Sensitized Photooxidation of Histidine and Its Derivatives. Products and Mechanism of the Reaction*

Motowo Tomita, Masachika Irie,† and Tyunosin Ukita

ABSTRACT: In connection with the chemical modification of protein, the photooxidation of histidine and *N*-benzoylhistidine was investigated. It was confirmed that histidine is photooxidized to aspartic acid and urea *via* several intermediate compounds. The isolation of the intermediates as pure compounds, however, was difficult because of their instability during the purification. In the photooxidation of *N*-benzoylhistidine also, besides the final products, many kinds of intermediate products were detected and among them 17 compounds were isolated in pure state and characterized. The structures of five compounds were determined, and those of other five compounds were proposed. The same products were obtained when riboflavin or rose bengal instead of methylene blue was used as the photosensitizer in the photooxidation of *N*-benzoylhistidine though their relative yields were slightly different. The reaction of the photooxidation of *N*-benzoylhistidine was explained by the following proposed mechanism given in Scheme I. Thus, the substrate was first converted into a cyclo peroxide by a 1,4-cycloaddition of the singlet oxygen. The cyclo peroxide ring then was ruptured to give the hydroxyl compounds such as XXI and XXII.

The subsequent nucleophilic additions at the C_4 or C_5 position of XXI or XXII, respectively, gave several intermediate compounds such as, III and XXIII, or dimers; XV (XVI) or XI (XII). Further photooxidation of the *N*-benzoylhistidine moiety of some of these dimers might give rise to monomeric compounds which were finally converted into *N*-benzoylaspartic acid and *N*-benzoylasparagine, as is discussed in the text.

It is well known that some of the amino acid residues such as histidine, tryptopan, methionine, and tyrosine in a protein can specifically be oxidized by the irradiation of visible light in the presence of a suitable photosensitizer (Weil *et al.*, 1951; Mclaren and Shugar, 1964; Yamagata *et al.*, 1962; Rippa and Pontremoli, 1968; Hoffee *et al.*, 1967), and that this reaction does not cause the cleavage of the peptide bond (Kenkare and Richards, 1966). The photooxidation of histidine residues in enzyme proteins has occasionally been applied to the investigation of the reaction mechanism of the enzymes (Weil *et al.*, 1951; Westhead, 1965; Martinez-Carrion, 1967). However, the mechanism of the photooxidation of histidine residue in a protein and even of the monomeric histidine has not precisely been elucidated yet.

With respect to the photooxidation of histidine, it has been reported that aspartic acid and urea were the final products of the reaction (Hara, 1960). The facts that the amino acid analysis of a photooxidized protein which contained histidine residues did not show any increase in amount of aspartic acid and that Kenkare and Richards (1966) detected two unknown products in the amino acid analysis of photooxidized ribonuclease A suggested a complicated reaction mechanism which should involve several intermediate compounds before reaching the final products. The present authors carried out a precise investigation on the reaction mechanism of the photooxidation of histidine and its derivatives and obtained several new observations which will be reported in this paper. Histidine, *N*-benzoylhistidine, and *N*-benzoylhistidinamide were used as the substrates of the photooxidation, and among them, *N*-benzoylhistidine was found to be the most suitable compound for the investigation of the reaction mechanism, because the products obtained from this compound could be detected by ultraviolet absorption and easily be extracted with organic solvents. In this paper, the properties and structures of some of the intermediates and final products, together with a precise kinetic study of the total photooxidation of *N*-benzoylhistidine will be described.

The paper also describes the differences in the rate of productions of the intermediates and final products observed when the photosensitizer was altered from methylene blue to rose bengal. The observed results will be discussed with respect to the different efficiencies of these two photosensitizers to produce $^1\Delta_g$ state and $^1\Sigma_g^+$ state singlet oxygens (Kearns *et al.*, 1967; Foote, 1968).

Experimental Section

All melting points were uncorrected. Infrared absorption spectra were measured by a Model DS 402-G spectrometer (Japan Spectroscopic Co. Ltd.) and ultraviolet absorption spectra were taken by a Cary spectrophotometer Model 11. Nuclear magnetic resonance spectra were taken in dimethyl sulfoxide-d_6 using a JNM-3H-60 spectrometer (Japan Electron Optics Laboratory) with tetramethylsilane as an internal standard. Mass spectra were measured by a JMS-OIS spectrometer (Japan Electron Optics Laboratory). The pK_a values were measured by a Radiometer Automatic titrator type TTTIc.

Materials. L-Histidine was obtained from Ajinomoto C. Ltd.; methylene blue, riboflavin, and rose bengal were pur-

* From the Faculty of Pharmaceutical Sciences, University of Tokyo, Tokyo, Japan. *Received June 25, 1969.* A preliminary communication is by Tomita *et al.* (1968).

† Present address: Faculty of Pharmaceutical Sciences, University of Kyoto, Kyoto, Japan.

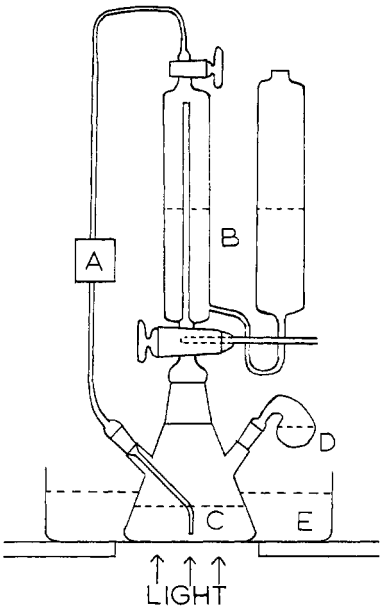


FIGURE 1: Apparatus for estimation of oxygen uptake. (A) Aeration pump, (B) gas buret, (C) reaction solution, (D) photosensitizer, and (E) water bath.

chased from Wako Pure Chemical Industries Ltd. and used without further purifications. *N*-Benzoylhistidine and *N*-benzoylhistidinamide were synthesized by the methods of Fischer (1899) and Bergmann *et al.* (1939), respectively.

Photooxidation Procedure. A solution (300 ml) containing 36 mmoles of L-histidine (or its derivatives) and 8 mg of photosensitizer was adjusted to pH 11.0 with 2 N NaOH unless otherwise indicated. The solution was photooxidized at 35–40° in a beaker. Aeration was effected by rapid stirring of the solution during the reaction. The illumination was provided by a 300-W tungsten lamp (Iwasaki Electric Co.) at a distance of 13 cm above the surface of the solution.

Estimation of Oxygen Consumed. The apparatus used for the photooxidation is illustrated in Figure 1. A solution of 18 mmoles of *N*-benzoylhistidine in 150 ml of H_2O was adjusted to pH 11.0 with 2 N NaOH solution and placed in the reaction vessel (a three-necked flat-bottomed flask of 300-ml volume). The illumination was performed at 20–23° for 30 min in an oxygen atmosphere. A solution of 8 mg of photosensitizer in 5 ml of H_2O was placed in the side arm D. The reaction was started by addition of this solution into the reaction vessel. This was performed by turning the side arm up. Starting from the time of addition of the sensitizer, the consumption of oxygen was read on the gas buret. This apparatus was used only for oxygen consumption experiment.

Paper electrophoresis was performed in 0.02 M phosphate buffer (pH 6.2) on Toyo Roshi No. 53 paper at 20 V/cm using ε-mono-DNP-Lys and DNP-Gly as markers. Each 20-μl aliquot of the irradiated solution was applied on the paper and run for 2 hr. The spots of the products and *N*-benzoylhistidine were detected by ultraviolet absorption. For the quantitative determination, the products were extracted from the spots with water and estimated spectrophotometrically at 230 mμ using a Beckman spectrophotometer.

Paper Chromatography. The paper chromatography of the photooxidized mixture was performed ascendingly on Toyo Roshi No. 53 paper in the following solvent systems: 2-propanol–concentrated ammonium hydroxide–water (8:2:1) (solvent 1) and 1 M ammonium formate–ethanol (3:7) (solvent 2). *N*-Benzoylhistidine and its photooxidation products were located by scanning over an ultraviolet lamp. The starting material was distinguished from the photooxidation products by Pauly's reagent.

