Alkaline Bromine Oxidation of Amino Acids and Peptides: Formation of α-Ketoacyl Peptides and Their Cleavage by Hydrogen Peroxide*

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Hypobromite oxidation in strong alkali gave nitriles of one less carbon atom from a number of amino acids and from the amino terminal residue in dipeptides. The carboxyl terminal residue of a dipeptide was liberated as the free amino acid on acidification of the oxidation mixture. Hypobromite oxidation in weak alkali (pH 9.4) again gave nitriles of one less carbon atom from amino acids but brought about an oxidative deamination of the free amino group in di- and higher peptides to give *N*-(α-ketoacyl) peptides. These latter compounds were cleaved by hydrogen peroxide in alkali to give a fatty acid of one less carbon atom from the α-ketoacyl moiety and, after acidification, the carboxyl terminal amino acid or peptide residue.

Although the oxidation of amino acids and proteins by *N*-bromosuccinimide and *N*-bromoacetamide has been the subject of several recent investigations (Chappelle and Luck, 1957; Konigsberg *et al.*, 1960; Stevenson and Luck, 1961; Patchornick *et al.*, 1960 and earlier works cited therein), little has appeared on the action of hypobromite (or alkaline bromine) on amino acids, peptides, and proteins since the extensive studies of Goldschmidt and collaborators in the late 1920's and early 1930's. Goldschmidt *et al.* (1927) studied the oxidation of amino acids and dipeptides with hypobromite under two sets of conditions: in 0.1 N potassium hydroxide and in "neutral" solution. The latter involved oxidation with a hypobromite solution prepared by admixture of 2 moles of potassium hydroxide with 1 mole of bromine (Br_2). When the hypobromite in such solutions was destroyed with hydrogen peroxide, the alkali concentration was about 0.001 N (Goldschmidt and Steigerwald, 1925; Goldschmidt, 1927). Under either condition, simple aliphatic amino acids (I) were converted to the nitriles of one less carbon atom (II) and carbon dioxide, with the consumption of 2 moles of hypobromite.

$$\underset{\text{I}}{H_2NCH(R)COO^-} + 2\,OBr^- + OH^- \longrightarrow \underset{\text{II}}{RC{\equiv}N} + CO_3^- + 2\,Br^- + 2\,H_2O$$

With dipeptides the reaction followed different courses under the two conditions. Under "neutral" conditions (A) the dipeptide (III) consumed about 2.5 moles of hypobromite with the oxidative deamination of the free amino group and the formation of the α-ketoacyl amino acid (IV) (isolated as its phenylhydrazone).

$$\underset{\text{III}}{H_2NCH(R)CONHCH(R')COO^-}$$

$$\xrightarrow{A:\ 2.5\ OBr^-} \tfrac{1}{2}\,N_2 + \underset{\text{IV}}{RCOCONHCH(R')COO^-} + 1.5\,H_2O + 2.5\,Br^-$$

$$\xrightarrow[OH^-]{B:\ 2\ OBr^-} 2\,Br^- + \underset{\text{II}}{RC{\equiv}N} + [{}^-OOCNHCH(R')COO^-]_{\text{V}} + 2\,H_2O \xrightarrow{H^+} CO_2 + \underset{\text{VI}}{{}^+H_3NCH(R')COOH}$$

In quite alkaline solution (B), the dipeptide consumed 2 moles of hypobromite with the formation of the nitrile of one less carbon atom (II) from the amino terminal amino acid; presumably the carbamino acid (V) was formed as an intermediate. The intermediate was resistant to further oxidation with hypobromite, but upon destruction of the excess hypobromite and acidification, the free amino acid was liberated.

On the basis of the above results with dipeptides, Goldschmidt *et al.* (1927) suggested that the alkaline hypobromite oxidation might be applied to the elucidation of the sequence of amino acids in proteins. The amino terminal amino acid would be determined from the nitrile split off during oxidation in alkaline solution. After the destruction of excess oxidizing agent, the solution would be acidified to liberate the free amino group of the amino acid next to the original amino terminal amino acid. The oxidation procedure would be repeated to give, in theory, the next to the original amino terminal

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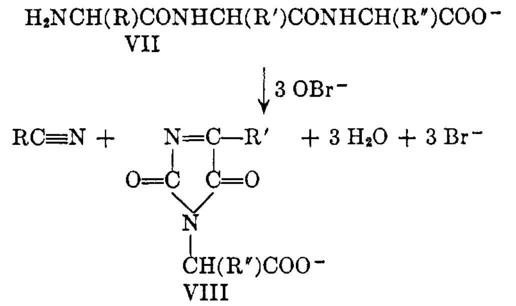
amino acid as a nitrile, and so on.

The proposed sequential method of degradation from the amino terminal end met with difficulties when it was found by Goldschmidt and Strauss (1929, 1930a) that a third reaction took place during the oxidation of tri- and tetrapeptides under *strongly alkaline* conditions. The tri- (VII) and tetrapeptides consumed 3 moles of hypobromite in the oxidation under alkaline conditions, again with the formation of a nitrile (II) from the amino terminal amino acid, but with production of a dehydrohydantoin (VIII) (instead of a carbamino compound) from the rest of the molecule. The formation of the stable dehydrohydantoin from tri- and higher peptides precluded the use of this series of reactions as a sequence method. Goldschmidt and Strauss did not report on the reaction of tri- and higher peptides with hypobromite under the "neutral" conditions.



