Photochemistry of 5-Bromo-1,3-dimethyluracil in Aqueous Solution*

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ABSTRACT: 5,5′-Di-1,3-dimethyluracil (IV), 1,3-dimethyluracil (III), *sym*-dimethyloxamide (II), *sym*-dimethylurea (VI), methylamine, ammonia, 5-carboxy-1,3-dimethyluracil (VII), and acetic acid formed by ultraviolet irradiation (mainly 254 mμ) of 5-bromo-1,3-dimethyluracil in aqueous solution were quantitatively isolated and identified. Based on the isolation of the first two photoproducts, a free radical reaction mechanism was suggested for this photochemical reaction. The homolysis of the C–Br bond to form uracil radicals and bromine atoms may be responsible for the enhanced irradiation sensitization of bromouracil–deoxyribonucleic acid (BU–DNA) *in vivo*, and the coupled product, 5,5′-di-1,3-dimethyluracil, may serve as a model for the study of radiation and photobiology.

Bacteriophages (Stahl *et al.*, 1961; Sauerbier, 1961), bacterial (Greer, 1960; Kaplan *et al.*, 1962) and mammalian cells (Djordjevic and Szybalski, 1960) containing 5-bromouracil or 5-iodouracil in place of thymine in their deoxyribonucleic acid (DNA) are much more sensitive to irradiation than the phages and cells with normal DNA. [The references cited in a previous paper (Ishihara and Wang, 1966) are pertinent to this work.] The mechanism by which this occurs remains unknown. The irradiation sensitization produced by these 5-halogenouracils could be due either to their greater chemical reactivity or to the inability of existing cellular mechanisms to repair the type of damage (irradiation product) produced. In either case, the isolation and identification of the products of halogenated uracils and the study of their chemical mechanisms is essential to a complete understanding of the enhanced irradiation sensitivity.

The photochemical behavior of methylpyrimidines is quite similar to that of their analogs in nucleic acids. Hence, methylpyrimidines have been successfully used as model compounds for chemical studies related to photobiology (Moore and Thomson, 1955; Wang *et al.*, 1956; Wang, 1959a). These chemical studies might also yield information about the effect of methyl groups on the photochemical reactions of pyrimidines. In this study, 5-bromo-1,3-dimethyluracil (I)[1] was used as the model compound. It is hoped that this study will assist our further work with 5-bromouracil, the nucleoside, and the nucleotide.

In a previous paper (Ishihara and Wang, 1966), we have reported the isolation and identification of *sym*-dimethyloxamide (II), 1,3-dimethyluracil (III), and 5,5′-di-1,3-dimethyluracil (IV) from the irradiation of BDMU. The probable mechanism for the formation of DMU and DMU-DMU through common DMU radicals (V) was considered. The possible importance of the free-radical mechanism and the formation of coupled products, such as DMU-DMU, in irradiation and photobiology was discussed. The present paper deals with further studies on the photochemistry of DMU in aqueous medium, including the quantitative determination of five other products which were not reported in the preceding paper.

Experimental Procedures

Melting points were determined on a Fisher-Johns block and are uncorrected. Infrared spectra were measured on a Perkin-Elmer Model 21 recording spectrophotometer. Ultraviolet spectra were measured on a Beckman Model DK-1 recording spectrophotometer. Microanalyses were performed by Mr. J. Walter at the Johns Hopkins University. Paper chromatography was carried out on Whatman No. 1 filter paper unless otherwise stated and the ratios given for the eluents are by volume. Evaporation of the solvents was carried out by a rotary evaporator below 40°.

The irradiation apparatus has been described previously (Wang, 1958). General Electric germicidal

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[1] Abbreviations used in this work: BDMU, 5-bromo-1,3-dimethyluracil; DMU, 1,3-dimethyluracil; DMU-DMU, 5,5′-di-1,3-dimethyluracil; CDMU, 5-carboxy-1,3-dimethyluracil; OH-DMU, 5-hydroxy-1,3-dimethyluracil; DMU·, 1,3-dimethyluracil radical.

tubes (G15T8) which emit mainly 254-mμ wavelength light were used as light source.

Chemically pure samples (BDMU I) prepared according to the method of Wang (1959b) were used [λ_{max}^{HOH} 283 mμ (ε 8.66 × 10³), λ_{min}^{HOH} 241 mμ (ε 1.76 × 10³), mp 184–185°].