Separation of the Reaction Mixture into Groups A *and* B. The photooxidation mixture of *N*-benzoylhistidine (8.0 g) was treated with active charcoal to remove the photosensitizer, and the mixture was filtered. The filtrate was applied to a column (2.7 × 25 cm) of Dowex 50W-X4 (H^+) (20–50 mesh) and the column was eluted with water to obtain the effluent containing acidic and neutral compounds (group A). The column was then eluted with 2 N NH_4OH and the effluent which contained basic compounds (group B) was collected. Each effluent was concentrated by evaporation *in vacuo* at room temperature and the concentrated solution was lyophilized.

Ion-Exchange Column Chromatographies of Groups A *and* B *on Dowex 1-X4.* An aqueous solution of 5.04 g of group A, which was adjusted to pH 9.0 with 2 N NaOH solution, was applied to a column (2.7 × 29 cm) of Dowex 1-X4 (formate) (200–400 mesh). The column was eluted with a linear gradient of 1–3 M HCOOH, and each 8.5-ml fraction was collected. An aliquot (10 μl) of each fraction was taken and diluted with 5.0 ml of water, and the optical density at 230 mμ of the solution was measured. The combined fractions of the peaks separated were concentrated to approximately 50 ml by evaporation *in vacuo* at room temperature and subsequently lyophilized to remove the solvent. The product was recrystallized from methanol or aqueous methanol. The group B was similarly separated by column chromatography as group A except that the column was eluted with a linear gradient of 0–2 M HCOOH.

Column Chromatography on Cellulose Powder of the Mixture of Compounds III and IV. The mixture of III and IV (500 mg) which was obtained by Dowex 1 column chromatography of group A was dissolved in a small amount of a solvent: 2-propanol–concentrated NH_4OH–H_2O (10:1:2), and the solution was applied to a column (3.0 × 41 cm) of cellulose powder. The column was eluted with the same solvent as above. Each 2.9-ml fraction was collected and the amount of the products contained in each fraction was estimated by spectrophotometry at 230 mμ. Each fraction obtained was evaporated *in vacuo* at room temperature and the residue was dissolved in water. The solution was adjusted to pH 2.2 by addition of Dowex 50W-X4 (H^+), and filtered, and the filtrate was evaporated *in vacuo* to dryness.

Separation of Group A *Compounds by Silica Gel Column Chromatography.* The group A compounds (5.0 g) were dissolved in 15 ml of methanol, and to the solution was added 5.0 g of silica gel. The suspension was well mixed and from the mixture, methanol was evaporated *in vacuo*. The residue was ground well and applied on the top of a column (2.7 × 20 cm) of silica gel. The column was eluted stepwisely by successive use of solvents: chloroform–ethyl acetate (1:1), ethyl acetate, ethyl acetate–acetone (1:1), and acetone. During each elution, every 50-ml effluent was taken and the amount of the products contained in the effluent was determined spectrophotometrically at 230 mμ. Product I, VIII, and XVII were eluted with ethyl acetate, II, III, and IV with

ethyl acetate–acetone, and V and VI with acetone. In this column chromatography, the recovery in weight of the fractionated compounds was 85% of the material applied.

Quantitative Determination of N-Benzoylhistidine and the Reaction Products. An aliquot (100 μl) of the irradiated solution was applied to an analytical column (1.0 × 2.0 cm) of Dowex 50W-X4 (H^+) (200–400 mesh). Group A was eluted with about 20 ml of water, and group B with 20 ml of 2 N NH_4OH. The both groups were adjusted to 25 ml with water and the products in each group were estimated spectrophotometrically at 230 mμ. The content of *N*-benzoylhistidine in the irradiated mixture was determined by Pauly's coloration as modified by Macpherson (1946).

The total yield of the two products, V and VI, which colored with Ehrlich's reagent (Fink *et al.*, 1956) was determined by the following method: An aliquot (100 μl) of the irradiated solution was added to 1 ml of Ehrlich's reagent, and the mixture was allowed to stand for 24 hr at room temperature. To the solution was added 3 ml of methanol and the absorbance was determined at 640 mμ.

Methylation of I, II, IV, V, and XIV. The product (I, II, IV, V, or XIV) was treated with diazomethane in dimethylformamide and the solvent was evaporated *in vacuo* at 40°. The methylated compound (I′, II′, IV′, V′, or XIV′) was recrystallized from ligroin (I′), acetone (IV′), or methanol (II′, V′, and XIV′).

Preparation of XVIII from XVII. The product XVII (300 mg) was dissolved in absolute methanol and the solution was saturated with dry hydrogen chloride on an ice bath. After the solution was allowed to stand for 12 hr at 5°, methanol was evaporated *in vacuo* at room temperature and the residue was applied to a column (1.1 × 20 cm) of silica gel. The effluent obtained by elution with ethyl acetate was evaporated *in vacuo*, and the residue was recrystallized from methanol.

Decomposition of XI, XII, XIII, XIV, or XV with HCl or NaOH. Each 250 mg of XI, XII, XIII, XIV, or XV was dissolved in 50 ml of 0.1 N HCl (or 1 N NaOH) in a 50-ml volumetric flask at room temperature. The solution was warmed at 95° on a water bath. At various time intervals, 100-μl aliquots of the solution were applied to the analytical columns (1.0 × 2.0 cm) of Dowex 50W-X4 (H^+). The column was washed with 20 ml of water to elute acidic and neutral compounds, and subsequently eluted with 20 ml of 2 N NH_4OH to obtain basic compounds. The both effluents were adjusted to 25 ml with water and estimated spectrophotometrically at 230 mμ. The contents of *N*-benzoylhistidine in the reaction mixture at various time intervals were determined by Pauly's coloration as modified by Macpherson (1946).

Results

Characterization of the Photooxidation Products of Histidine. As a preliminary experiment, photooxidation was performed using 300 ml of aqueous solution containing 9 mmoles of histidine. The products were analyzed by paper chromatography and paper electrophoresis. In the early stage of the reaction, at least three new photooxidation products other than aspartic acid and urea were detected, the spots of which colored pink with ninhydrin reagent. The mobilities of these spots in paper electrophoresis (at pH 6.2) fall in between those of monobasic acid and neutral compound. All the efforts to isolate these products as pure compounds, however, failed due to their instability during the purification. Aspartic acid and urea as final products were detected on the paper chromatography by spraying ninhydrin reagent and Ehrlich's reagent (Fink *et al.*, 1956), respectively. The yield of aspartic acid began to increase after 6-hr photooxidation with simultaneous decrease of the above-mentioned intermediate products. After 10-hr irradiation and successive hydrolysis of the photooxidation mixture with HCl, aspartic acid was obtained in a yield of 36%. These observations indicated that histidine was photooxidized to give aspartic acid *via* several unstable intermediate compounds.

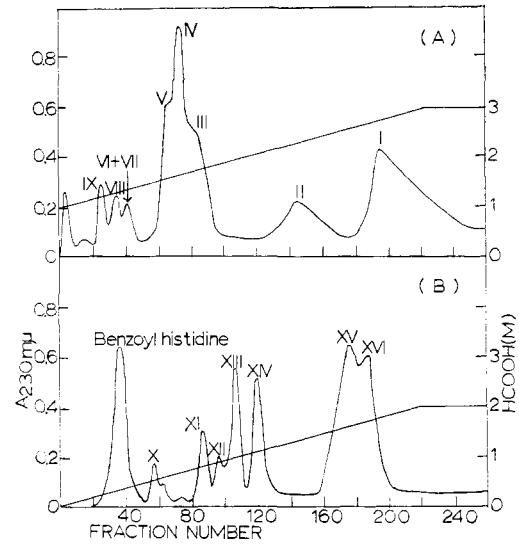


FIGURE 2: Separations of the products belonging to groups A and B by Dowex 1-X4 (formate) column chromatography. (A) Group A compounds (5.0 g) prepared from 6-hr photooxidation mixture of *N*-benzoylhistidine were loaded on a Dowex 1-X4 (formate) column and eluted by a gradient of increasing formic acid concentration. Column size, 2.7 × 29 cm. Fractions, 8.5 ml each. (B) Group B compounds (5.0 g) prepared from 6-hr photooxidation mixture of *N*-benzoylhistidine were applied to a Dowex 1-X4 (formate) column and eluted as described in part A. Column size, 2.7 × 29 cm. Fractions, 10.0 ml each.

