

# Oxidative Degradation of Imidazoles by Bromine or *N*-Bromosuccinimide\*

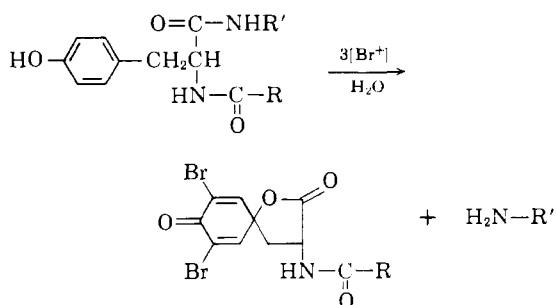
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**ABSTRACT:** The reaction of bromine or of *N*-bromosuccinimide with imidazole or with a number of its 4(5)-substituted derivatives, in aqueous media, leads to oxidative degradation of the heterocyclic ring. The products formed are ammonia, glyoxal (or the corresponding substituted glyoxal), and probably formamide. From studies of the rate of consumption of oxidant,

it is concluded that, as a result of intramolecular carboxyl participation, a labile lactone is formed as an intermediate in the oxidation of imidazole-4(5)-propionic acid (and its analogs).

No evidence of such participation is observed, however, in the case of the corresponding ester or amide.

The action of bromine or of *N*-bromosuccinimide (NBS)<sup>1</sup> on tyrosine-containing peptides has been shown to result in a coupled phenol oxidation-peptide cleavage reaction (Schmir *et al.*, 1959; Schmir and Cohen, 1961; Wilson and Cohen, 1963a,b) which has been utilized for the selective splitting of complex sequences (Wilson and Cohen, 1963b). Among the other functional groups which are present in a natural polypeptide, and which may be subject to oxidative attack, is the imidazole ring of histidine (Schmir and Cohen, 1961; Shaltiel and Patchornik, 1963). Although we have shown that, in mixtures of simple tyrosine and histidine



derivatives, the phenolic system is attacked preferentially (Schmir and Cohen, 1961), there is little assurance and considerable doubt that the same selectivity would be observed in the three-dimensional matrix of a protein. The purpose of the present investigation was to examine the course of oxidation of the imidazole ring by positive halogen reagents and to explore factors which

TABLE 1: Oxidative Degradation of Imidazole with NBS.

| Oxidant | Medium <sup>a</sup>     | TBI <sup>b</sup> | TSC <sup>c</sup> | NH <sub>3</sub> <sup>d</sup> |
|---------|-------------------------|------------------|------------------|------------------------------|
| 1 NBS   | Buffer <sup>e</sup>     | 24               | 10               | 14                           |
| 2 NBS   | Buffer                  | 30               | 25               | 35                           |
| 1 NBS   | 0.01 N HBr <sup>f</sup> | 20               | 12               | 28                           |
| 2 NBS   | 0.01 N HBr              | 25               | 23               | 50                           |
| 1 NBS   | 0.1 N HBr               | 13               | 27               | 60                           |
| 2 NBS   | 0.1 N HBr               | 18               | 60               | 130                          |
| 1 NBS   | 0.5 N HBr               | 5                | 35               | 78                           |
| 2 NBS   | 0.5 N HBr               | 10               | 72               | 155                          |

<sup>a</sup> Solutions were 0.1 M in imidazole and contained 20% acetonitrile. <sup>b</sup> Per cent of imidazole converted to tribromoimidazole (TBI) (based on dry weight of crude product). <sup>c</sup> Per cent of imidazole oxidized to glyoxal, based on recovery of its bithiosemicarbazone (TSC). <sup>d</sup> Ammonia determinations by ninhydrin assay (ammonium chloride standard); high values are owing possibly to further oxidation of formamide to carbamic acid by NBS. <sup>e</sup> Acetate buffer (0.2 M, pH 4.5). <sup>f</sup> In HCl solution, mixed chlorobromoimidazoles were obtained.

might be utilized to control the reaction.<sup>2</sup> Moreover there exists an independent interest in selective methods of modifying or degrading the imidazole ring in polypeptides and proteins in which this ring is thought to participate in an enzymatic process.<sup>3</sup>

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<sup>1</sup> Abbreviation used in this work: NBS, *N*-bromosuccinimide.

<sup>2</sup> A preliminary report of this work has appeared in a review article by Witkop (1961).