Irradiation of BDMU and Partial Fractionation of the Irradiation Mixture

An aqueous solution of BDMU (1 mM, 10 l.) was irradiated for 1.5 hr at room temperature. During irradiation, the absorbancy of the solution at 283 mμ decreased to *ca.* 45% of the original and the pH decreased from 6.0 to *ca.* 3.0.

The irradiated solution was adjusted to pH 9.0 with 0.1 N NaOH solution, and was concentrated to 10 ml. The distillate, or *distillate fraction*, was collected in a condenser containing 10 ml of 4 N HCl.

The solution concentrated to 10 ml as above was extracted continuously with ether for 5 days. The *ether fraction* obtained was kept for the analysis of neutral photoproducts.

The aqueous solution that remained after extraction with ether was evaporated to dryness. The residue, *aqueous fraction*, was retained for identification of the acidic photoproducts.

Isolation and Identification of Photoproducts

Distillate Fraction. A. QUALITATIVE IDENTIFICATION OF THE VOLATILE BASES. The distillate fraction was concentrated to a small volume and chromatographed in solvent systems A [1-butanol–acetic acid–water (4:1:5)] and B [1-butanol–acetic acid–water (2:1:1)] by descending technique. The dried chromatograms were sprayed with ninhydrin (0.1% in acetone). Heating revealed two spots in both systems: a violet spot (R_F 0.38 in system A, 0.60 in B) and a yellow one (R_F 0.23 in A, 0.46 in B) which coincided well with parallel runs using authentic samples of methylamine hydrochloride (violet) and ammonium chloride (yellow), respectively (Alcántara and Wang, 1965). Their identification was established by comparing the infrared spectra of the eluted substances from the chromatograms with authentic samples of methylamine hydrochloride and ammonium chloride.

B. QUANTITATIVE DETERMINATION OF THE TOTAL VOLATIVE BASES. An aqueous solution of BDMU (1 mM, 1 l.) was irradiated for 1.5 hr. The irradiated solution was acidified with 1 ml of concentrated HCl and was then evaporated to dryness. The residue was dissolved in 10 ml of water and aliquots of 3 ml were used for the determination of the total volatile bases according to the modified method of Archibald (1943) and Varner *et al.* (1953). After 20 min of distillation, 2 ml of ethanol was added to the sample chamber. Distillation was continued for 15 min and the content in the receiver was then titrated to pH 5 with 0.01 N HCl. This process was repeated until the content in the receiver showed a pH no greater than 6 (addition of <1 drop of 0.01 N HCl lowered the pH to <5). Each 3-ml aliquot of the sample was titrated with 2.01 ml of 0.01 N HCl, indicating 0.067 mequiv of volatile bases should be formed from 1 l. of 1 mM BDMU solution.

Ether Fraction. The dried residue (983 mg) from the ether extract was chromatographed on an alumina column. Elution of BDMU, *sym*-dimethyloxamide, DMU, DMU-DMU, and an unidentified compound was carried out with anhydrous ether, followed by anhydrous ether containing 0.5, 1.0, and 1.5% of ethanol as reported previously (Ishihara and Wang, 1966).

The sample remaining on the column after elution with 1.5% ethanol in anhydrous ether was washed off with 1 l. of methanol. The methanolic solution was evaporated to dryness, and the organic products were extracted from the residue three times with 10-ml portions of chloroform. After removal of the chloroform, the dried residue, 217 mg, was redissolved in 1 ml of chloroform. This solution was mixed with 1 g of alumina, treated as above, and placed on an alumina column. A shorter column (30 g, 1 × 30 cm) was used in order to facilitate further elution. At a flow rate of about 1 ml/min, fractions of 25 ml were collected. The elution was initiated with 1.5% ethanol in anhydrous ether until tube 55. The combined eluates of the first 21 test tube fractions contained a small amount (2.4 mg) of a mixture of the four compounds identified in the previous fractions; the combined weight from tubes 22 to 55 was 17.2 mg, the identity of the product(s) remaining unknown. The dried residue from fractions 56 to 85 (eluted with 2% ethanol) and from 86 to 115 (3% ethanol) weighed 57.3 mg. Part of this could be sublimed at 50°, 0.05 mm of pressure; the sublimate (25.2 mg) melted at 102°. It was identified by melting point, mixture melting point, and infrared spectrum as *sym*-dimethylurea (VI). Further elution with stepwise increase of ethanol concentration gave several unidentified compounds.