Isolation of the Photooxidation Products of N-Benzoylhistidine. As it was found that the photooxidation products of histidine other than aspartic acid and urea were unstable and difficult to be isolated, *N*-benozylhistidine was used as the starting material of the photooxidation in order to isolate the intermediate compounds and determine their chemical structures. The photooxidation products of *N*-benzoylhistidine were separated by ion-exchange column chromatography using Dowex 50W-X4 (H^+) into two groups, groups A and B. The group A contained the products having no basic residues and group B the products which had basic residues. The compounds involved in group A were further separated by column chromatography through Dowex 1-X4 (formate) and the chromatographic pattern is given in Figure 2.

In a spearate experiment, the group A was chromatographed through silica gel by stepwise elution with different organic solvents. With ethyl acetate, the products I, VIII, and XVII, with ethyl acetate–acetone (1 :1), II, III, and IV, and with acetone, V and VI were eluted, respectively. On setting aside the mixture of I and XVII, the latter compound appeared as

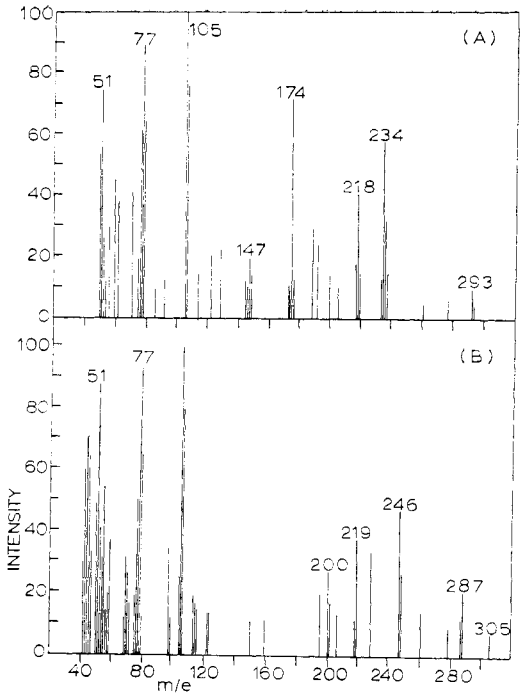


FIGURE 3: Mass spectra of II′ (A) and V′ (B).

TABLE I: Yields and Some Properties of the Products Obtained from *N*-Benzoylhistidine.

Product	Yield (%)	Mp (°C)	Crystal Form	R_F Value in Solvent 1	2
I	15	179	Needles	0.37	0.57
II	4	217	Needles	0.38	0.77
III	3			0.40	
IV	8	187	Needles	0.58	0.70
V	3	170 (dec)	Prisms	0.44	0.72
VI	<1	180 (dec)	Prisms	0.44	0.67
VII	<1	190 (dec)	Needles	0.44	0.74
VIII	1	280 (dec)	Needles	0.40	0.69
IX	<1			0.80	0.83
X	<1				
XI	3	173 (dec)	Prisms	0.35	0.56
XII	1			0.35	0.52
XIII	6	193 (dec)	Prisms	0.35	0.58
XIV	5	157 (dec)	Prisms	0.37	0.60
XV	14	170 (dec)	Prisms	0.35	0.64
XVI	9			0.35	0.67
XVII	2	202 (dec)	Needles	0.66	0.75

crystals. As the compound XVII was unstable in aqueous solvents, XVII was not obtainable from any fraction of Dowex 1 column chromatography. After evaporation *in vacuo* of acetone from the mixture of V and VI, and subsequent recrystallization from methanol, V was obtained as crystals. The fraction which was obtained by Dowex 1 (formate) column chromatography and which contained III and IV was subjected to cellulose column chromatography. By this procedure III was separated as an amorphous powder which gave each single spot in paper chromatography and paper electrophoresis. The peak fraction in Figure 2 which contained VI and VII was rechromatographed through Dowex 1-X4 (formate) to separate these two products.

Figure 2 also presents the chromatographic elution pattern through Dowex 1-X4 (formate) of the products contained in the group B fraction. This pattern clearly indicates that the group B contained at least seven products which were represented by X, XI, XII, XIII, XIV, XV, and XVI. The products XI, XIII, XIV, and XV were recrystallized from aqueous methanol after removal of the solvent *in vacuo* from the corresponding fractions.

The yields in per cent of the products were determined from absorbancies at 230 mμ of the fractions obtained by Dowex 1 column chromatography after 6-hr irradiation of *N*-benzoylhistidine (Table I).

Characterization of the Photooxidation Products of N-Benzoylhistidine. The properties of I and IV were listed in Tables I, II, III, and IV, and these compounds were found to be *N*-benzoylaspartic acid and, *N*-benzoylasparagine, respectively, by comparison with authentic compounds (Fischer, 1899). The methyl esters of I and IV were also identical with dimethyl *N*-benzoylaspartate and methyl *N*-benzoylasparaginate synthesized by authentic methods (Fischer, 1899), respectively.

Compound II gave infrared absorptions characteristic of the amido carbonyl and carboxyl groups, and when II was methylated with diazomethane to II′, the absorption of carboxyl group shifted to that of methyl ester (Table III). The ultraviolet and nuclear magnetic resonance spectra of II indicated the presence of a benzoyl group. Compound II moved as a monoanion in electrophoresis run at pH 6.2. On acid hydrolysis, II was decomposed to aspartic acid and urea. The peak of *m/e* 234 observed in the mass spectrum of the methyl ester (II′) corresponded to a fragment produced by loss of a ureido ($NHCONH_2$) group from II′ (molecular ion *m/e* 293) (Figure 3). From these data the structures of *N*-benzoylaspartylurea and its methyl ester were proposed to II and II′, respectively. The mass spectrum of V′ (methyl ester of V) gave a peak of molecular ion, *m/e* 305, which coincided with the molecular formula of $C_{14}H_{15}N_3O_5$ and the peak *m/e* 287 indicated that V′ has a structure which should be readily dehydrated (Figure 3).

The peaks *m/e* 77 and 105 indicated the presence of benzoyl group in V′. But the molecular extinction coefficient at 228 mμ of V′ (ε 6400) which was calculated for the molecular weight of 305, was much smaller than those of I, II, and IV (ε *ca.* 10,200). This small value of molecular extinction coefficient of V′ could be explained by a steric hindrance which inhibits the planarity of benzoylamido group in V. This assumption is supported by the similar smaller extinction at 230 mμ of *N,N*-dimethylbenzamide than those of *N*-methylbenzamide or benzamide (Edward and Meacock, 1955).

The peaks in the nuclear magnetic resonance spectrum of V′ are given in the Table III. On deuteration of V′ the signals at τ 3.5 and 2.5 disappeared while that at τ 4.8 remained unchanged. The infrared spectrum of V indicated the presence of carbonyl groups other than that of the benzoyl residue.

TABLE II: Molecular Formula and Elemental Analysis Data.