<sup>3</sup> To date, selective degradation of the imidazole ring in proteins has been achieved only by photooxidation (Weil and Buchert, 1953). For examples of selective chemical modification of imidazole in proteins, see review by Westheimer (1962).

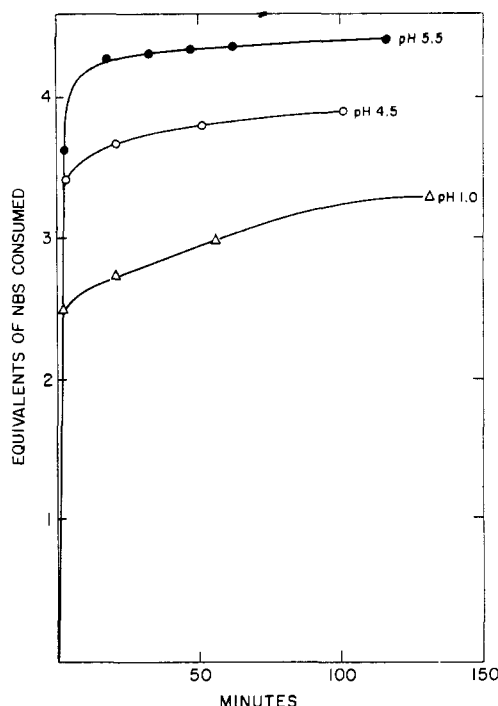


FIGURE 1: Effect of *pH* on rate of consumption of NBS by imidazole. Acetate buffer, 0.2 M, *pH* 4.5, containing 20% acetonitrile; six equivalents of NBS added.

In recent discussions on the chemistry of imidazoles, an emphasis on their lack of reactivity toward oxidants such as chromic acid (Acheson, 1960; Albert, 1959; Katritzky and Lagowski, 1960; Wiley, 1953; Loudon, 1957) has somewhat overshadowed the fact that imidazole rings are easily oxidized<sup>4</sup> and ruptured by permanganate (Wyss, 1877; Pinner and Schwarz, 1902; Jowett and Potter, 1903), periodate,<sup>5</sup> peracids (Botwinnik and Prokofjev, 1937), hydrogen peroxide (Radziszewski, 1884; Von Euler and Hasselquist, 1958; Pinner and Schwarz, 1902; Nofre *et al.*, 1961; Inoue, 1953), ozone (Langenbeck, 1924), and ultraviolet light (Weil *et al.*, 1951; Sluyterman, 1961; Schreiber and Pfenningsdorf, 1961). However, the only previous indication that halogen is capable of degrading the imidazole ring follows from the isolation of ammonium bromide upon bromination of the heterocyclic base (Balaban and Pyman, 1922).

The reaction of imidazole with bromine or with NBS in aprotic solvents leads, even with limited amounts of reagent, to the tribromo derivative exclusively.<sup>6</sup> In aqueous media, however, the formation of tribromoimidazole is accompanied by ring degradation, leading to the formation of glyoxal, ammonia,

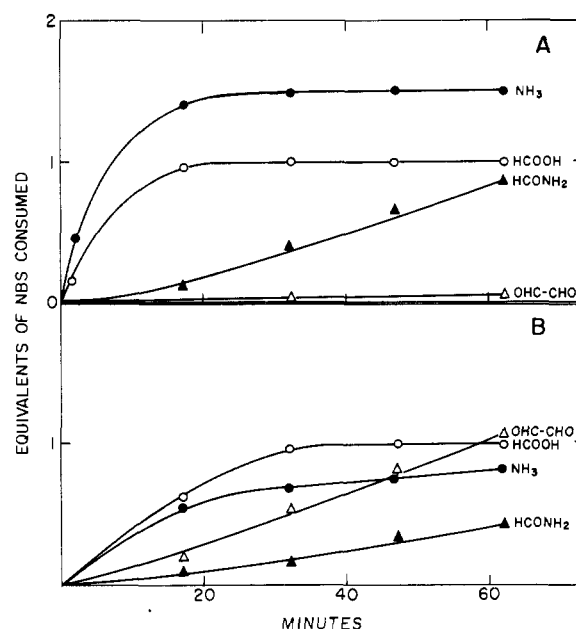


FIGURE 2: Rates of consumption of NBS by degradation products of imidazole. (A) Acetate buffer, 0.2 M, *pH* 4.5, containing 20% acetonitrile and three equivalents of NBS; (B) 0.1 N HCl, containing 20% acetonitrile and three equivalents of NBS. Ammonia is oxidized according to the equation:  $2\text{NH}_3 + 3\text{Br}^+ \rightarrow \text{N}_2 + 3\text{HBr} + 3\text{H}^+$  (Mellor, 1928).