Our interest in these reactions stemmed from observations made in another connection. Namely, a di- or tripeptide was treated with hypobromite in a buffered solution at pH 9.4 (similar to Goldschmidt's "neutral" conditions) and the excess hypobromite was removed with *hydrogen peroxide*. Upon paper chromatography of the reaction mixture, we detected the carboxyl terminal amino acid (instead of the α-ketoacyl amino acid) or the carboxyl terminal dipeptide (instead of the dehydrohydantoin) from the oxidation of the corresponding di- or tripeptide. The present report describes our investigation of these reactions. In addition to the peptides, we investigated the oxidation of a number of amino acids. Some of these results are discussed in the present communication.

Oxidation of Amino Acids.—The consumption of hypobromite per mole of compound was determined in the presence of excess hypobromite as a function of time at 0° in buffered solution at pH 9 4 and also in N potassium hydroxide. Comparison of the results obtained with certain selected amino acids shows that the rate of oxidation was generally faster in N potassium hydroxide (Fig. 1) than at pH 9.4 (Fig. 2). With the exception of the simple aliphatic amino acids, the compounds were oxidized to different degrees at the two pH values, the extent of oxidation generally being greater in N potassium hydroxide than at pH 9.4. The amount of oxidant consumed per mole of compound was used as a guide in determining the products of the reaction. In the present studies, investigation of the products was largely confined to those amino acids and pH conditions where the oxidant consumption was about 2 moles per mole of compound. Investigation of the products formed from those amino acids which consumed an excessive amount of the reagent was left for the future.

The products identified in the reaction mixture from the oxidation of a number of amino acids are shown in Table I. In several instances gas-liquid chromatography was used as an aid in identification and also to estimate the yields. Like previous workers (Goldschmidt *et al.*, 1927; Friedman and Morgulis, 1936), we found that the simple aliphatic amino acids (alanine, valine, and leucine) were oxidized to the corresponding nitriles of one less carbon atom and the same products were obtained in essentially the same yield at pH 9.4 as in N potassium hydroxide. We did not analyze for the presence of aldehydes, which have been reported (Friedman and Morgulis, 1936) to be formed in small amounts in the hypobromite oxidation of some amino acids. Aspartic acid behaved differently at the two pH values. At pH 9.4 it gave a small yield of cyanoacetic acid, while in strong alkali the main product identified was dibromoacetamide. This probably arose from the expected product, cyanoacetic acid, by bromination of the α-carbon, followed by decarboxylation and partial hydrolysis of the nitrile to an amide. The rather unusual fact that an amide hydrolyzed more slowly than the nitrile (and thus accumulated) may be due to steric interference by the bromine atoms on the α-carbon atom (Cason *et al.*, 1953).

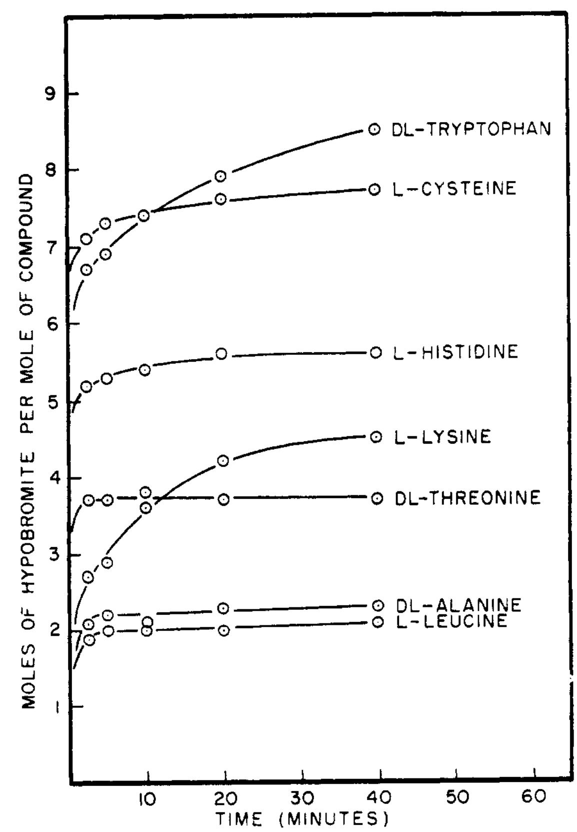


FIG. 1.—Rate of consumption of hypobromite by a number of amino acids during oxidation at 0° in N potassium hydroxide.