Product	Mol Formula	Calcd (%)			Found (%)		
		C	H	N	C	H	N
I	$C_{11}H_{11}NO_5$	55.69	4.67	5.91	55.70	4.87	5.72
II	$C_{12}H_{13}N_3O_5$	51.61	4.69	15.05	51.75	4.88	14.56
II′	$C_{13}H_{15}N_3O_5$	53.24	5.16	14.33	53.48	5.22	14.33
IV	$C_{11}H_{12}N_2O_4$	55.93	5.12	11.86	56.13	5.23	11.89
V	$C_{13}H_{13}N_3O_5$	52.01	5.03	13.00	51.89	5.05	13.53
V′	$C_{14}H_{15}N_3O_5$	55.08	4.95	13.71	55.42	5.21	13.50
XIII	$C_{26}H_{24}N_6O_8 \cdot 2H_2O$	55.12	4.59	14.84	55.00	4.56	14.90
XIV	$C_{26}H_{24}N_6O_8 \cdot 2MeOH$	54.90	5.27	13.72	55.06	5.01	13.72
XIV′	$C_{27}H_{26}N_6O_8 \cdot H_2O$	55.86	4.83	14.48	56.30	4.97	14.42
XV	$C_{26}H_{26}N_6O_8 \cdot H_2O$	54.93	4.93	14.79	54.83	5.12	14.85
XVII	$C_{13}H_{11}N_3O_5$	53.87	3.95	14.68	53.98	3.83	14.53
XVIII	$C_{14}H_{13}N_3O_5$	55.44	4.29	13.86	55.52	4.37	13.49

When V was methylated to V′, the absorption at 1705 cm^{-1} of V shifted to 1735 cm^{-1} with the simultaneous appearance of a new peak at 3440 cm^{-1} corresponding to that of hydroxyl group, which has not been observed in the case of V due to the possible hydrogen bonding with carboxyl group. In the electrophoresis at pH 6.2 V migrated as a monoanion, while V′ behaved as a neutral compound. These results indicated that V should have the structure of 2-oxo-4-benzoyl-6a-hydroxyperhydropyrrolo[2,3-*d*]imidazole-5-carboxylic acid.

The peaks in the infrared spectrum of XVII showed, as are given in the Table III, the presence of a carbonyl group and the the absence of a carboxyl group. The behavior of XVII in electrophoresis at pH 6.2 also indicated that this compound is not a carboxylic acid. The signals in nuclear magnetic resonance spectrum of XVII closely resembled those of I, II, and IV in the high magnetic field. The mass unit 56.5 as a metastable ion in the mass spectrum of XVII indicated the presence of C_6H_5CO group, and the lack of the molecular peak and appearance of the peak at *m/e* 245 must be caused by elimination of carbon dioxide from this compound (Figure 4). Furthermore, XVII was considered to be fairly unstable in aqueous solution as XVII was not found in the effluent from the Dowex 1 column chromatography.

A derivative of XVII, XVII′, which was produced by methylation of XVII with diazomethane, gave a higher R_F value in thin-layer chromatography than that of XVII, but this compound could not be obtained in crystalline form.

On treatment of XVII with methanolic HCl, a product XVIII, having mp 163°, was obtained as prisms. The analyses indicated that XVIII had a molecular formula of $C_{14}H_{13}N_3O_5$. The infrared spectrum of this compound revealed, as shown in Table III, the peaks corresponding to the carbonyl group which somewhat shifted to shorter wave numbers than those found for XVII. The mass spectrum of XVIII indicated the presence of a benzoyl group and the peak *m/e* 244 in the spectrum should be caused by demethoxy carbonylation of XVIII (Figure 4). The nuclear magnetic resonance spectrum of XVIII lacked the two-proton multiplet at τ 7.2 which was observed in the spectrum of XVII, but it showed a new three-proton singlet at τ 6.3 and a one-proton doublet at τ 4.4. In the ultraviolet spectrum of XVIII a new absorption maximum at 275 mμ was observed besides that of the benzoyl group at 228 mμ, and in alkaline medium the absorption maximum at 275 mμ showed a hyperchromicity. The pK_a value of XVIII, as estimated by both the absorption and titration, was 8.0. From the results described above, the structures of *N*-benzoyl-β-2,4-dioxoimidazolidinylalanine spirolactone and 5-(2-benzamide-2-methoxycarbonylethylidenyl)hydantoin were proposed for XVII and XVIII, respectively.

Compound III was obtained as ammonium salt which traveled as a monocarboxylic acid in the electrophoresis run at pH 6.2. On passage of the ammonium salt through a

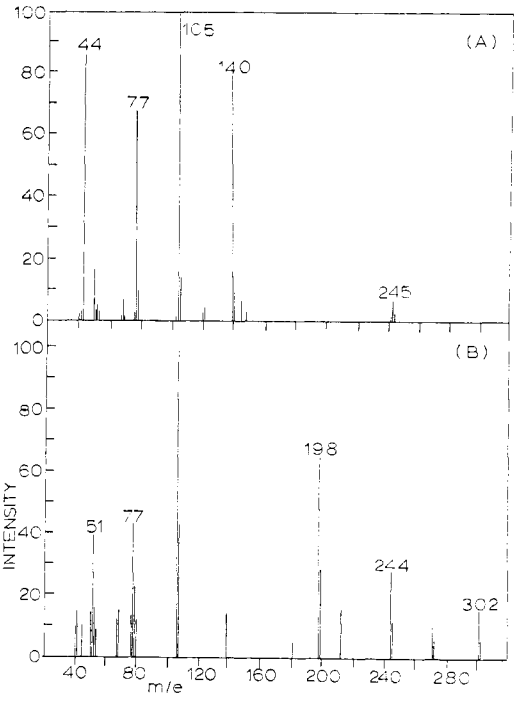


FIGURE 4: Mass spectra of XVII (A) and XVIII (B).

TABLE III: Physical Properties of the Products, I, II, II′, V, V′, XVII, and XVIII.

Compound	I	II	II′	V	V′	XVII	XVIII
Ultraviolet spectra (Solvent) λ_{max}, mμ (ϵ)	(H_2O) 228 (10,200)	(H_2O) 228 (10,500)	(MeOH) 228 (9,700)	(H_2O) 228 (5,000)	(MeOH) 228 (6,400)	(MeOH) 230 (10,900)	(MeOH) 230 (15,500) 275 (7,000)
Infrared spectra (KBr) ν_{max} (cm^{-1})	3,350, 1,655, 1,520 (CONH) 1,705 (COOH)	3,350, 1,655, 1,525 (CONH) 1,700 (COOH) ($NHCONH_2$)	3,360, 1,655, 1,530, 3,240 (CONH) 1,750 (COOMe) 1,695 ($NHCONH_2$) 1,320, 1,100	3,380, 1,080 (—OH) 3,260, 1,430, 1,710 (COOH) 1,690 (NHCONH) 1,620 (CON<)	3,440, 1,070 (—OH) 1725, 3,280 (COOMe) (NHCONH) 1,650 (CON<)	3,340, 1,650, 1,530, 1,280, 1,180 (CONH) 1,780, 960 (γ-lactone) 1,805, 1,760, 1,730 (hydantoin)	3,370, 1,640, 1,535, 3,160 (CONH) 1,730, 3,050 (COOMe) 1,780, 1,730, 1,695 (hydantoin)
Nuclear magnetic resonance (dimethyl sulfoxide-d_6) τ value	7.20 (1 H, d) (CH_2) 5.30 (1 H, m) (CH<) 2.80–2.10 (5 H, m) (PhCO) 1.25 (1 H, d) (CONH)	7.10 (2 H, d) (CH_2) 5.20 (1 H, m) (CH<) 2.90–2.05 (8 H, m) (PhCO and $NHCONH_2$) 1.25 (1 H, d) (CONH) −0.25 (1 H, s) (COOH)	7.10 (2 H, d) (CH_2) 6.35 (3 H, s) (COOMe) 5.10 (1 H, m) (CH<) 2.90–2.05 (8 H, m) (PhCO and $NHCONH_2$) 1.25 (1 H, d) (CONH)	7.70 (2 H, m) (CH_2) 5.60 (1 H, m) (CH<) 4.80 (1 H, s) (CH<) 4.60 (8 H, broad) (HDO) 2.60 (7 H, s) (PhCO and (NHCONH)	7.65 (2 H, m) (CH_2) 6.35 (3 H, s) (COOMe) 5.50 (1 H, m) (CH<) 4.75 (1 H, s) (CH<) 2.55 (7 H, s) (PhCO and NHCONH) 3.50 (1 H, s) (OH)	7.15 (2 H, d) (CH_2) 5.00 (1 H, m) (CH<) 2.65–2.00 (5 H, m) (PhCO) 0.90 (1 H, s) (NHCO) 0.65 (1 H, d) (NHCO) −1.40 (1 H, s) (CONHCO)	6.30 (3 H, s) (COOMe) 4.60 (1 H, m) 4.40 (1 H, d) (>CHCH=) 2.75–2.00 (5 H, m) (PhCO) 0.90 (1 H, d) (CONH) −0.65 (1 H, s) (CONH) −1.20 (1 H, s) (CONHCO)

TABLE IV: Physical Properties of the Products, IV, VIII, XIII, XIV, XIV′, and XV.

