had been hydroxylated. Our results indicate that proline hydroxylation in this plant system takes place on completed, released polypeptide chains.

Hydroxyproline occurs in many plant glycoproteins (Pusztai and Watt, 1969) and is especially abundant in the protein component of the plant cell wall (for a review, see Lamport, 1970). This wide distribution contrasts with animal cells, where the imino acid has been found specifically in only two proteins, collagen and elastin (Gross, 1963). However, the scheme for assembly of hydroxyproline-containing macromolecules is similar in both plant and animal cells: the imino acid is synthesized by the enzymatic hydroxylation of peptidylproline (Sadava and Chrispeels, 1971; Rosenbloom and Prockop, 1968); the completed macromolecule is then glycosylated (Chrispeels, 1970a,b; Rosenbloom *et al.*, 1968) and subsequently secreted into the intercellular matrix (Olson 1964; Takeuchi and Prockop, 1969).

A matter of some controversy from studies on collagen biosynthesis is the question of whether proline hydroxylation occurs before or after release of the macromolecule from a protein-synthesizing ribosomal complex. Several laboratories have reported that when collagen-synthesizing tissues were incubated in radioactive proline, polyribosomal complexes which contained radioactive hydroxyproline could be isolated (Manner *et al.*, 1967; Fernández-Madrid, 1967; Goldberg and Green, 1967; Miller and Udenfriend, 1970; Lazarides *et al.*, 1971); in one recent study, no hydroxyproline was found (Bachra and van der Eb, 1970). However, protocollagen-like molecules, containing little or no hydroxyproline, have been isolated after release from the ribosomal complex had occurred (Rosenbloom *et al.*, 1967), indicating that hydroxylation must take place after release. Moreover, if the proline hydroxylase were inhibited for several hours, thus allowing an accumulation of released, nonhydroxylated chains, reversal of this inhibition led to maximal hydroxylation (Juva *et al.*, 1966). These latter two experiments support the hypothesis of hydroxylation after release of a completed polypeptide.

To study this problem in plants, we designed experiments using disks of carrot root. *In vivo* experiments showed that proline hydroxylation could occur after release of the polypeptides from the ribosomal complex. Actual isolation of polyribosomes showed no significant hydroxyproline in the nascent chains.

Materials and Methods

Organism. We performed our experiments on disks (1 cm in diameter and 0.5 mm thick) of phloem parenchyma from

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