and probably formamide. The extent of the competitive reactions may be followed by (1) recovering and weighing the tribromoimidazole; (2) recovering glyoxal as its bithiosemicarbazone or osazone; (3) determining the ammonia liberated (ninhydrin method). The results thus obtained under various reaction conditions are presented in Table I.<sup>7</sup> The formation of tribromoimidazole is suppressed, while that of glyoxal is enhanced, by increasing the acidity of the medium. Our efforts to analyze rate data (Figure 1) were thwarted by several factors: (1) the extreme rapidity with which the first 2–4 moles of oxidant were consumed; (2) the degradation of tribromoimidazole itself by NBS (compound IV  $\rightarrow$  V);<sup>8,9</sup> (3) the competitive consumption of NBS by the various degradation products (Figure 2). Because the rates of oxidation of these degradation products vary with *pH* (Figure 2), both the initial and the overall rates of NBS consumption by imidazole show a dependence on *pH* (Figure 1).

The oxidative degradation of various 4(5)-substituted imidazoles (compound VII) follows the same course as that of imidazole and leads to the corre-

<sup>4</sup> For extensive reviews of the chemistry of imidazoles, see Hofmann (1953) and Schipper and Day (1957).

<sup>5</sup> L. A. Cohen, unpublished observations.

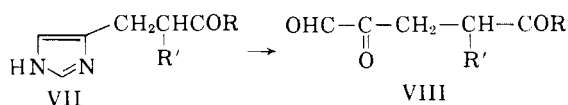
<sup>6</sup> Cf. the rapid formation of tribromoaniline upon bromination of aniline (Francis *et al.*, 1925).

<sup>7</sup> The course of the reaction and yields of products were essentially the same for either NBS or bromine.

<sup>8</sup> Compound V is presumed to be the primary degradation product of compound IV.

<sup>9</sup> The oxidation level of tribromoimidazole precludes its consideration as a source of glyoxal, the latter arising by direct ring oxidation.

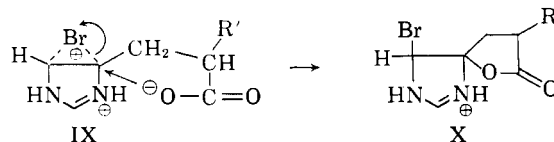
sponding five-carbon ketoaldehydes (compound VIII).



- (a) R = OH, R' = H  
 (b) R = OH, R' = C<sub>6</sub>H<sub>5</sub>CONH  
 (c) R = OH, R' = phthalimido  
 (d) R = OCH<sub>3</sub>, R' = C<sub>6</sub>H<sub>5</sub>CONH  
 (e) R = NHCH<sub>2</sub>COOH, R' = H

In these instances, however, no ring-brominated materials could be detected as alternative products. The ketoaldehydes (compounds VIIa and b) were recovered as bithiosemicarbazones or as quinoxaline derivatives. The effects of reaction conditions on cleavage yield are summarized in Table II. Because of

instability precluded the isolation of reaction intermediates, we consider the degradation of compounds VIIa, b, and c to differ from that of imidazole itself by the fact that the side-chain carboxyl is utilized in an intramolecular displacement reaction (compound IX → X). Further consumption of NBS, owing to oxidation of ammonia and formamide, depends on the relatively



slow breakdown of the intermediate lactone (compound X). As in the case of imidazole (Figure 1), increased acidity leads to a somewhat lower overall rate of NBS