TABLE I

PRODUCTS OBTAINED IN THE HYPOBROMITE OXIDATION OF AMINO ACIDS

Amino Acid Oxidized	pH[a]	OBr^- Consumed[b] (mole/mole)	Oxidation Product	Yield (%)
Alanine	9.4	2.0	Acetonitrile[c]	70
Alanine	14	2.2	Acetonitrile[c]	70
Valine	9.4	2.1	Isobutyronitrile[c]	50
Valine	14	2.3	Isobutyronitrile[c]	50
Leucine	9.4	2.0	Isovaleronitrile[c]	70
Leucine	14	2.1	Isovaleronitrile[c]	89
Aspartic acid	9.4	2.6	Cyanoacetic acid[d,e]	23
Aspartic acid	14	2.4	Dibromoacetamide[f]	33
Asparagine	14	2.5	Cyanoacetamide[e,g]	40
Glutamic acid	14	2.4	β-Cyanopropionic acid[c,h]	58
Lysine	14	4.2	Glutaronitrile[c,i]	45

[a] pH 14 refers to N potassium hydroxide. [b] After a 20-minute reaction time. [c] Identified by behavior in gas-liquid chromatography. [d] M.p. 150–160°. Meisenheimer and Schwarz (1906) give 169°. [e] Infrared spectrum was identical with authentic sample. [f] M.p. 153–156°. Steinkopf (1905) gives 155–156°. Contains an amide peak at 6.0 μ. *Anal.*: Calcd. for $C_2H_3NOBr_2$: N, 6.46; Br, 73.69 Found: N, 6.18; Br, 72.05. [g] M.p. and mixed m.p. with authentic sample was 120–121°. [h] After the nitrile was refluxed in 5.7 N hydrochloric acid for 3 hours, it gave succinic acid; m.p. and mixed m.p. with authentic sample was 184–186°. [i] The nitrile was hydrolyzed to give glutaric acid; m.p. and mixed m.p. with authentic sample was 94–95°.

In contrast to aspartic acid, asparagine gave the normal product, cyanoacetamide, on oxidation in strong alkali. The failure of asparagine to undergo a Hoffman rearrangement (at least to any marked degree) should be noted. Glutamic acid upon oxidation in strong alkali also gave the normal oxidation product, β-cyanopropionic acid, in fair yield. When lysine was treated with excess hypobromite in N potassium hydroxide, over 4 moles of hypobromite were consumed and a 45% yield of the dinitrile (glutaronitrile) was obtained. The isolation of this dinitrile indicates that oxidation of the ε-amino group took place as well as the normal oxidation of the α-amino and carboxyl groups. Oxidation of aliphatic amines of five or more carbon atoms to nitriles is not unexpected (Mowry, 1948). The consumption of about 2 moles of hypobromite upon oxidation of lysine at pH 9.4 indicates the probable formation of δ-amino-valeronitrile or δ-cyano-α-amino-valeric acid under these conditions, but the product has not been isolated and identified.

Although a number of naturally occurring amino acids remain to be investigated, from those so far studied it appears that the hypobromite oxidation (especially at pH 9.4) may give unique and volatile products from each amino acid. The volatility of the products is of interest from the point of view of gas-liquid chromatography. Application of this technique to the amino acid analysis of proteins is dependent upon conversion of the amino acids to volatile derivatives. Hypobromite oxidation may afford a means of obtaining volatile derivatives from the amino acids. In this connection it may be noted that Bayer (1958) has reported on the use of gas-liquid chromatography to separate the aldehydes produced by hypochlorite oxidation of the simple aliphatic amino acids.

Oxidation of Peptides.—Several dipeptides, a tripeptide, and a tetrapeptide were made up (McGregor and Carpenter, 1961) in amounts large enough for investigation of the reaction under a variety of conditions. As noted above, Goldschmidt and co-workers (1927) had investigated the hypobromite oxidation of dipeptides in "neutral" and alkaline solution. Their reports on tri- and higher peptides, however, were restricted to oxidations under quite alkaline conditions. Our results on the rate and extent of consumption of hypobromite at 0° and pH 9.4 or in N alkali are shown for several selected peptides in Figures 3 and 4. As was true with the amino acids, the rate as well as the extent of oxidation appeared greater in strong alkali than at pH 9.4. In agreement with Goldschmidt (reaction B, above) we found that oxidation of dipeptides in *strong alkali* consumed approximately 2 moles of hypobromite and gave a nitrile of one less carbon atom from the amino terminal amino acid. After destruction of the excess hypobromite and acidification of the medium, the carboxyl terminal amino acid was liberated in free form Thus, alanyl-leucine, alanyl-valine, and alanyl-methionine gave acetonitrile and leucine, valine, or methionine sulfoxide respectively. With a number of other dipeptides, where no attempt was made to determine the nitrile, the carboxyl terminal amino acid was detected after acidification of the oxidation mixture, *e.g.*, alanine from leucyl-alanine (McGregor, 1959).

The tri- and tetrapeptides consumed 3 to 4 moles

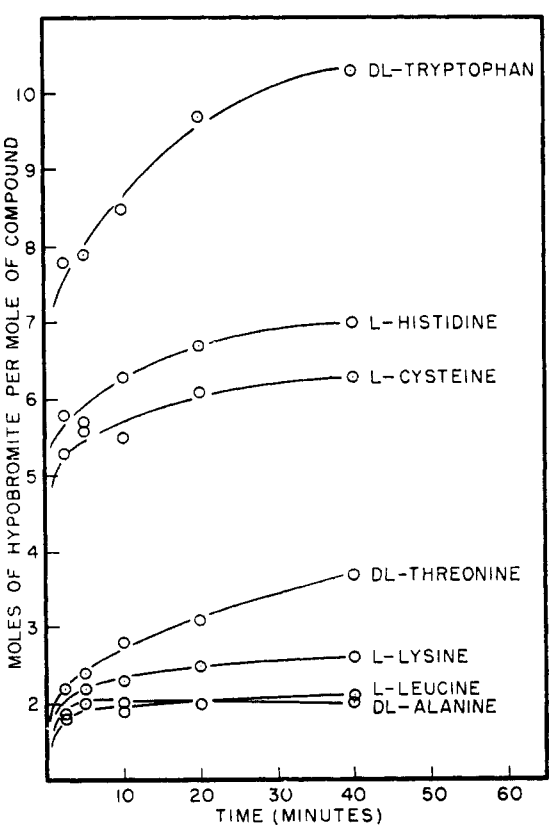


FIG. 2.—Rate of consumption of hypobromite by a number of amino acids during oxidation at 0° at pH 9.4.