Aqueous Fraction. The solution was extracted continuously with chloroform for 24 hr in order to remove the last trace of neutral products. The residue from the aqueous layer was dissolved in 1 ml of distilled water, acidified with 0.7 ml of 2 N H_2SO_4 with cooling, mixed with 1 g of Celite 535, and quantitatively transferred onto the top of a Celite column (16 g, 0.6 × 45 cm). The column was prepared by mixing 16 g of Celite 535 with 12.5 ml of 0.5 N H_2SO_4 as stationary phase, followed by slurrying with the mobile phase, 1-butanol–chloroform (10:90) (Phares *et al.*, 1952).

The column was eluted at an approximate flow rate of 30 ml/hr with 10% 1-butanol in chloroform up to tube 45, and then, from tube 46 on, with 35% 1-butanol in chloroform. The mobile phase was equilibrated with the same volume of stationary phase prior to use. Fractions of 5 ml, collected in test tubes containing 3 ml of distilled water, were titrated to pH 8.0 with 0.05 N NaOH. During titration, the organic and the aqueous phases were mixed by bubbling a stream of N_2. On the basis of titration, the test tube fractions were combined to form five large fractions. The organic layers of the combined fractions were discarded, and the aqueous layers were evaporated to dryness. The residues were retained for

TABLE I: Titration and Isolation Results.

Fraction No.	Test Tube No.	Net Titer (ml)	Calcd		Found	
			mequiv	mg	mg	Identifn
1	6–12	2.39	0.120	22.5	18.2	CDMU
2	13–18	1.31	0.066	8.9[a]	8.5	Acetic acid
3	24–32	0.40	0.020	—	1.9	—
4	51–61	2.95	0.148	—	15.8	—
5	64–69	0.82	0.041	—	4.7	—

[a] Calculated as $CH_3COONa \cdot 3H_2O$.

identification. Table I summarizes the results (average of three runs).

Fraction 1: 5-Carboxy-1,3-dimethyluracil (VII, CDMU; 1,3-Dimethylisoorotic Acid). The residue was dissolved in 3 ml of water and acidified with 1 ml of 4 N HCl. The solution was extracted three times with 5-ml portions of chloroform. The combined chloroform extracts were evaporated to dryness. The residue obtained was dissolved in 5 ml of hot water and decolorized with a small amount of charcoal. The filtrate, concentrated to *ca.* 0.5 ml, on cooling yielded 18.2 mg of colorless crystals (mp 190–192°). After recrystallization from water, 15 mg of colorless needles (mp 193.5–194°) was obtained. Infrared and ultraviolet spectra were identical with those of the authentic CDMU (Alcántara and Wang, 1965).

Fraction 2: Acetic Acid. A portion (*ca.* 5 mg) of the residue was dissolved in 1 ml of water and acidified with 1 ml of 2 N H_2SO_4 with cooling. The mixture was heated to boiling, and the distillate was collected in an ice-cooled receiver containing 4 ml of 4 N NH_4OH. After 10 min, the content in the receiver was evaporated to dryness and the residue was chromatographed. Single spots obtained in two solvent systems, in 1-butanol saturated with 1.5 N NH_4OH (R_F 0.11) (Reid and Lederer, 1952) and in 95% ethanol–concentrated NH_4OH, 100:1 (R_F 0.34) (Kennedy and Barker, 1951), had the same R_F values as those of an authentic sample of ammonium acetate of parallel runs. The infrared spectrum of the sodium salt of the acid was identical with the spectrum of an authentic sample of sodium acetate.

Fractions 3–5: Unidentified Acids. The amounts of these fractions, based on titration and weighing, are given in Table I. However, attempts to characterize these compounds were unsuccessful.

Experiments Related to Mechanistic Study

The Absence of 5-Hydroxy-1,3-dimethyluracil (VIII, OH-DMU) in the Irradiated Solution. A. METHOD. An aqueous solution of OH-DMU becomes bright blue immediately after the addition of a few drops of ferric chloride solution (0.5% in acetone) at room temperature. Although the color disappears gradually, it was estimated that 100 μg/ml of OH-DMU could be detected unequivocally. This color test apparently is specific for 5-hydroxy compounds because it gives also a positive reaction for 5-hydroxyuracil and negative tests for the starting material, BDMU, and its photoproducts such as DMU and DMU-DMU up to a concentration of 20 mg/ml. 1,3-Dimethylbarbituric acid (10 mg/ml) becomes light gold-brown.

This color reaction was also used on paper chromatograms by spraying ferric chloride reagent onto the dried paper sheet. The sensitivity of this test is 10 μg/spot.