Compound	IV	VIII	XIII	XIV	XIV′	XV
Ultraviolet spectra (solvent)	(MeOH)	(MeOH)	(H_2O)	(H_2O)	(H_2O)	(H_2O)
λ_{max}, mμ	288	228	224	224	224	224
(ϵ)	(10,200)	(10,800)	(23,800)	(22,400)	(18,700)	(21,800)
Infrared spectra (KBr)	3,420, 3,350, 1,650	3,350, 1,780, 1,720,	3,420, 1,680, 1,620,	3,300, 1,650, 1,530,	3,290, 1,655, 1,530,	3,390, 1,650,
ν_{max} (cm^{-1})	(CONH) ($CONH_2$)	1,705 (hydantoin)	1,540 (CONH)	1,370 (CONH)	1,355 (CONH)	1,525 (CONH)
	1,705 (COOH)	1,650, 1,535 (CONH)	1,740 (COOH)	1,740 (COOH)	1,740 (COOMe)	1,740 (COOH)
Nuclear magnetic resonance	7.40 (2 H, d) (CH_2)	7.40 (1 H, m)	6.95 (3 H, m) (CH_2)	6.90 (4 H, m) (CH_2)	7.40 (4 H, m) (CH_2)	
(Dimethyl sulfoxide-d_6)	6.10 (3–4 H, broad)	2.70–2.10 (6 H, m)	5.15 (16 H, broad)	5.60 (16 H, broad)	6.30 (3 H, s)	
τ value	(HDO)	(PhCO)	(HDO)	(HDO)	(COOMe)	
	5.35 (1 H, m)	1.30 (3 H, m)	2.90–2.00 (16 H, m)	2.90–2.00 (15 H, m)	5.30 (2 H, m) (CH<)	
	(CH<)	−0.80 (1 H, s)	(PhCO and un-	(PhCO and un-	3.40 (1 H, s) (imid-	
	2.70–2.10 (5 H, m)		identified proton)	identified proton)	azole NH)	
	(PhCO)		1.30 (2, H, d)	1.30 (2 H, d)	2.90–2.10 (15 H, m)	
	1.50 (1 H, d)		(CONH)	(CONH)	(PhCO and un-	
	(CONH)				identified proton)	
					1.30 (2 H, d) (CONH)	

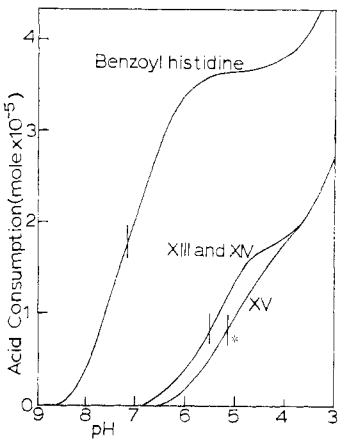


FIGURE 5: pH titrations of *N*-benzoylhistidine, XIII, XIV, and XV. Ten milligrams each of the compound was used. (*) pK_a value.

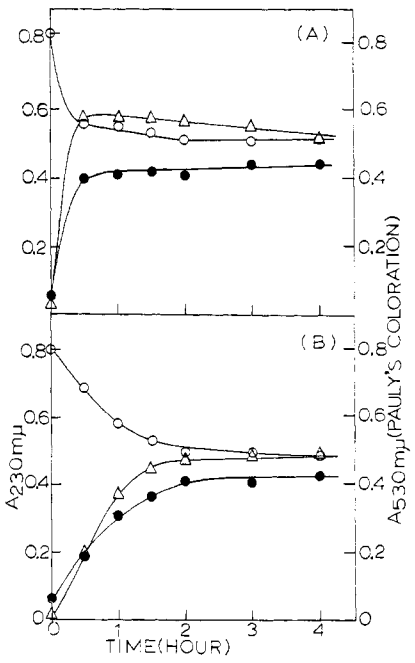
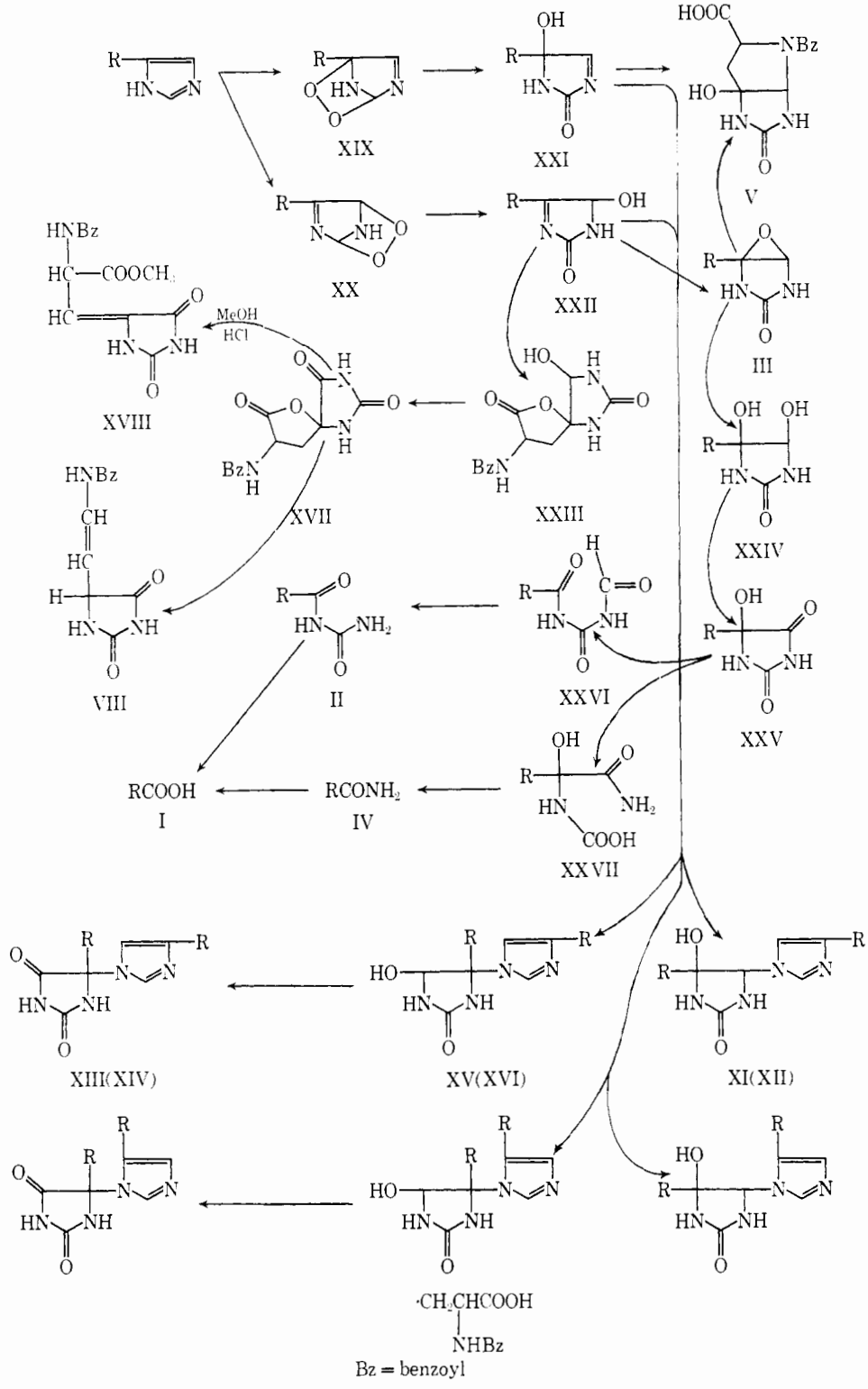


FIGURE 6: Time course of the degradation of XIV with 0.1 N NaOH (A) or HCl (B) at 95°. Total amount of the products which belonged to group A (●), and those of XIV plus *N*-benzoylhistidine (○), and Pauly's coloration estimated at 530 mμ (△).

column of Dowex 50W-X4 (H^+) and subsequent concentration of the effluent, this compound was converted into V in a yield of 30%, indicating that III has a close chemical structure to that of V. Compound III revealed only one absorption maximum of benzoyl residue at 228 mμ in the ultraviolet range. Thus, a tentative structure of *N*-benzoyl-β-(2-oxo-4,5-epoxyimidazolidyl)alanine, was given to III.