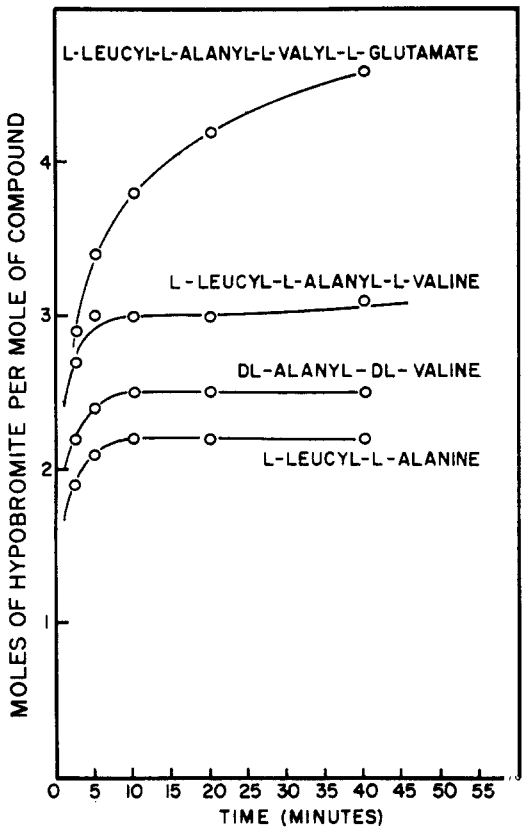


FIG. 3.—Rate of consumption of hypobromite during oxidation of several peptides at 0° in N potassium hydroxide.

of hypobromite in strongly alkaline reaction. After decomposition of the excess hypobromite with either hydrogen peroxide or sodium sulfite, followed by acidification, no ninhydrin-reacting materials were found in the reaction mixture. This is the result that would be expected if, as stated by Goldschmidt and Strauss (1929, 1930a) (see above), the oxidation with hypobromite of tri- and tetrapeptides in strongly alkaline solution proceeds to the formation of dehydrohydantoins.

When dipeptides were oxidized at pH 9.4 and the excess hypobromite was destroyed with *hydrogen peroxide*, the carboxyl terminal amino acid was detected in the reaction mixture. This made it appear that the oxidation at pH 9.4 was taking the same course as in strongly alkaline solution. However, no nitrile was detected as one of the oxidation products of a dipeptide at pH 9.4. Furthermore, if the excess hypobromite was removed from the oxidation at pH 9.4 with *sodium sulfite* instead of *hydrogen peroxide*, the carboxyl terminal amino acid was not detected on paper chromatography of the reaction mixture. Instead a substance was present on the chromatograms near the solvent front which gave a delayed ninhydrin color reaction, the color appearing after the papers had been kept at room temperature for several days. By making use of this delayed ninhydrin color reaction as a test, it was possible to isolate in crystalline form the product of the hypobromite oxidation at pH 9.4 of alanyl-valine. The properties of this product indicate that it is pyruvoyl-valine (IV, R = methyl; R′ = isopropyl). Thus when sodium sulfite was used instead of hydrogen peroxide to remove the excess hypobromite, the results of hypobromite oxidation of dipeptides were in agreement with those reported under the "neutral" condition by Goldschmidt *et al.* (1927) (reaction A).

A similar result was obtained in the oxidation of a tripeptide at pH 9.4. Leucyl-alanyl-valine gave a crystalline compound with the properties of *N*-(α-ketoisocaproyl)-alanyl-valine. Furthermore, upon oxidation at pH 9.4, leucyl-alanyl-valyl-glutamic acid, as well as several dipeptides, gave noncrystalline products whose properties (ultraviolet absorption and chromatographic behavior before and after hydrolysis) were those of an α-ketoacyl peptide structure (McGregor, 1959). Thus the main products of the hypobromite oxidation at pH 9.4 of tri- and tetrapeptides, as well as dipeptides, appear to be the α-ketoacyl compounds rather than the dehydrohydantoins observed to be formed by Goldschmidt and Strauss (1929, 1930*a*) upon oxidation of these higher peptides in strongly alkaline solution.