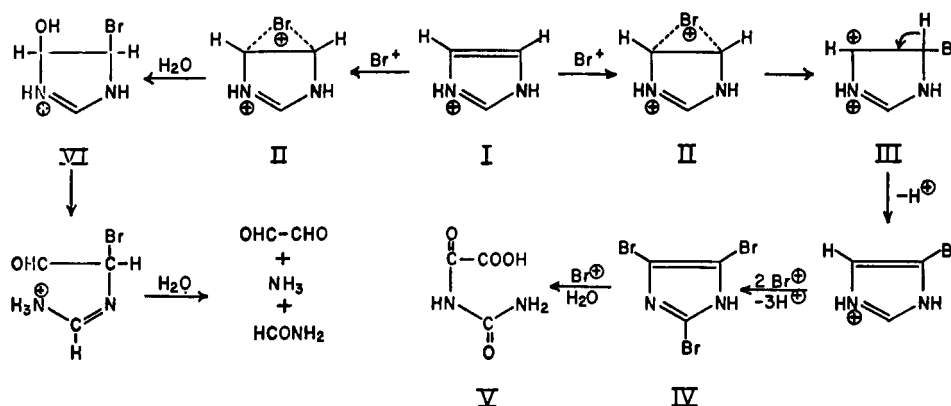


TABLE II: Oxidative Degradation of 4(5)-Substituted Imidazoles.

| Substrate | Medium           | Oxidant <sup>a</sup> | TSC <sup>b</sup> | Quin <sup>c</sup> | NH <sub>3</sub> <sup>d</sup> |
|-----------|------------------|----------------------|------------------|-------------------|------------------------------|
| VIIa      | 0.01 N HCl       | 1 NBS                |                  |                   | 75                           |
| VIIa      | 0.01 N HCl       | 3 NBS                |                  |                   | 136                          |
| VIIa      | 0.01 N HCl       | 2 Br <sub>2</sub>    |                  |                   | 142                          |
| VIIa      | 1 N HCl          | 2 NBS                | 29               | 18                | 130                          |
| VIIa      | H <sub>2</sub> O | 1 NBS                | 36               | 14                | 62                           |
| VIIb      | 1 N HCl          | 1 NBS                | 23               | 58                | 85                           |

<sup>a</sup> Oxidant added as acetonitrile solution (20% final concentration). <sup>b</sup> Per cent oxidation of substrate to ketoaldehyde, based on recovery of its bithiosemicarbazone (TSC). <sup>c</sup> Per cent oxidation of substrate to ketoaldehyde, based on recovery of its quinoxaline (Quin) derivative. <sup>d</sup> Ammonium chloride was used as colorimetric standard for ninhydrin assay; high values are owing to further oxidation of formamide to carbamic acid by NBS.

incomplete precipitation of the derivatives, as well as the formation of by-products during derivative formation, the results of ninhydrin assay provide the more reliable measure of the extent of ring degradation. As is shown in Figure 3, imidazole consumes 3.5 equivalents of NBS almost instantaneously. On the other hand, compounds VIIa, b, and c consume only one equivalent of oxidant in a rapid reaction. Although

consumption by compounds VIIa, b, and c. This effect is more likely due to the pH dependence in the reactivities of ammonia and formamide (Figure 2) than to a variation in the stability of the lactone (compound X) with pH.

Imidazole-4(5)-acetic acid consumes NBS at a rate corresponding to that of imidazole itself (Figure 3b), suggesting that partial stabilization of an intermediate

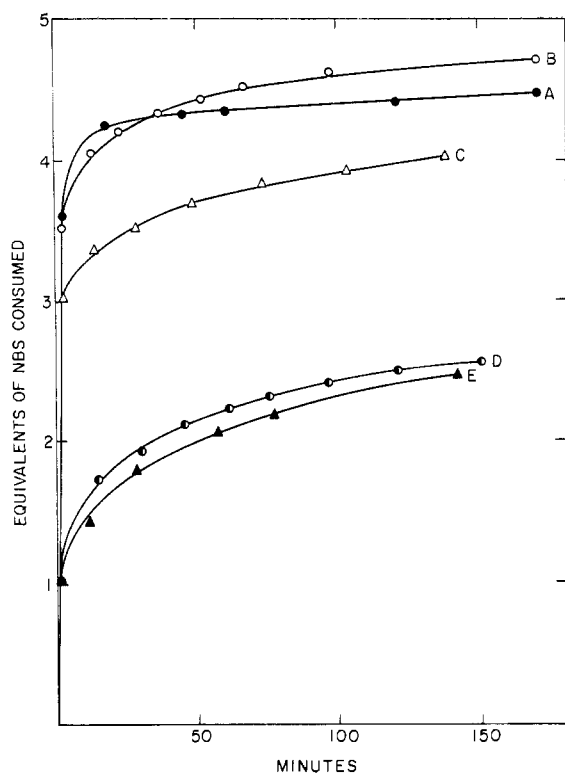
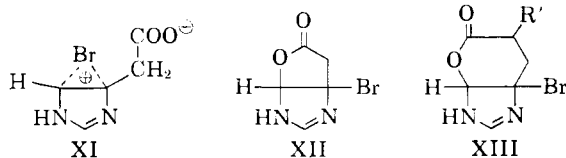