B. PROCEDURE. Dried ether extract (1050 mg) from 10 l. of irradiated solution was dissolved in 10 ml of hot water. While hot, the solution was divided into two equal portions for the following experiments.

One 5-ml portion was cooled in ice water. After 2 hr the crystalline material (chiefly BDMU) was removed by filtration and the filtrate was applied on six sheets of Whatman No. 3MM paper (23 cm in width) to develop with isoamyl alcohol saturated with water by ascending technique. Most of the colored material remained at or near the origin after 24 hr of development. The area corresponding to OH-DMU (R_F 0.40) was cut out and the substances were eluted three times with 100-ml portions of hot water. The combined eluates were evaporated to dryness, and the residue was dissolved in 3 ml of hot ethanol. After cooling in ice water for 2 hr, crystalline material (chiefly DMU-DMU) was removed by suction filtration. The filtrate was then rechromatographed on one sheet of Whatman No. 1 paper as above. Again, the area corresponding to OH-DMU was cut out and eluted three times with 20-ml portions of hot water. After evaporation to dryness, the remainder was taken up in 1 ml of water. To a 0.5-ml aliquot of this solution was added 2 drops of the ferric chloride solution and the other 0.5-ml portion was spotted on a paper strip for chromatography as above. The dried chromatogram was sprayed with ferric chloride solution. In both cases, the color tests were negative.

C. CONTROL TEST. To the other 5-ml portion of the sample solution was added 200 μg of OH-DMU while hot, and the resulting solution was treated in an identical manner as above. The ferric chloride test was posi-

tive both in solution and on the chromatogram.

Test for Aldehydes in the Irradiated Solution. The concentrated irradiated solution without any treatment was mixed with an equal volume of saturated 2,4-dinitrophenylhydrazine solution in 2 N HCl. However, no yellow or orange phenylhydrazone precipitate was obtained.

The Irradiation of DMU-DMU (IV). An aqueous solution of DMU-DMU (0.3 mM, 100 ml) was irradiated for 1.5 hr in the usual manner. The irradiated solution was evaporated to dryness. The residue was dried over P_2O_5 *in vacuo* and was taken up in 1 ml of chloroform, 0.3 ml of which was applied on a paper strip and developed with 1-butanol–0.25 N NH_4OH (86:14) by descending technique. The starting material appeared as a wide heavy band (R_F 0.6–0.3 region), while two very faint bands were located near the origin with R_F values of 0.08 and 0.05, respectively. The dried chromatogram was redeveloped three times with the same solvent to separate the two bands near the origin more clearly. The compound having an R_F value of 0.05 was tentatively identified as CDMU by comparing the chromatographic mobility with an authentic sample. After elution with water, the quantity was estimated by its ultraviolet absorbancy to be *ca.* 0.2% of DMU-DMU. The identity of the other band (R_F value, 0.08) was not clear

Results and Discussion

Our preliminary spectroscopic study indicated that BDMU and 5-bromouracil undergo similar photochemical changes with even biological doses of ultraviolet (Ishihara and Wang, 1966). Also, it is well known that halogen atoms are split by the action of visible or ultraviolet light from carbonyl compounds (Gould, 1959). Thus, one would expect that the absorption of 254-mμ light gives the BDMU molecules sufficient energy to break the C–Br bonds forming uracil radicals (DMU·) and bromine atoms.

Generally, radical reactions result in the formation of many products. Since this is a radical reaction, one would expect the photochemical changes of BDMU to give a number of photoproducts. In fact, this was found to be the case, so that it was necessary to separate the mixture into several fractions prior to the isolation of individual compounds. After a number of preliminary experiments, the fractionation method described in the experimental details was used.

Volatile Bases. The decrease in pH during irradiation indicated the formation of acidic photoproducts. The pH of the solution was adjusted to 9 with 0.1 N NaOH in order to prevent the loss of any volatile acids during evaporation, and later, to facilitate the separation of their sodium salts from the neutral photoproducts.

The *distillate fraction* collected in HCl solution contained volatile bases which were characterized as methylamine hydrochloride and ammonium chloride. The formation of ammonia is probably a secondary process resulting from the demethylation of methylamine (Michael and Noyes, 1963; Alcántara and Wang, 1965), which should be a photodecomposition product of BDMU. The volatile bases amounted to 0.067 mequiv and, when calculated as methylamine, 2.08 mg would be obtained from 1 mmole of BDMU.