Hydrogen Peroxide Cleavage of α-Ketoacyl Peptides.—The similarity of structure between the α-ketoacyl peptides and α-diketones indicated that the former might be cleaved by alkaline hydrogen peroxide in a manner similar to the α-diketones (Bunton, 1949).

$$\underset{\text{IV}}{\text{RCOCONHCH(R')COO}^-} + \text{H}_2\text{O}_2 + 2\,\text{OH}^- \longrightarrow \text{RCOO}^- + [^-\text{OOCNHCH(R')COO}^-] + 2\,\text{H}_2\text{O}$$

$$[^-\text{OOCNHCH(R')COO}^-] \xrightarrow{\text{H}^+} \text{CO}_2 + {}^+\text{H}_3\text{NCH(R')COOH}$$

Such a reaction would explain the differences in reaction products that we observed when hydrogen peroxide was used instead of sodium sulfite to remove the excess hypobromite from the reaction mixture. The rate and extent of cleavage of pyruvoyl-valine and α-ketoisocaproyl-alanyl-valine by hydrogen peroxide in 0.1 N potassium hydroxide at 37° was determined by measuring the formation of the amino acid or peptide produced in the cleavage. These experiments showed that in the presence of excess hydrogen peroxide, pyruvoyl-valine or α-ketoisocaproyl-alanyl-valine was converted to valine or alanyl-valine in yields of 70 and 60% respectively. The reaction was essentially complete after 30 minutes under these conditions. At this time determination of fatty acids by gas chromatography showed the presence of acetic acid (60%) or isovaleric acid (80%) as products of the hydrogen peroxide treatment of pyruvoyl-valine or α-ketoisocaproyl-alanyl-valine respectively. The oxidation of α-ketoacyl peptides with hydrogen peroxide was accompanied by a decrease in the absorbancy of the band at 240 mμ, which is attributed to the α-ketoamide group (Errera and Greenstein, 1947).

Ninhydrin Color Reaction.—The response of these α-ketoacyl peptides to the ninhydrin color test in the procedure of Moore and Stein (1954) is of interest. Although in most cases the amount

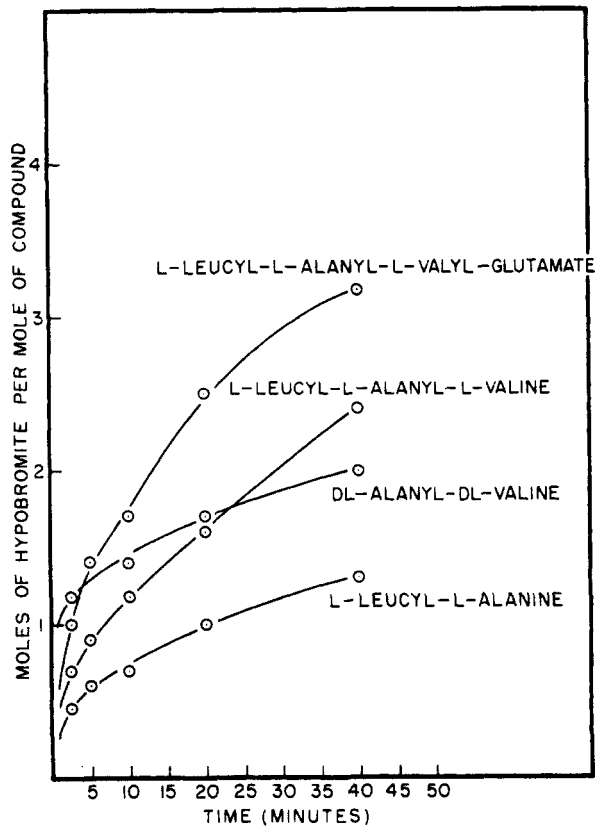


FIG. 4.—Rate of consumption of hypobromite during oxidation of several peptides at 0° at pH 9.4.

of color produced when the ninhydrin test is performed in solution is proportional to the free α-amino groups (Moore and Stein, 1954), it has been found that certain peptides can yield an amount of color in considerable excess of that predicted on the basis of the free α-amino group (Dowmont and Fruton, 1952; Yanari, 1956). A similar observation was made here with several peptides. In addition some of the α-ketoacyl peptides also gave a ninhydrin color reaction although they do not contain a free α-amino group. When the color test was performed by the method of Moore and Stein (1954) and the color yield was compared with that of alanine, the following alanine equivalents were obtained: alanyl-valine (1.6), pyruvoyl-valine (0.95), leucyl-alanyl-valine (1.17), and α-ketoisocaproyl-alanyl-valine (0.58). The rate of production of color was quite a bit slower for the α-ketoacyl compounds than for those bearing a free α-amino group. As a consequence it was possible to analyze for each type of compound in a mixture by using a combination of short (2 minutes) and long (30 minutes) heating times in the color development reaction.

DISCUSSION

The oxidation reactions described above have some obvious implications for work in protein structure: The amino terminal amino acid can be determined from the nitrile released on hypobromite oxidation in strong alkali; sequential degradation of the peptide chain from the amino terminal end may be brought about by repeated alternate oxications with hypobromite at pH 9.4 followed by cleavage with hydrogen peroxide. At each stage the amino terminal residue would be determined by the fatty acid released in the hydrogen peroxide treatment.

However, the complications arising from the numerous possibilities for oxidation of the side-chains of polyfunctional amino acids will probably limit the application of these reactions to small peptides. *N*-Bromosuccinimide has an action on amino acids (Stevenson and Luck, 1960) and simple peptides (Heyns and Stange, 1955) that is similar to the hypobromite oxidation at pH 9.4. However, even in acid solution, *N*-bromosuccinimide can bring about a splitting of peptide bonds in proteins containing tryptophan (Ramachandran and Witkop, 1959). Presumably, similar reactions would take place with hypobromite, greatly complicating its use in a sequential method.