FIGURE 3: Rates of consumption of NBS by 4(5)-substituted imidazoles. Acetate buffer, 0.2 M, pH 4.5, containing 20% acetonitrile and six equivalents of NBS. (A) Imidazole; (B) imidazole-4(5)-acetic acid; (C) compound VIId; (D) compound VIIb or VIIc; (E) compound VIIa.

by formation of a fused-ring system (compound XI  $\rightarrow$  XII) does not occur. In accord with experience in the tryptophan series (Witkop, 1961), the intermediate lactone derived from compound VIIa has been formulated as compound X rather than as XIII.

The reactivity of the methyl ester of *N*-benzoyl-histidine (compound VIId) approaches that of imidazole (Figure 3C), since the stabilizing influence of an intermediate lactone is lacking. Although esters have been shown to serve as nucleophiles in intramolecular addition to olefins (Craig and Witt, 1950; Craig, 1952; Arnold *et al.*, 1953), the reaction conditions were such that the ester function faced relatively little competition from other nucleophiles. In the present instance, the poor nucleophilicity of the ester group, coupled with the relative instability of the bromonium ion intermediate, permits attack by water and subsequent ring degradation to assume predominance.



Imidazole-4(5)-propionylglycine (compound VIIe)

consumes NBS at a rate comparable to that of the propionic ester (compound VIId) rather than of the propionic acid. Accordingly, it may be anticipated that peptide bond cleavage, as a result of amide participation, will not be significant. Indeed, an examination of the products of oxidation of the amide with NBS failed to reveal glycine in detectable quantities.<sup>10</sup> The same amide, in 50% acetic acid, has been found to undergo cleavage with three equivalents of NBS to the extent of 25%, simple histidine peptides behaving in an analogous manner (Shaltiel and Patchornik, 1963). Participation and peptide bond cleavage under the latter reaction conditions may be a result of the decreased water content of the medium.

### Experimental<sup>11</sup>

**Materials.** Imidazole-4(5)-acetic acid was obtained by hydrolysis of 4(5)-cyanomethylimidazole (Bauer and Tabor, 1957). *N*-Benzoyl-L-histidine and its methyl ester were prepared according to the method of Gerngross (1919). *N*-Phthaloyl-DL-histidine was prepared by fusion of L-histidine with phthalic anhydride (Helferich and Böshagen, 1959). Imidazole-4(5)-propionic acid was prepared by hydrogenolysis of  $\alpha$ -chloroimidazolepropionic acid with 5% rhodium on alumina.<sup>12</sup>

**Imidazole-4(5)-propionylglycine (Compound VIIe).** A solution of 379 mg (1 mmole) of ethyl 1-*p*-tolylsulfonfyl-imidazole-4-propionylglycinate<sup>13</sup> in 3 ml of ethanol and 3 ml of 1 N sodium hydroxide was warmed at 55° for 35 minutes. Following removal of ethanol *in vacuo* and chilling of the residual aqueous solution, there was obtained by filtration 55 mg (28%) of ethyl *p*-toluenesulfonate, mp 32–33°. The filtrate was adjusted to pH 5–6 with 1 N hydrochloric acid, diluted to 60 ml with acetone, and chilled overnight. The crystalline product (120 mg, 61%, mp 210°) was recrystallized twice from 90% ethanol to give needles, mp 211–212°. For analysis, the compound was dried at 80° for 18 hours.

**Anal.** Calcd for  $C_8H_{11}N_3O_3$  (mw 197.19): C, 48.72; H, 5.62; N, 21.31. Found: C, 48.73; H, 5.46; N, 21.45.

**Oxidative Degradation of Imidazole (Typical Procedure).** To a solution of 680 mg (0.01 mole) of imidazole in 80 ml of acetate buffer (0.2 M, pH 4.5) was added, with stirring, a solution of 3.56 g (0.02 mole) of NBS in 20 ml of acetonitrile. The mixture was stored at room temperature until it gave a negative reaction with starch-iodide paper (0.5–3 hours). After the reaction mixture had been chilled in ice for several hours, the precipitate of tribromoimidazole was collected by

<sup>10</sup> The NBS degradation of ribonuclease gave no indication of the cleavage of histidyl peptide bonds, under comparable conditions (Wilson and Cohen, 1963b).