Neutral Products. Neutral photoproducts from the ether fraction were separated on an Alumina column. The starting material, BDMU, was recovered from the first fraction in *ca.* 12% of the starting weight. It would seem, then, that 88% of BDMU should have undergone photochemical changes. However, the observed spectral decrease was only *ca.* 55%. This discrepancy apparently is due to the fact that some photoproducts have an ultraviolet absorbancy similar to that of the starting material. Consequently, conclusions based solely on spectral change can be misleading. Estimation of the quantum yields or interpretation of photochemical reactions requires additional knowledge of the photoproducts and reaction mechanisms (Wang, 1962).

The identification of DMU and DMU-DMU serves as evidence for the free-radical mechanism. DMU must be formed through radical displacement, *i.e.*, the DMU· abstracts H· from surrounding organic molecules (discussed below). DMU-DMU must be formed through radical coupling, *i.e.*, the combination of two DMU· radicals. Since these compounds should be two of the primary products, the other products may be considered as secondary from these primary products.

sym-Dimethyloxamide (II) must have been formed from 1,3-dimethylparabanic acid (IX) by decarbonylation. Both oxamide and parabanic acid were reported as photoproducts from extensively irradiated uracil (Conrad, 1954). The formation of parabanic acid derivatives from a six-member ring compound by contraction, however, is not well understood and is a subject of interest to us.

The isolation of *sym*-dimethylurea suggests the cleavage of the bonds between N_1–C_6 and N_3–C_4. Since the bond energy of C–N is rather low (47 kcal/mole), this is likely to occur.

Acidic Products. The acidic photoproducts were separated on a Celite 535 column according to the method of Phares *et al.* (1952) which was used successfully for the separation of acidic photoproducts from 1,3-dimethylthymine in this laboratory (Alcántara and Wang, 1965). Acetic acid is one of the two acidic photoproducts thus far identified. Since the molecular structures of BDMU and DMU do not contain the CCH_3 group, the formation of acetic acid requires an unstable intermediate, such as malonic acid which decarboxylates easily to give acetic acid.

The isolation of CDMU was indeed unexpected. This requires the addition of one carbon atom to the ring structure. One of the conceivable pathways is the photodecomposition of intermediates with a two carbon atom or longer side chain at position 5 of the pyrimidine ring. In this regard, DMU-DMU seems to be a likely candidate. However, the irradiation of this coupled product resulted in the formation of only 0.2% of CDMU, while under the same irradiation condition, the ratio of CDMU/DMU-DMU in the irradiated

TABLE II: Products from Irradiation of BDMU.

Fraction	Product	mg	mequiv
Ether	BDMU (I)	258.5	1.2
	sym-Dimethyloxamide (II)	13.5	0.11
	DMU (III)	135.2	0.97
	DMU-DMU (IV)	75.1	0.27
	sym-Dimethylurea (VI)	25.2	0.29
Distillate	Methylamine	20.8	0.67
	Ammonia	as CH_3NH_2	
Aqueous	CDMU (VII)	18.2	0.12
	Acetic acid	8.5	0.07
		as $CH_3COONa \cdot 3H_2O$	
	Aldehyde	No 2,4-D precipitate	
	5-Hydroxy-DMU (VIII)	Not detected by ferric chloride test	

solution of BDMU was *ca.* 25%. These results clearly suggest that DMU-DMU is not an intermediate in the formation of CDMU. In the absence of other intermediates with substituents on C-5, the alternative pathway is the CO_2 fixation on DMU. Indeed, this type of fixation has recently been reported in a similar system (Getoff, 1965).

Furthermore, if CDMU is formed by photooxidation, 5-formyl-1,3-dimethyluracil should be present as an intermediate. In a similar case, it was shown that the photooxidation of thymine derivatives resulted in the formation of CDMU and 5-formyl derivative (Alcántara and Wang, 1965). Therefore, the absence of 5-formyl-1,3-dimethyluracil in the irradiated solution of BDMU renders the photooxidation hypothesis even less likely.