EXPERIMENTAL

Melting points are uncorrected. Elementary analyses were performed by the Microchemical Laboratory, Chemistry Department, University of California, Berkeley. The dipeptides, DL-alanyl-DL-leucine, DL-alanyl-DL-valine, and DL-alanyl-DL-methionine, were purchased from Mann Research Laboratories. The other peptides used in this study were synthesized in this laboratory (McGregor and Carpenter, 1961). With the exception of β-cyanopropionic acid, which was synthesized according to the procedure of Gresham *et al.* (1952), the remaining nitriles used as standards in the gas-liquid chromatography were of commercial origin (Eastman Organic Chemicals or Matheson, Coleman and Bell).

Gas-Liquid Chromatography of the Nitriles.—The nitriles were subjected to gas-liquid chromatography on the Aerograph Model 110-C equipped with 5-ft, commercially packed columns of Carbowax (column A), LAC-446 (column B), or silicone (GESF-96) (column C). Helium was used as the carrier gas at a flow rate of 60 ml per minute. The retention times for authentic samples of nitriles were determined under the same conditions as those used for testing the products of the oxidation, with agreement in retention times within ±2%. Quantitation of the area under the peak was done by the peak height—half-band width method (Pecsock, 1959). In the cases of acetonitrile, isobutyronitrile, and isovaleronitrile, a known amount of *n*-butyronitrile was added to the unknown to serve as an internal standard. The recoveries were corrected for losses suffered in a standard sample of nitrile which had been carried through the whole procedure. The correction amounted to as much as 50% in the case of acetonitrile. The retention times for the nitriles obtained on the various columns (A, B, or C) and temperatures were as follows: acetonitrile (column B) at 27° was 30 minutes; acetonitrile (column B) at 32° was 22 minutes; *n*-butyronitrile (column B) at 27° was 87 minutes; *n*-butyronitrile (column C) at 28° was 32 minutes;

isobutyronitrile (column B) at 40° was 24 minutes; isovaleronitrile (column C) at 28° was 57 minutes; β-cyanopropionic acid (column A) at 73° was 15 minutes; glutaronitrile (column A) at 75° was 19 minutes.

Determination of Rate and Degree of Hypobromite Oxidation. For the reactions at pH 9.4, 5 ml of a 0.02 to 0.04 M solution of the amino acid or peptide in N potassium carbonate was added to 5 ml of a 0.37 M solution of bromine (Br_2) in N potassium carbonate (0.1 ml of bromine in 5 ml of N potassium carbonate). For the reaction at high pH, N potassium hydroxide was substituted for the potassium carbonate in the above mixtures. The reactions were performed in an ice-bath with precooling of reactants before mixing. At specified time intervals, 1-ml aliquots were removed and added to a mixture of 10 ml of 10% potassium iodide and 2 ml of 5.7 N hydrochloric acid. The acidic mixture was left in the dark for 5 minutes and then titrated with 0 1 N sodium thiosulfate to the starch-iodine end-point. For calculation of the stoichiometry of the reaction, the oxidant was considered to be hypobromite.

Hypobromite Oxidations for Product Identification.—The oxidations for product identification were performed under conditions similar to those described above with the use of larger amounts of material: 25–250 ml of about 0.07 M solution of the amino acid or peptide in either N potassium carbonate (pH 9.4 oxidations) or N potassium hydroxide (high pH oxidations) were mixed with an equal volume of 0.38 M bromine (Br_2) in either N potassium carbonate or N potassium hydroxide. The reactions were stopped after 20 minutes (except for threonine, 2.5 minutes) at 0° by addition of sodium sulfite and acidification of the mixture to pH 7 to destroy the excess hypobromite. This mixture will be referred to hereafter as the *neutral solution.*

Aliquots were removed from the neutral solution for determination of the ninhydrin color yield by the quantitative method of Moore and Stein (1954). Other aliquots were removed for qualitative determination of amino acids or peptides by paper chromatography using as solvent systems either 1-butanol, acetic acid, water (4:1:1) (Solvent I) (Reed, 1950) or 1-butanol, acetic acid, pyridine, water (30:6:20:24) (Solvent II) (Waley and Watson, 1953). The remainder of the neutral solution was used for the isolation of the nitriles.

For the relatively volatile nitriles (acetonitrile, isovaleronitrile, and isobutyronitrile), the *neutral solution* was extracted at least three times with equal volumes of methylene chloride (or ether). The combined extracts were dried over magnesium sulfate and concentrated to a small volume, a short condenser being used to minimize loss of nitrile. A known amount of *n*-butyronitrile was added and the solution was made up to a known volume (to contain about 10 mg per ml) for investigation by gas-liquid chromatography.

For the other nitriles the *neutral solution* was concentrated to dryness *in vacuo* at a bath temperature under 35°. Neutral products were extracted directly with ether or ethyl acetate. Acidic products were extracted after acidification of the residue. The organic solvent was removed *in vacuo* to give the crude product as either crystals or an oil. The oils were identified by gas-liquid chromatography and the crystals by melting point, infrared spectra, and, where necessary, analysis. For a summary of the results with the amino acids see Table I. The results with the peptides are given below.