As is indicated in Table IV, VIII gave absorptions of a hydantoin group in infrared, and a benzoyl group in ultraviolet regions, respectively. This compound moved as a neutral compound in electrophoresis run at pH 6.2 and revealed the presence of double bond in the bromination test. From these results a tentative structure of 5-(2-benzamidovinyl)hydantoin was given to VIII.

SCHEME I



Compound XIV had a pK_a of 5.6 which was estimated by pH titration (Figure 5), and the compounds obtained from the peaks XIII and XV gave pK_a values of 5.6 and 5.1, respectively. Further, these three compounds as well as XI and XII liberated *N*-benzoylaspartic acid and *N*-benzoylhistidine on treatment with 0.1 N NaOH or HCl solution (Figure 6). Thus XI–XV were assumed to be produced by condensation of *N*-benzoylhistidine with its photooxidation products.

The facts that the analysis of XIII gave the same mo-

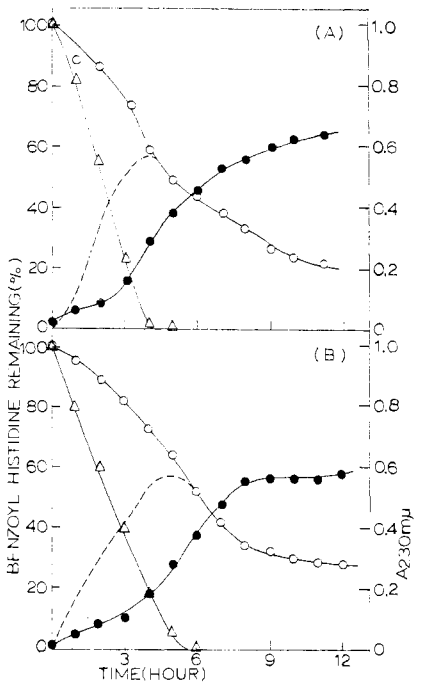


FIGURE 7: Time course of photooxidation of *N*-benzoylhistidine and formation of groups A and B. Photosensitizer: rose bengal (A), methylene blue (B). (●) Group A products, (○) sum of group B products and *N*-benzoylhistidine, (△) *N*-benzoylhistidine determined by Pauly's coloration, and (– – –) the amount of group B products obtained by (○) minus (△).

lecular formula, $C_{26}H_{24}N_6O_8$, as that of XIV and physical properties similar to those of XIV lead us to propose its structure to be an isomer of XIV, as shown in Scheme I.

An assumed structure of XV is presented in Scheme I, from its molecular formula of $C_{26}H_{26}N_6O_8$ and physical characteristics.

Time Course of the Photooxidation of N-Benzoylhistidine. The photooxidation of *N*-benzoylhistidine using methylene blue as the sensitizer was investigated in detail. The per cent in the amount of imadazole residue which remained in the irradiated mixture was estimated with Pauly's reagent and plotted as a function of reaction time (Figure 7). The aliquots of the reaction mixture were taken at time intervals and the products which belonged to group A were separated from those which belonged to group B by ion-exchange column chromatography using Dowex 50W-X4 (H^+). The yield of the total products contained in these two groups were determined by the measurement of ultraviolet absorbance and plotted against the reaction time.

As is represented in Figure 7B the yield of the products belonging to group B reached a maximum after 5-hr reaction, then gradually descreased, while the yield of the products belonging to group A still increased even after 8 hr. This observation indicated that the compounds in group B should have been converted into the compounds in group A by photooxidation. As was described above, group B contained at least six dimeric compounds, XI, XII, XIII, XIV, XV, and XVI, the structures of which were assigned as shown in Scheme I. The dimeric compounds must have been degradated into monomeric products by further photooxidation. Figure 7B also shows that the time-dependent lowering of the curve

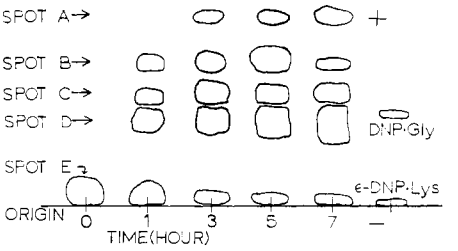


FIGURE 8: Electrophoresis of the photooxidation mixture. The reaction mixtures obtained by different time irradiation were applied to paper electrophoresis at 20 V/cm and pH 6.2. Markers: ε-dinitrophenyllysine (DNP-Lys) and dinitrophenylglycine (DNP-Gly).

for group B compounds turned near horizontal after 8 hr and thereafter approximately 30% of the group B compounds remained resistant against photooxidation. That is, a part of group B compounds might not be convertible to the group A compound.

On electrophoresis of the photooxidation mixture at pH 6.2, five spots were detected by absorption of ultraviolet ray (Figure 8). These spots were represented as spot A, B, C, D, and E in the order from the farthest to the closest to the origin. The compounds contained in each spot were extracted with water and estimated spectrophotometrically at 230 mμ. In Figure 9 the amount of the compounds corresponding to each spot is plotted against the photooxidation time. Table V indicates the correspondency of 17 isolated products to the spots obtained by the electrophoresis. The spot A contained only one product, benzoylaspartic acid (I). The spot B contained two compounds, XV and XVI. Figure 9A indicates that the yield of these compounds in spot B against reaction time showed a maximum at 5-hr reaction and a decrease thereafter.

Spot C contained XI, XII, XIII, and XIV which also belonged to group B. These four compounds should be classified into two types: the one that is stable and the other unstable to the prolonged photooxidation, because the time-

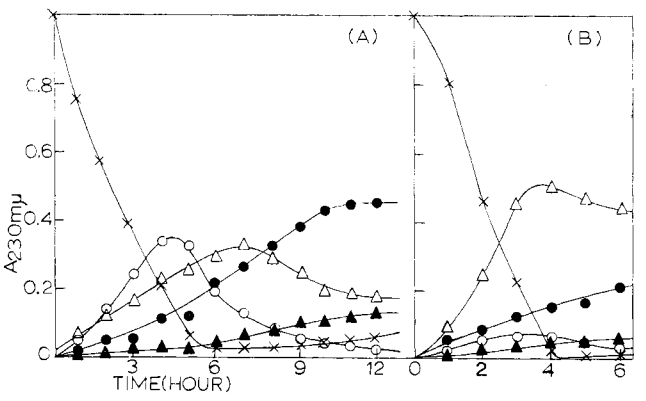


FIGURE 9: Time course of photooxidation of *N*-benzoylhistidine. Photosensitizer: methylene blue (A) and rose bengal (B). The photooxidation products were separated into spots A, B, C, D, and E by paper electrophoresis as described previously (Figure 8), and their amounts were determined by the measurement of ultraviolet absorbancy at 230 mμ. (▲) Spot A, (○) spot B, (△) spot C, (●) spot D, and (×) spot E.

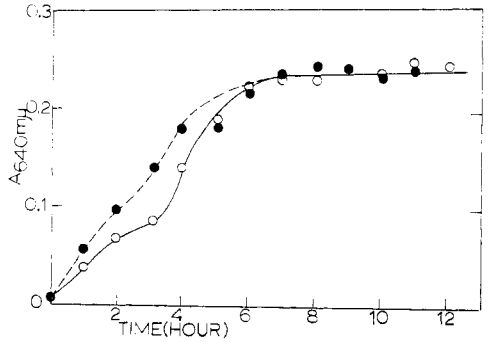


FIGURE 10: Formation of the products colored by Ehrlich's reagent. (○) In the presence of methylene blue and (●) in the presence of rose bengal. For experimental conditions, see text.