In view of our concern with the photochemistry of nucleic acids and the reaction mechanism, a question arose as to whether DMU· radicals react with the solvent HOH. The formation of DMU-DMU and CDMU indicated that DMU· radicals do not react appreciably with water. Otherwise, DMU would probably be the only intermediate since there is an abundance of solvent molecules. On the other hand, DMU is one of the major products under this experimental condition. If DMU· radicals should abstract H· from water, there would be a resulting formation of HO· which would in turn couple with DMU· radicals to form OH-DMU. The absence of this hydroxy compound, as evidenced by the negative ferric chloride test, suggests that water does not participate in this chain reaction. Considering the irradiation of DNA containing 5-bromouracil, the absence of water participation would favor the formation of coupled products of the bases as the terminal step. The possible formation of interstrand coupled products could explain the finding that BU-DNA shows an enhanced ability for interstrand linking (Opara-Kubinska *et al.*, 1963). Accordingly, the significance of the formation of coupled products should be examined in biological systems.

Scheme I

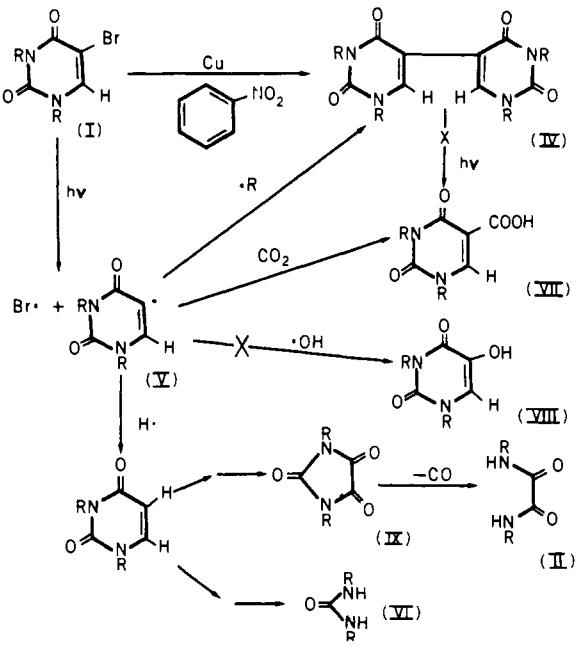

Table II and Scheme I summarize the above results. Note that the amounts of the products are expressed from 10 mmoles or 2.19 g of BDMU. In considering the yields of these products, one must be aware that besides the BDMU recovered, one Br atom should also be subtracted from the original weight. This is because all the photoproducts do not contain Br. In addition, decarbonylation, condensation, decarboxylation, etc., would further decrease the weight of recoverable material. The yield, therefore, could not be ascertained without a more complete understanding of the reaction.

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Photochemistry of 5-Bromouracil in Aqueous Solution*

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ABSTRACT: 5,5′-Diuracil, uracil, glyoxaldiurene, barbituric acid, oxalic acid, isoorotic acid, parabanic acid, urea, ammonia, and glyoxal formed by the ultraviolet irradiation (mainly 254 mμ) of 5-bromouracil in aqueous solution were quantitatively isolated and identified. The photochemical process is therefore a free radical reaction and both 5,5′-diuracil and uracil are formed through the uracil radicals as in the case of photolysis of 5-bromo-1,3-dimethyluracil, albeit their secondary products are different. 5,5′-Diuracil type of coupled products may be of importance in radiation and photobiology.

In a previous paper (Ishihara and Wang, 1966a), we have reported the isolation and identification of 5,5′-diuracils as photoproducts from the irradiation of 5-bromouracil derivatives. This type of coupled product results from the formation of a single bond between two uracil radicals. The possible importance of the formation of coupled products between purines and pyrimidines in deoxyribonucleic acid (DNA) molecules was discussed in relation to radiation and photobiology.

The preceding paper (Ishihara and Wang, 1966b) deals with studies on the photochemistry of 5-bromo-1,3-dimethyluracil (BDMU)[1] in aqueous medium, including isolation, identification, and quantitation of nine compounds from this irradiation reaction. Also reported was evidence for the possible absence of any aldehydes or 5-hydroxy-1,3-dimethyluracil as photoproducts. Actually, BDMU has been used as a model compound in order to facilitate our further work with biologically important compounds such as 5-bromouracil (BU), its nucleosides, and its nucleotides.

This paper describes our studies of the photochemistry of BU in aqueous solution, including the isolation and identification of 11 compounds in the irradiation mixture. In addition, there is evidence for the possible absence of four conceivable photoproducts which are of importance in considering the photochemical mechanisms. In general, both BDMU and BU form free radi-

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[1] Abbreviations used in this work: BDMU, 5-bromo-1,3-dimethyluracil; BU, 5-bromouracil; U-U, 5,5′-diuracil; DMU-DMU, 5,5′-di-1,3-dimethyluracil.