Hypobromite Oxidation of Peptides in N Potassium Hydroxide—Acetonitrile, identified by gas-liquid chromatography, was extracted from the *neutral solution* after oxidation in N potassium hydroxide of alanyl-leucine, alanyl-valine, and alanyl-methionine. Leucine, valine, and methionine sulfoxide were present on paper chromatograms of the neutral solution from the oxidation of the respective alanyl peptides Quantitative ninhydrin analyses of the components in the neutral solution indicated that the free amino acids were present in about 90% of the theoretical yield from the dipeptides.

When leucyl-alanyl-valine or leucyl-alanyl-valyl-glutamic acid was oxidized in the strong alkali as described above, no ninhydrin-reactive substances could be detected in the neutral solution either by the quantitative method or by chromatography on paper.

Hypbromite Oxidation of Peptides at pH 9.4.—Alanyl-leucine and alanyl-valine were oxidized at pH 9.4 and extracts of the *neutral solution* were investigated for acetonitrile by gas-liquid chromatography with negative results. When the quantitative ninhydrin color test was performed on these neutral solutions, they gave a color yield equivalent to that expected for the production of 1 mole of amino acid from the original peptide. However, no free amino acids were detected when paper chromatograms of the neutral solution were developed with ninhydrin. When the papers were allowed to stand at room temperature for several days, however, faint violet spots developed near the solvent front. Thus, from the oxidation of alanyl-valine, a substance with R_F values of 0.91 in Solvent I and 0.81 in Solvent II was detected by the formation of violet-colored spots several days after spraying with ninhydrin. When the material was eluted from the paper, hydrolyzed in acid, and rechromatographed, valine was the only ninhydrin-reactive material in the hydrolysate.

Leucyl-alanyl-valine as well as leucyl-alanyl-valyl-glutamic acid gave about the same color yield in the quantitative ninhydrin reaction before and after oxidation at pH 9.4. Upon paper chromatography, no free amino acids or peptides were detected. However, from the oxidation of the tripeptide, a material with R_F values of 0.90 (Solvent I) and 0.85 (Solvent II) was detected several days after the paper had been sprayed with ninhydrin.

Pyruvoyl-DL-valine.—DL-Alanyl-DiL-valine (250 mg.) was oxidized with hypobromite at pH 9.4 as described above. The *neutral solution* was concentrated to a small volume, acidified with a slight excess of hydrochloric acid, and reduced to dryness *in vacuo* at a bath temperature of 35°. The residue was extracted with three 20-ml portions of hot

ethyl acetate. The combined extracts were dried over magnesium sulfate and concentrated to dryness *in vacuo* to give 180 mg (72%) of an oil which crystallized from ethanol and petroleum ether, m.p. 95–97°. Wieland *et al.* (1958) report 96°.

Anal. Calcd. for $C_8H_{13}NO_4$: C, 51.33; H, 7.00; N, 7.48. Found: C, 51.03; H, 6.68; N, 7.27.

The compound had an absorption peak at 240 mμ, which has also been noted by Errera and Greenstein (1947) for pyruvoyl-glycine. The infrared spectra showed peaks at 5.8 and 8.5 μ which were absent in the starting dipeptide and which can be ascribed to aliphatic ketones. In the quantitative ninhydrin reaction the compound gave a color yield equal to 0.95 that of alanine, whereas the yield of the starting material was 1.6 that of alanine. On paper chromatography in Solvent I it gave a substance at R_F 0.90 with a delayed ninhydrin reaction. After hydrolysis of the compound (5.7 N hydrochloric acid, 110° for 24 hours), only valine was detected on paper chromatograms.

N - (α - Ketoisocaproyl) - L - alanyl - L - valine.—L-Leucyl-L alanyl-L-valine (McGregor and Carpenter, 1961) (250 mg) was oxidized at pH 9.4 and the product was isolated as for pyruvoyl-DL-valine. An oil (206 mg) was obtained which was crystallized from ethanol to give 150 mg. (60%) of needle-shaped crystals; m.p. 186–188°.

Anal.: Calcd. for $C_{14}H_{24}N_2O_5$: C, 55.98; H, 8.05; N, 9.33. Found: C, 55.84; H, 7.92; N, 9.30.

The compound exhibited absorption peaks in the ultraviolet at 240 mμ and in the infrared at 5.8 and 8.55 μ which were absent in the starting tripeptide but which were present in pyruvoyl-valine. On paper chromatography of the compound in Solvent I, a substance was detected at R_F 0.90 (Solvent I) by a delayed ninhydrin color reaction Upon hydrolysis of the compound, only alanine and valine were detected on paper chromatograms of the hydrolysate

Hydrogen Peroxide Cleavage of α-Ketoacyl Compounds—Pyruvoyl-DL-valine (9.4 mg, 0.05 mmole) or α-ketoisocaproyl-L-alanyl-L-valine (15 mg, 0.05 mmole) was dissolved in 10 ml of 0.1 N potassium hydroxide and allowed to react with 1 ml of 30% hydrogen peroxide at 37°. Aliquots (2 ml) were removed at various time intervals, acidified with glacial acetic acid (0.15 ml), and then brought back to neutrality with sodium bicarbonate. Excess hydrogen peroxide was destroyed by incubation with catalase (0.03 ml of a 5% solution) for 20 minutes at 37°. Aliquots (0.05 ml) were removed from this solution for paper chromatography and for quantitative ninhydrin analysis by the method of Moore and Stein (1954) modified in that the heating time was cut to 2 minutes at 100°. The short heating time virtually eliminated the production of color from the starting materials. The color yields were compared with those obtained from samples of valine (1.18 mg) or alanyl-valine (1.88 mg) which had been carried through the whole procedure with the exception that the hydrogen peroxide was omitted. Paper chromatography showed that the hydrogen peroxide treatment of pyruvoyl-valine or α-ketoisocaproyl-alanyl-valine liberated valine or alanyl-valine respectively. The quantitative ninhydrin analyses indicated that these had reached their maximum yields of 70 and 60%, respectively, in 30 minutes.