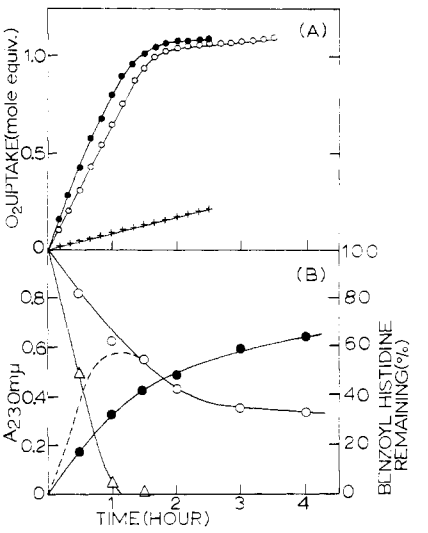


FIGURE 11: Relation of oxygen consumption to the formation of the intermediates. (A) Time course of the oxygen consumption in the presence of methylene blue (○), rose bengal (●), or riboflavins (– + –). (B) Time courses of the disappearance of *N*-benzoylhistidine and production of compounds belonging to groups A and B in the presence of methylene blue as photosensitizer. The experiment was performed under the conditions applied for the estimation of oxygen consumption during the photooxidation of *N*-benzoylhistidine (see Experimental Section). (●) Group A products, (○) sum of group B products and *N*-benzoylhistidine, (△) Pauly's coloration estimated at 530 mμ, and (– – –) the amount of group *B* products obtained by (– ○ –) minus (– △ –).

course curve, after reaching a maximum at 7 hr, once lowered and then stayed constant after 10 hr.

In order to isolate the products in spot C which were stable against the prolonged photooxidation, spot C was prepared from 10-hr photooxidated mixture, extracted, and analyzed according to the method given in the Experimental Section. The results indicated that among the four compounds in spot C, XIII and XIV were stable and XI and XII were unstable for the prolonged photooxidation. As a conclusion, the analysis of the products by electrophoresis indicated that group B was consisted of two photochemically stable products, XIII and XIV, and four unstable compounds, XI, XII, XV, and XVI. Spot D was found to contain products II, III, IV, V, and X. The Figures 7 and 9, which showed the time courses of the formation of various products, indicate that the compounds contained in spot D should be produced mainly *via* dimeric products which belonged to both spots B and C, though direct productions of these compounds from the precursors (XXI and XXII) could not be neglected. The conversion of compounds contained in spots B and C into II and IV is also reasonable in the structural relations as shown in Scheme I.

Spot E consists of *N*-benzoylhistidine, VI, VII, VIII, IX, and XVII. The curves given in Figure 9A indicate that the amount of the compounds in spot E decreased up to 6 hr starting from the zero time, and this decreasing rate is parallel to the disappearance of Pauly's coloration given in Figure 7, which indicated the disappearance of *N*-benzoylhistidine. The gradual ascent of the curve for spot E (Figure 9) after 6-hr reaction should be corresponded to the increasing amount of VI, VII, VIII, IX, and XVII, all of which belonged to group A and did not move in the electrophoresis at pH 6.2.

The time-course curve of the production of V plus VI, which was estimated by Ehrlich's coloration, is given in Figure 10. This curve, however, practically corresponds the rate of the production of V because the yield of V was remarkably higher than that of VI. As V is stable for further irradiation, this compound must be one of the final products. The yield of the sum of V and VI was approximately 5% as calculated from Ehrlich's coloration.

The rate of oxygen consumption in the photooxidation of *N*-benzoylhistidine is given in Figure 11. Using the same apparatus and conditions as applied for the estimation of oxygen consumption, the rates of decrease of *N*-benzoylhistidine and of formation of group A and B compounds were estimated. The results are given also in Figure 11.

Figure 11 revealed that the productions of group A and B compounds similarly proceeded as in the experiment given in Figure 7, though the rates of the corresponding curves were relatively higher in the case of the experiment given in Figure 11 than in Figure 7. After 1.5-hr reaction, approximately 1.0 mol equiv of oxygen to *N*-benzoylhistidine was consumed (Figure 11).

The Effect of the Photosensitizers on the Photooxidation. A precise investigation about the effect of the photosensitizers

TABLE V: Correspondency of the Electrophoretic Spots and the Products Belonging to Groups A and B.

	Spot				
Group	A	B	C	D	E
A	I[a]			II	VI, IX
				III	VII
				IV	VIII
				V	XVII
B		XV	XI		
			XII	X	
		XVI	XIII		BH[b]
			XIV		

[a] The numerals indicate the number of products. [b] BH, *N*-benzoylhistidine.

on products and their formation rates was performed. In Figure 11A the oxygen consumptions were plotted against reaction time in the presence of methylene blue, riboflavin, and rose bengal. The rates in oxygen consumption increased in the order of the sensitizer, riboflavin, methylene blue, and rose bengal. The alteration of the photosensitizer to riboflavin or rose bengal from methylene blue did not give any difference in the kinds of the photooxidation products. The yield of the mixture of XV and XVI, estimated from the color density of the extract from the spot B in Figure 8 was markedly small when rose bengal was used as the sensitizer.

The time courses of the formations of photooxidation products under the presence of rose bengal as sensitizer were estimated and given in Figure 7A, 9B, and 10. The comparison of the results with those obtained for the reactions under the presence of methylene blue (Figure 9) indicated that the maximal yield of spot B compounds was higher than 30% for methylene blue and approximately 7% for rose bengal as sensitizers. In contrast, the yield of spot C compounds was higher when rose bengal was used as sensitizer than when methylene blue was used. The relative rates of other reactions were similar for either kinds of these sensitizers, though in general the reactions proceeded more rapidly with rose bengal.

Dependence of the Reaction Rate upon the Kind of Substrate. As is shown in Figure 12, under the same reaction condition, no difference was observed among the rates of the photooxidations in which histidine, *N*-benzoylhistidine, or *N*-benzoylhistidinamide was used as the substrates, and the result indicated that the substitution of hydrogen at the free α-amino or α-carboxyl groups of histidine did not give any influence on the rate of this photooxidation reaction. Histidine and the derivatives were not photooxidized in the absence of photosensitizer and the reaction was terminated by removal of the photosensitizer.

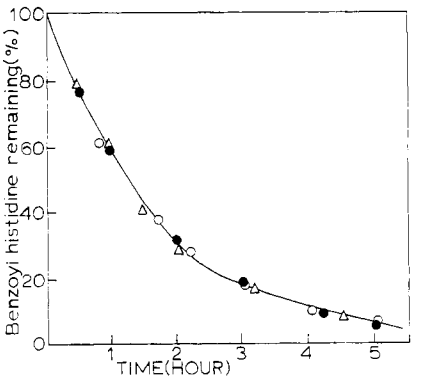


FIGURE 12: Time course of photooxidation of histidine and its derivatives in the presence of methylene blue. Histidine or its derivative (18 mmoles) in 200 ml of water, and 8 mg of methylene blue were used. The other irradiation conditions were as described in Experimental Section. The amount of histidine and its derivatives was determined by Pauly's coloration according to the method of Macpherson. (○) Histidine, (●) *N*-benzoylhistidine, and (△) *N*-benzoylhistidinamide.

Discussions

The reaction mechanisms of the sensitized photooxidations reported in several recent articles (Foote *et al.*, 1968; Kopecky and Reich, 1965; McKeown and Waters, 1966) involve the participation of the singlet oxygen. The prediction of the products to be produced and the explanations of the reaction mechanism were achieved to some extent in the cases of the photooxidations of hydrocarbons. As for the similar reactions in heterocyclic compounds, however, practically no reports are available except those of pyrrole (Mayo and Reid, 1962), tryptophan (Benassi *et al.*, 1967), imidazole (Wasserman *et al.*, 1968), and some purine derivatives (Van Vunakis and Simmon, 1964; Matsuura and Saito, 1968) and even in these reports no information was encountered with respect to the influence of the nitrogen atoms present in their structures to the photooxidation.