<sup>11</sup> Melting points are uncorrected. Microanalyses were performed by the Analytical Service Laboratory of this Institute under the direction of Mr. H. G. McCann.

<sup>12</sup> T. W. Beiler, unpublished experiments.

<sup>13</sup> For a description of the preparation of this compound, see paper VI of this series (Schmir *et al.*, 1965).

pressure filtration.<sup>14</sup> The precipitate was washed with water and air-dried (735 mg, mp 216–219°). Following recrystallization from boiling water, the compound melted at 221–222° (Balaban and Pyman, 1922).

*Anal.* Calcd for  $C_3H_5N_2Br_3$ : Br, 78.66. Found: Br, 78.45.

A 1-ml aliquot of the filtrate was withdrawn and diluted to 100 ml with water. One ml of the resulting solution was assayed for ammonia by the ninhydrin method (Moore and Stein, 1948). To the original filtrate was added 2 g of sodium acetate trihydrate followed by a hot solution of 2.73 g (0.03 mole) of thiosemicarbazide in 20 ml of water. The mixture was stored overnight at room temperature, and the precipitate was collected, washed with water, and air-dried. Purification by suspension in boiling water afforded 510 mg of pale-yellow granules which decomposed >250° without melting.

*Anal.* Calcd for  $C_4H_8N_6S_2$ : C, 23.52; H, 3.95; N, 41.14; S, 31.39. Found: C, 23.31; H, 3.70; N, 41.40; S, 31.60.

In an analogous experiment, the filtrate was treated with 4 g of sodium acetate trihydrate and a filtered solution of 1.5 g of phenylhydrazine hydrochloride in 20 ml of water. The mixture was stored at room temperature for several hours and then chilled. The crystalline osazone was collected by filtration, washed with water, and air-dried (585 mg, 25%, mp 155–160°). After two recrystallizations from benzene-ligroin, there was obtained 234 mg of the osazone, mp 169–171°; mixed mp with an authentic sample (Neuberg and Rosenthal, 1914), 169–171°. Infrared spectra of the two samples in chloroform were identical.

*Oxidative Degradation of Imidazole-4(5)-propionic Acid (Compound VIIa).* (A) ISOLATION OF GLYOXAL-PROPIONIC ACID AS ITS BISTHIOSEMICARBAZONE. To a stirred solution of 1.4 g (0.01 mole) of compound VIIa in 50 ml of water was added, in one portion, a solution of 1.78 g (0.01 mole) of NBS in 15 ml of acetonitrile. After several minutes, the acetonitrile was removed *in vacuo* and a solution of 2 g sodium acetate trihydrate and 2.73 g thiosemicarbazide in 15 ml water was added to the reaction mixture. Crystals began to separate after 5 minutes. After the solution had stood 6 hours at 25°, the precipitate was collected (0.89 g, mp 223–224°). Upon acidification of the mother liquor, an additional 0.1 g was obtained, to give a total yield of 36%. The material was recrystallized from water as pale yellow needles, mp 225–226°.<sup>15</sup>

*Anal.* Calcd for  $C_7H_{12}N_6O_2S_2$ : C, 30.42; H, 4.38; N, 30.42; S, 23.20. Found: C, 30.47; H, 4.28; N, 29.80; S, 23.70.

(B) ISOLATION OF GLYOXALPROPIONIC ACID AS QUIN-  
OXALINE-2-PROPIONIC ACID. To a stirred solution of 1.177 g (8.4 mmoles) of compound VIIa in 40 ml of water was added, in one portion, a solution of 1.5 g

of NBS in 15 ml of acetonitrile. After several minutes, the acetonitrile was removed *in vacuo* and a solution of 0.91 g of *o*-phenylenediamine in 15 ml of warm ethanol was added. After 2 hours at 25°, the mixture was heated on a steam cone for 20 minutes. Ethanol was removed *in vacuo*, and the solution was adjusted to pH 2.5–3 with 6 N hydrochloric acid and evaporated to dryness. The residue, after drying in a desiccator, was extracted three times with hot ethyl acetate. The extract was evaporated to dryness, yielding 1.21 g of an oily solid. The material was extracted three times with hot ligroin; upon cooling of the ligroin extract, a crystalline precipitate, 0.24 g (14%), mp 107–112°, was obtained. After two recrystallizations from hot ligroin, the product melted at 115–117° (Jones *et al.*, 1950).