In another set of experiments pyruvoyl-DL-valine (10 mg, 0.05 mmole) or *N*-(α-ketoisocaproyl)-L-alanyl-L-valine (15 mg, 0.05 mmole) was dissolved in 10 ml of 0.1 N potassium hydroxide. Hydrogen peroxide (1 ml of 30%) was added and the flasks incubated at 37° for 30 minutes. The solvent was removed in a rotary evaporator. In the case of pyruvoyl-valine the residue was dissolved in 0.5 ml of water and acidified with 0.2 ml of 85% phosphoric acid. The resulting solution, along with three 0.25-ml washes, was absorbed on a column of diatomaceous earth and anhydrous sodium sulfate similar to that described by James and Martin (1952). The fatty acid was eluted with ether. In the case of *N*-(α-ketoisocaproyl)-alanyl-valine, the acidified residue was extracted directly with ether. The ether solutions were subjected to gas-liquid chromatography on a 5-ft column with helium at 60 ml per minute used as the carrier gas. The stationary phase was made up as follows: 70% 60–80 mesh fire brick and 30% mixture of stearic acid–phosphoric acid–Dow-Corning Silicone 550 (10:10:80 by weight). Controls of acetic acid (retention time of 7.7 minutes at 105°) and isovaleric acid (retention time of 13.5 minutes at 132°) were carried through the whole procedure. From pyruvoyl-valine a material was obtained at the retention time of acetic acid (7.8 minutes at 105°) which amounted to 60% of the theoretical yield. From *N*-(α-ketoisocaproyl)-L-alanyl-L-valine a material was obtained at the retention time of isovaleric acid (13.5 minutes at 132°) which amounted to 80% of the theoretical yield. The fatty acids were missing from samples of the α-ketoacyl peptides that had been carried through the whole procedure with the exception that the hydrogen peroxide was omitted.

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The Relation of Free Sulfhydryl Groups to Chromatographic Heterogeneity and Polymerization of Bovine Plasma Albumin*

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Heterogeneity of bovine plasma albumin has been examined with respect to chromatographic behavior, molecular weight, and free sulfhydryl content. Fractionation of different components of bovine plasma albumin was achieved by taking advantage of their ability to displace one another on DEAE-cellulose. The heterogeneity so disclosed is chiefly related to the well-known heterogeneity of bovine plasma albumin with regard to molecular weight (monomers, dimers, and higher polymers) and free sulfhydryl content (mercaptalbumin and non-mercaptalbumin). There are at least two chromatographically distinct non-mercaptalbumins, the larger fraction being adsorbed more tightly to DEAE-cellulose than mercaptalbumin. The smaller non-mercaptalbumin fraction is the least tightly bound component of all and hence is best demonstrated by frontal analysis. A small fraction of monomer of undetermined sulfhydryl titer was not separable from the dimers and higher polymers, which are bound more tightly than the bulk of the monomers. Further heterogeneity within the more tightly binding non-mercaptalbumin component was revealed by its incomplete reduction to mercaptalbumin by β-mercaptoethanol. Similar heterogeneity was observed in the dimer component, which is only partly reduced to monomer by the same reagent. Within the dimer component produced by ethanol treatment, and probably within that normally occurring, there are chromatographic differences which reflect the heterogeneity of the constituent monomers; these differences are increased by hybridization. While all the ethanol-produced dimers were found to be dissociable by β-mercaptoethanol, free sulfhydryl groups were not required for their formation.

That crystalline preparations of plasma albumin are heterogeneous in several respects has long been recognized. The presence of small amounts of more rapidly sedimenting materials is a common observation, as is the presence of a sulfhydryl titer of less than one equivalent per mole. Heterogeneity has also been reported in chromatographic behavior (Boman and Westlund, 1956; Sober *et al.*, 1956; and Tiselius *et al.*, 1956) and in starch gel electrophoresis (Saifer *et al.*, 1961).

The nature of the intermolecular bond in the normally occurring dimer is not known, nor is it known whether the normally occurring dimer is an artifact of purification or, if it is, to what extent. Some studies have been carried out with synthetic dimers produced by various treatments. Thus, the dimer formed at low pH, most recently studied by Williams and Foster (1960), appears to be held together at first by pH-reversible secondary valences and only with time stabilized by formation of intermolecular disulfides by exchange or oxidation. The initial reversible bond is probably similar to that formed in 3–5 M urea as reported by Gutter *et al.* (1957). The reversible dimerization in the presence of Hg^{++} (Hughes, 1947) or bifunctional organic mercurials (Edsall *et al.*, 1954) is fairly well understood. The formation of a disulfide dimer by oxidation of the sulfhydryl groups on two monomers has been reported by Straessle (1954) on treatment of solid mercury dimer in ethanol with iodine.

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