Previously, aspartic acid and urea were reported to be the photooxidation products of histidine (Hara, 1960), but the report hardly contributed to the explanation of the reaction mechanism, because the yield of the aspartic acid reported was only 2%.

Several compounds identified or obtained this time as the products in the total photooxidation of *N*-benzoylhistidine could be arranged as shown in Scheme I. Two endoperoxides (XIX and XX) could be produced as the first intermediate products, by a 1,4 cycloaddition of singlet oxygen on the conjugated double bond of *N*-benzoylhistidine. The disappearance of immidazole residue estimated by coloration with Pauly's reagent mainly corresponded to this 1,4-cycloaddition reaction. The endoperoxides must be very unstable and they should subsequently be converted into XXI and XXII, respectively, by the cleavage of the O–O bonds. As the C_4 position of imidazole residue in the structure of XXI and the C_5 position in that of XXII might easily be attacked by several nucleophiles, these compounds in the reaction mixture should immediately be converted into the next derivatives, which are represented by the structure, III, V, XXIII, XXIV, XV, and XVI in Scheme I.

Among these products, V must be produced by the intramolecular attack of nonbonding electron pair of amido nitrogen at the C_4 position of XXI. The lactone (XVII) should be produced *via* an intermediate compound, XXIII, which was formed by an initial intramolecular attack of carboxylate at the C_5 position of XXII. Subsequent dehydrogenation of the secondary hydroxyl group of XXIII can give rise to XVII. Compound XVIII must be produced from XVII with methanolic HCl by methanolysis and a subsequent dehydration.

A similar dehydrogenation as that involved in the conversion of XXIII into XVII should give a hydroxyimidazolidinedione (XXV) from a dihydroxyl compound (XXIV), which was produced by the nucleophilic attack of OH^- of the C_4 position of XXI. This imidazolidinedione was assumed to be a key intermediate compound to give the three subsequent products which were isolated from the reaction mixture as crystalline compounds: *N*-benzoylaspartylurea (II), *N*-benzoylaspartic acid (I), and *N*-benzoylasparagine (IV). Thus, as is shown in Scheme I, prototropic ring rupture of XXV at C_4–C_5 bond, subsequent deformylation and liberation of urea must successively give II and I.

The ring rupture at C_2–N_3 bond of XXV and subsequent prototropic degradation of XXVII gave *N*-benzoylasparagine (IV). As II did not give *N*-benzoylasparagine by acid (or alkaline) hydrolysis, the route of the production of IV from XXV must be different from that of the production of II.

The other four products obtained as crystals, XI, XIII,

XIV, and XV, gave the molecular weights corresponding to dimeric products. From their chemical and physical properties, the occurrence of these compounds could most likely be a nucleophilic attack at C_4 or C_5 position of XXI (or XXII) by imidazole residue of the starting *N*-benzoylhistidine.

The oxygen consumption and the productions of group A and B compounds (Figure II) showed that rate of the disappearance of Pauly's coloration is not necessarily parallel to that of the oxygen consumption during the first 100-min reaction. Thus at the first 1-hr reaction when approximately 0.65 mol equiv of oxygen was consumed, 93% of imidazole residue already disappeared, while the formation of group B compounds reached a maximum. The results indicated that the production of dimers (group B compounds), which gave no Pauly's reaction, does not need the consumption of equimolecular amount of oxygen by the starting material. Figure 11 shows a slight consumption of oxygen that was still observed after 100-min reaction time when total of the starting *N*-benzoylhistidine already disappeared. It is difficult to give an exact explanation which kind of reaction actually corresponded to the oxygen consumption observed after the disappearance of Pauly's reaction. However, from the facts that the conversions of XV (or XVI) into XIII (or XIV) and XXIII into VIII *via* XVII require each 0.5 mol equiv of oxygen and that, as is seen in Figure 9, the amount of spot E, which contained VIII and XVII, gradually increases in the final phase of the reaction, it should not be unreasonable to attribute the oxygen consumption observed after 1-hr reaction to the oxidation of secondary hydroxyl groups at the C_4 position of XV (or XVI) and XXIII into carbonyl groups. At any rate, it is obvious that the photooxidation of *N*-benzoylhistidine, besides the first cycloaddition of oxygen to the imidazole residue, involves a dehydrogenation reaction.

Kearns *et al.* (1967) reported that in the sensitized photooxidation, the type of the oxidation depends upon the sensitizers used. Thus methylene blue produced only $^1\Delta_g$ oxygen, rose bengal both $^1\Delta_g$ and $^1\Sigma_g^+$ oxygens, while riboflavin revealed a specific dehydrogenation activity in addition to the production of the singlet oxygens.

The results of our research, however, indicated that in the photooxidation of *N*-benzoylhistidine the difference in the kind of photosensitizer did not give any influence on the kind of the oxidation and the mechanism in the first step of the oxidation reaction is common for any of the photosensitizers tested: it involves the common occurrences of two endoperoxides (XIX and XX) by participation of $^1\Delta_g$ state oxygen. A sensitizer-dependent difference observed was the amount of the intermediate compounds, XV and XVI, at a definite reaction time, which were smaller when rose bengal was used than when methylene blue was used as the sensitizer (Figure 9). This difference should most reasonably be explained by that when rose bengal was used, the intermediate compounds XV and XVI could more rapidly be oxidized to XIII and XIV by $^1\Sigma_g^+$ singlet oxygen which is specifically produced by this photosensitizer in addition to the usual triplet and $^1\Delta_g$ singlet oxygens.

References

Benassi, C. A., Scoffone, E., Galliazzo, G., and Iori, G. (1967), *Photochem. Photobiol. 6*, 857.

Bergmann, M., Fruton, J. S., and Pollok, H. (1939), *J. Biol. Chem. 127*, 643.

Edward, J. T., and Meacock, S. C. R. (1955), *Chem. Ind. (London)*, 1576.

Fink, R. M., Cline, R. T., and McGaughen, C. (1956), *Anal. Chem. 28*, 4.

Fischer, E. (1899), *Chem. Ber. 32*, 2451.

Foote, C. S. (1968), *Science 162*, 963.

Foote, C. S., Wexler, S., and Ando, W. (1968), *J. Am. Chem. Soc. 90*, 975.

Hara, H. (1960), *Nogei Kagaku Kaishi 34*, 493.

Hoffee, P., Lai, C. Y., Pugh, E. J., and Horecker, B. L. (1967), *Proc. Natl. Acad. Sci. U. S. 57*, 107.

Kearns, D. R., Khan, A. U., Hollins, R. A., and Padlick, P. (1967), *J. Am. Chem. Soc. 89*, 5455.

Kenkare, U. W., and Richards, F. M. (1966), *J. Biol. Chem. 241*, 3197.

Kopecky, K. R., and Reich, H. J. (1965), *Can. J. Chem. 43*, 2265.

Macpherson, H. T. (1946), *Biochem. J. 40*, 470.

Martinez-Carrion, M. (1967), *J. Biol. Chem. 242*, 1426.

Matsuura, T., and Saito, I. (1968), *Tetrahedron Letters*, 3273.

Mayo, P. D., and Reid, S. T. (1962), *Chem. Ind. (London)*, 1576.

Mckeown, E., and Waters, W. A. (1966), *J. Chem. Soc.*, 1040.

McLaren, A. D., and Shugar, D. (1964), Photochemistry of Proteins and Nucleic Acids, New York, N. Y., Macmillian.

Rippa, M., and Pontremoli, S. (1968), *Biochemistry 7*, 1514.

Tomita, M., Irie, M., and Ukita, T. (1968), *Tetrahedron Letters*, 4933.

Van Vunakis, H., and Simmon, M. I. (1964), *Arch. Biochem. Biophys. 105*, 197.

Wasserman, H. H., Stiller, K., and Floyd, M. B. (1968), *Tetrahedron Letters*, 3277.

Weil, L., Gorgon, W. G., and Buchert, A. R. (1951), *Arch. Biochem. Biophys. 46*, 266.

Westhead, E. W. (1965), *Biochemistry 4*, 2139.

Yamagata, S., Takahashi, T., and Egami, F. (1962), *J. Biochem. (Tokyo) 52*, 261.