*Anal.* Calcd for  $C_{11}H_{10}N_2O_2$ : C, 65.33; H, 4.99; N, 13.86. Found: C, 65.30; H, 5.14; N, 13.85.

*Oxidative Degradation of N-Benzoyl-L-histidine (Compound VIIb).* (A) ISOLATION OF *N*-BENZOYL-4-KETO-L-GLUTAMIC- $\gamma$ -SEMIALDEHYDE AS ITS BISTHIOSEMICARBAZONE. To a stirred solution of 1.1 g (4 mmoles) of *N*-benzoyl-L-histidine monohydrate in 50 ml of water and 5 ml of 1 N hydrochloric acid was added, in one portion, a solution of 712 mg (4 mmoles) of NBS in 6 ml of acetonitrile. After several minutes, there was added 3 g of sodium acetate trihydrate followed by a solution of 1.1 g of thiosemicarbazide in 10 ml of hot water. After the mixture had been kept at room temperature for 20 minutes, it was acidified to pH 3 with 6 N hydrochloric acid. After storage for 9 hours, the product was collected (367 mg, 23%). For purification, the material was suspended in boiling water and dimethylformamide was added dropwise until solution was complete. After the solution had cooled, the crystals which had separated were collected and washed with water, alcohol, and ether, and melted at 234–235°. Following recrystallization from a mixture of acetic acid, dimethylformamide, and water, the product was washed with water and ethanol. After the material had been dried at 100° *in vacuo* for 20 hours, it melted at 240–241° (decomp). Further attempts to purify the derivative led to partial decomposition.

*Anal.* Calcd for  $C_{14}H_{17}N_7O_3S_2$ : C, 42.52; H, 4.33; N, 24.80; S, 16.22. Found: C, 42.59; H, 4.60; N, 23.72; S, 17.51.

(B) ISOLATION OF *N*-BENZOYL-4-KETO-L-GLUTAMIC- $\gamma$ -SEMIALDEHYDE AS *N*-BENZOYL-3-(2-QUINOXALYL)-L-ALANINE. To a stirred solution of 1.1 g (4 mmoles) of *N*-benzoyl-L-histidine monohydrate in 25 ml of water and 5 ml of 1 N hydrochloric acid was added, in one portion, a solution of 712 mg (4 mmoles) of NBS in 6 ml of acetonitrile. After several minutes, there was added 1 g of sodium acetate trihydrate followed by a solution of 450 mg of *o*-phenylenediamine in 6 ml of warm ethanol. After the mixture had been stored 1 hour at room temperature, it was adjusted to pH 2 with acid and the crystalline precipitate (560 mg, mp 220–221° decomp) was collected. After 24 hours, an additional 187 mg of material was deposited from the filtrate; total yield, 58%. After two recrystallizations from abs

<sup>14</sup> Suction filtration could not be used owing to the volatility of glyoxal.

<sup>15</sup> Neuberg and Collatz (1930) report mp 223–225° decomp.

ethanol, the derivative was obtained as silky needles, mp 223–224° decomp.<sup>16</sup>

*Anal.* Calcd for  $C_{18}H_{15}N_3O_3$ : C, 67.27; H, 4.71; N, 13.08. Found: C, 67.52; H, 4.92; N, 12.93.

*Determination of Rates of Consumption of NBS.* A solution of 0.2 mmole of the substrate in 16 ml of acetate buffer (0.2 M, pH 4.5) was adjusted to the desired pH using a Beckman Zeromatic pH meter. To the solution was added 4 ml of acetonitrile containing 3–6 equivalents of NBS. The reaction mixture was stored in a glass-stoppered flask in a constant-temperature room (25°) and was protected from intense light. One-ml aliquots were withdrawn at various time intervals and pipetted into flasks containing 1 ml of 10% potassium iodide and 9 ml of 1 N sulfuric acid. The iodine liberated was titrated with sodium thiosulfate using a starch indicator. For reactions conducted in acidic media, acetate buffer was replaced by 0.1 N hydrochloric acid.

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<sup>16</sup> The racemate of this compound has been reported by Ried and Schiller (1953).